Université de Liège
Faculté de Médecine vétérinaire
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Service de Bactériologie vétérinaire et Maladies bactériennes animales

Emergence d'*Escherichia coli* O80:H2 entéropathogènes et Shigatoxinogènes chez les Veaux et les Humains en Belgique : Une Etude sur leur Isolement, Identification, (Phylo-)Génomique et Virulence *in vivo*

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Rie IKEDA

Thèse présentée en vue de l'obtention du grade de Docteur en Sciences vétérinaires

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At the time COVID-19 was ravaging the world, I began my Ph.D., still vividly remembering the desolate Brussels International Airport.

As you all know, there is a famous phrase from Philosopher: *"**Man is but a reed, the weakest in nature, but he is a thinking reed**."* – Blaise Pascal, *Pensées*. However, thinking was what I disliked most in this world. I must offer my apologies to Pascal." Why were tasks others did easily so hard for me? I do not want to THINK any more." This question haunted me for a long time, spending my days struggling in self-denial and conflict. At times, I even felt a kind of sense that I might never find an answer to it in my lifetime. Now, I find relief in being able to bring provisional closure to that question in the doctoral thesis.

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Philosophy, the discipline of continual questioning.

*Ph.D., **'Doctor of Philosophy'**, is not, at least to me, a tool to inflate one's self-importance, nor a symbol of authority and knowledgeable.*

It was a continuous series of questions, and the record of their pursuit, and finally to the ansewer for me.

*If Socrates were alive today, how would he respond to my answers? maybe he sais, "**Know thyself**".*

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Au moment où la COVID-19 faisait rage dans le monde, j'ai commencé mon doctorat, me souvenant encore vivement de l'aéroport international de Bruxelles, désert. Comme vous le savez, il existe une phrase célèbre :« ***L'homme n'est qu'un roseau, le plus faible de la nature, mais c'est un roseau pensant.*** » – Blaise Pascal, *Pensées*

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*Peut-être dirait-il : « **Connais-toi toi-même** »*

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かつて、パスカルは、"人間は考える葦である"と言いました。しかし、"考えること"、それも特に"論理的に思考すること"は、私が世界で最も嫌うことでした。周囲の人々は簡単にこなしていることが、なぜ自分にはこんなにも高い壁に見えるのだろう。そんな問いを抱え、自己否定と葛藤の中でもがく日々が続きました。5年の月日を経た今、博士論文という形で、その問いに対して一旦区切りをつけられたことに安堵しております。これは決して私ひとりの力で成し得たものではありません。ひとえに関わってくださったベルギーと日本、両国の皆様のおかげです。この場をお借りして、関係する全ての皆様に深甚なる感謝を申し上げます。これらの言葉を最後に筆を置き、謝辞の結びとさせていただきます。

“Philosophy”とは、常に問い続ける学問、すなわち哲学である。
Doctor of Philosophy、もちろん博士号のことである。少なくとも私にとっては、自己を大きく見せるための道具でも知識や権威の象徴でもなかった。
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「汝自身を知れ」と言うかもしれない。

池田 里絵

ABBREVIATION LIST

°C: Degree Celsius

µm: micrometer

µg: microgram

A/E: Attaching and Effacing

AF/R1, R2: Adhesive Factor Rabbit

Agg-STEC : Aggregative STEC

ANSES : Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail

APEC : Avian pathogenic *Escherichia coli*

attp/*attB* : Attachment site

ARSIA : Association Régionale de Santé et d'Identification Animale

BFP: Bundle forming pili

BLAST(N) :Basic Local Alignment Search Tool (Nucleotide)

BTB: Bromothymol Blue

CC: Clonal Complex

CDC: Centers for Disease Control and Prevention

cgMLST: Core Genome Multi Locus Sequence Typing

Cif: Cycle inhibition factor

ColBM: Colicin BM

ColV: Colicin V

CT-SMAC: Cefixim-Tellurite-Sorbitol-MacConkey

DA: Diffuse adherence

DEC: Diarrheagenic *Escherichia coli*

DHL: Deoxycholate hydrogen sulfide lactose

DNA: Deoxyribonucleic acid

E. coli: *Escherichia coli*

EAEC: Enteroaggregative *Escherichia coli*

EAF: EPEC adherence factor

EF1-dependent: Elongation Factor 1

Efa1/lifA: EHEC Factor for adherence 1/ Lymphocyte inhibitory factor A

ECDC: European Centre for Disease Prevention and Control

EFSA: European Food Safety Authority

EHEC: Enterohemorrhagic *Escherichia coli*

Ehly: Enterohemolysin

EIEC: Enteroinvasive *Escherichia coli*

EPEC: Enteropathogenic *Escherichia coli*

ESBL: Extended Spectrum -Lactamase

Esc: *E. coli* secretion protein

Esp: *E. coli* secreted protein

ETEC: Enterotoxigenic *Escherichia coli*

ExPEC: Extra-intestinal *Escherichia coli*

fliC: Flagellin encoding protein

flkA: Flagellar assembly-associated protein

fllA: Flagella-related protein

flmA: Flagellar motor-associated protein

fln: Flagellum formation or regulation-related protein

Gb3/CD77: Globotriaosylceramide/Cluster of Differentiation77 leukocyte antigen

Gb4: Globotetraosylceramide

HC: Hemorrhagic colitis

HGT: Horizontal gene transfer

HeLa: Henrietta Lacks

Hly: Hemolysin

HUS: Hemolytic uremic syndrome

Iha: IrgA homologue adhesin

InVS : Institut de Veille Sanitaire

K_2TeO_3: Potassium tellurite compound

KatG: Hydrogen peroxide detoxification

Kps: Capsular polysaccharide (K-antigen) biosynthesis protein

L: Lineage

LA: Localized Adherence

LAL: Localized-Like Adherence

LdaG: The Locus of Diffuse Adherence

LEE: Locus of Enterocyte Effacement

Lpf: Long Polar Fimbriae

LPS: Lipopolysaccharide

Map: Mitochondrial associated protein

ml: milliliter

min: Minutes

MIC: Minimal Inhibitory Concentration

ML: Maximum likelihood

MLST: Multi Locus Sequence Typing

NGS: Next Generation Sequencers

Nle: Non-LEE located

NM: Nonmotility

nm: nanometer

NMEC: Neonatal meningitis *Escherichia coli*

ORF: Open Reading Frame

Paa: Porcine Attaching and Effacing associated fimbriae

PAI (Pai): Pathogenicity Island

pap: Pyelonephritis-associated pili

PBP: Penicillin-Binding Protein

PCR: Polymerase Chain Reaction

PDG: Peptidoglycan

RDEC: Rabbit diarrheagenic *Escherichia coli*

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

RT: Real time

Saa: STEC autoagglutinating

SePEC: Septicaemic *Escherichia coli*

SL: Sub-Lineage

SLT: Shiga-Like Toxin

SNP: Single Nucleotide Polymorphism

SpLE1: Sakai prophage like element 1

SS: Salmonella-Shigella

ST: Sequence Type

STEC: Shigatoxigenic *Escherichia coli*

Stx/STX: Shiga toxin

T3SS: Type III Secretion System

TAI: Tellurite resistance and Adherence-conferring Island

$TeO3^{2-}$: Tellurite ion (trioxotellurate IV)

Ter: Tellurite resistance

Tir: Translocated intimin receptor protein

TMA: Thrombotic Microangiopathy

TSI: Triple Sugar Iron

TTP: Thrombotic Thrombocytopenic Purpura

UPEC: Uropathogenic *Escherichia coli*

UK: United Kingdom

USA: United States of America

UTI: Urinary Tract Infections

UV: Ultraviolet

VT: Vero Toxin

WGS: Whole Genome Sequencing

WHO: World Health Organization

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RÉSUMÉ

O80:H2 est un sérotype émergent d'*Escherichia coli* productrices de toxines de Shiga (STEC) chez l'homme depuis 2010 en Europe occidentale. STEC O80:H2 était précédemment considéré comme un sérotype « mineur », car bien moins fréquemment associé au syndrome hémolytique-urémique (HUS) que les sérotypes « majeurs » classiques, tels que O26:H11 et O157:H7. Les deux principales propriétés de virulence des STEC O80:H2 sont : (i) la production de toxines de Shiga (Stx) codées par des gènes (par exemple *stx1a*, *stx2a*, *stx2c*, *stx2d*, …) situés sur des prophages ; (ii) la formation de lésions d'attachement-effacement (A/E) via un système de sécrétion de type 3 (T3SS) et l'adhésine intimine codés par des gènes situés sur un îlot de pathogénicité « Locus of Enterocyte Effacement » (LEE). Etant donné que ces souches STEC produisent la lésion A/E, elles seront dénommées souches AE-STEC dans ce travail. Le gène *eae* qui code pour l'adhésine intimine appartient à un variant rare, *eaeXI* (*eae*□). Cependant, les STEC O80:H2 se distinguent des sérotypes majeurs par leur implication dans des infections systémiques, telles que bactériémie et septicémie, en plus de la colite hémorragique (HC) et du HUS, suite à la présence d'un plasmide pS88-like de type ColV porteurs de gènes codant pour des propriétés invasives. De plus, non seulement des STEC O80:H2, mais aussi des *E. coli* entéropathogènes (EPEC) O80:H2, productrices de lésions A/E, mais pas de Stx, ont isolées plus fréquemment de puis 2009 de jeunes veaux diarrhéiques, occasionnellement septicémiques, suggérant un réservoir animal potentiel. Cependant, bien que les bovins et d'autres ruminants asymptomatiques soient porteurs d'AE-STEC appartenant aux sérotypes majeurs, le réservoir des AE-STEC O80:H2 n'a pas été

identifié à ce jour. Leur association avec des bovins adultes sains et des aliments d'origine bovine n'a, en effet, été démontrée que de manière très limitée, quand elle a pu l'être.

Le but général de ce travail de thèse de doctorat était donc de récolter des informations pour une meilleure compréhension structurelle et fonctionnelle sur les populations d'*E. coli* O80:H2 en : (i) renouvelant les essais d'isolement de souches AE-STEC et/ou EPEC O80:H2 à partir de bovins adultes asymptomatiques à l'abattoir et en fermes (ETUDES 1 et 2) ; (ii) confirmant par des études génétiques, la structure génomique, les virulotypes et la phylogénie des AE-STEC et EPEC O80:H2 humains et veaux (ETUDE 3) ; (iii) évaluant les rôles respectifs de leurs différentes propriétés de virulence dans un modèle *in vivo* en larves du papillon de nuit *Galleria mellonella* (ETUDE 4).

Les ÉTUDES 1 et 2 étaient centrées sur des tentatives d'isolement d'AE-STEC ou EPEC O80:H2 à partir de bovins sains en utilisant des méthodes non spécifiques (ETUDE 1) ou basées sur des propriétés spécifiques (ETUDE 2 : non-fermentation du mélibiose et résistance au tellurite de potassium). Bien que plusieurs échantillons fécaux positifs à la PCR pour le sérotype O80 aient été obtenus à partir de bovins sains dans deux abattoirs et de vaches saines en fermes durant l'ETUDE 1, aucune des souches isolées à partir de ces matières fécales n'appartenait au sérotype O80:H2. Le séquençage du génome total a confirmé que ces *E. coli* O80:nonH2 appartenaient aux sérotypes O80:H6 et O80:H45, phylogénétiquement distants du sérotype O80:H2, et étaient non-AE-STEC non-EPEC. Dans l'ÉTUDE 2, aucun des 52 AE-STEC et EPEC O80:H2 humains et de veaux analysés ne renfermaient l'opéron *mel*, ni n'étaient positifs à la fermentation du mélibiose (seules les souches de veaux furent phénotypiquement testées). Inversement, les niveaux de résistance au tellurite de potassium et la présence de l'opéron *ter* étaient différents entre

les AE-STEC et EPEC humains et de veaux. La plupart des AE-STEC O80:H2 humains et de veaux ne renfermaient pas l'opéron *ter* et avaient des Concentrations Minimales Inhibitrices faibles contrairement à la plupart des EPEC de veaux (seules les souches de veaux furent phénotypiquement testées). L'application de ces deux propriétés sur des échantillons fécaux provenant d'un abattoir n'a cependant pas permis d'identifier de souches appartenant au sérotype O80:H2.

L'ETUDE 3 visait à clarifier : (i) les combinaisons de gènes associés à la virulence (virulotypes) et la structure phylogénétique de la population d'AE-STEC et EPEC O80:H2 humaines et de veaux isolées en Belgique, en augmentant le nombre de souches étudiées de 52 à 129 ; et (ii) la localisation des gènes de virulence dans deux AE-STEC O80:H2 de veaux *stx2f*+, en comparant leurs séquences génomiques totales. Sans tenir compte de la détection du gène *stx2f* dans ces deux souches AE-STEC de veaux à côté des gènes *stx1a*, *stx2a*, *stx2c* et *stx2d*, les virulotypes des souches AE-STEC et EPEC humaines et de veaux ont été confirmés par la détection : (i) du gène *eaeξ* situé sur le LEE et codant pour l'adhésine intimine ; (ii) de différents gènes localisés ou non sur le LEE et codant le T3SS (*espA*, *espB, espF*, *tir*/*nleA*, *nleB*, *nleC*) ; (iii) de gènes situés sur le plasmide pS88 (*cia*, *cvaA*, *etsC*, *iucC*, *hlyF*, *ompTp*, *iss*, *iroN*, *sitA*) ; (iv) de différents autres gènes associés à la virulence et localisés sur d'autres plasmides ou sur le chromosome (*cia*, *imm*, *espP*, *ehxA*, *efa1*..). La structure de la population de souches AE-STEC et EPEC O80:H2 humaines et de veaux isolées en Belgique a aussi été confirmée avec l'existence de deux lignées (L) dans un arbre phylogénétique basé sur les « Single Nucleotide Polymorphisms » (SNP). La lignée L1 comprenait toujours quatre sous-lignées (SL), dont les SL1.1 et SL1.2 regroupaient 96% des souches. De plus, deux profils de gènes associés à la virulence permettaient toujours de différencier les souches des

SL1.1 et SL1.2 sur la base de la présence / absence de gènes situés, en théorie, sur le plasmide pS88 (*etsC*, *iucC*) et ou non (*cma, iha*).

Le séquençage des génomes totaux des deux AE-STEC O80:H2 *stx2f*+ de veaux (SES0057 appartenant à SL1.1 et SES0108 appartenant à SL1.2) a été effectué par la méthode de « long-read sequencing » dans le but de clarifier les structures du LEE, des phages Stx2f et des plasmides pS88, et de confirmer la localisation des gènes *ets*, *iuc*, *cma* et *iha*. Les LEE de ces deux souches ont été confirmés comme étant identiques. En ce qui concerne les phages Stx2f, deux copies identiques étaient présentes dans chacune des deux souches. Une de ces copies était insérée dans le locus *ssrA* tmRNA et la seconde en amont dans un autre prophage (« prophage-in-prophage »), lui-même inséré dans le locus *thrW* tRNA. Le deux phages Stx2f de la souche SES0108 montraient >99% d'identité avec des phages Stx2f de deux *E. coli* appartenant à d'autres sérotypes, tandis que les deux phages Stx2f de la souche SES0057 étaient différents. Le plasmide de la souche SES0057 appartenant à SL1.1 ne portait pas les gènes *ets* and *iuc* qui n'avaient pas été détectés dans cette souche, mais de manière surprenante le gène *cma*, tandis que le plasmide pS88 de la souche SES0108 appartenant à SL1.2 portait les gènes *ets* and *iuc*, mais pas le gène *cma* qui n'avait pas été détecté dans cette souche. Enfin, le gène *iha* détecté uniquement dans la souche SES0057 était présent sur un élément intégré dans le chromosome, le "Sakai-prophage like element-1" (SpLE-1).

L'ÉTUDE 4 a évalué la contribution de différents facteurs à la virulence des AE-STEC et EPEC O80:H2 dans un modèle *in vivo* en larves du papillon de nuit *Galleria mellonella*. Trois souches AE-STEC *stx1a*+, trois souches AE-STEC *stx2d*+ et deux souches EPEC avec différents plasmides pS88, ainsi qu'une souche *E. coli* K12 DH10B de laboratoire transfectée avec un phage Stx2d, une souche *E. coli* K12 DH10B de

laboratoire transconjugante avec un plasmide pS88 porteur des gènes *ets* et *iuc*, et trois souches contrôles ont été testées. Les souches AE-STEC *stx2d*+ et la souche K12 DH10B transfectée avec le phage Stx2d ont montré la létalité statistiquement la plus élevée de toutes les souches, en dehors de la souche contrôle positif, suggérant que Stx2d est un facteur de virulence majeur de O80:H2 dans ce modèle. En revanche, la virulence des souches AE-STEC *stx1a*+ n'était pas statistiquement plus élevée que celle des souches EPEC, laissant ouverte la question sur le rôle de Stx1a dans ce modèle. De même, la contribution des différents plasmides pS88 à la virulence des six souches AE-STEC n'était pas statistiquement significative. Cependant, le plasmide pS88 porteur des gènes *ets* et *iuc* contribuait de manière statistiquement significative, quoique limitée, à la virulence de la souche EPEC et de la souche *E. coli* K12 DH10B transconjugante. Inversement, le rôle du plasmide pS88 non porteur des gènes *ets* et *iuc* à la virulence de la souche EPEC n'était pas statistiquement significatif. Ces résultats suggèrent que les plasmides pS88 ne contribueraient pas de manière importante à la virulence des AE-STEC et EPEC dans ce modèle en larves de *Galleria mellonella*.

En conclusion, les ETUDES 1 et 2 soulignent que la non-fermentation du mélibiose, mais pas la résistance au tellurite de potassium, peut aider à isoler des AE-STEC et EPEC O80:H2 de différentes sources, notamment à partir de porteurs sains chez lesquels elles sont probablement présentes en faibles concentrations. Dès lors, d'autres méthodologies, non seulement conventionnelles ou spécifiques basées sur l'utilisation de milieux de croissance contenant différents antiseptiques ou antibiotiques, mais aussi ne reposant pas sur l'utilisation de ces milieux de croissance, doivent être développées et évaluées dans des études futures. Dans l'ÉTUDE 3, la structure de la population des AE-STEC et EPEC O80:H2 belges humaines et de veaux, y compris la classification phylogénétique (deux

lignées, dont une est subdivisée en quatre sous-lignées) et les virulotypes (présence d'un LEE dans toutes les souches à une exception près, de différents gènes *stx* dans les STEC et d'un plasmide pS88), a été confirmée lorsque le nombre de souches étudiées est augmenté de 52 à 129. L'existence de deux profils génétiques selon l'appartenance à l'une des sous-lignées, basés sur la présence / absence de gènes ou groupes de gènes situés, en théorie, sur le plasmide pS88 (*ets* et *iuc*) ou non (*cma* et *iha*) a également été confirmée. Les caractéristiques et la structure génomique dus LEE, des phages Stx2f et des plasmides pS88, de deux AE-STEC *stx2f*+ de veaux appartenant aux deux sous-lignées avec profils génétiques différents ont aussi été clarifiées après séquençage et analyse de leurs génomes totaux. De manière surprenante le gène *cma* est situé sur le plasmide pS88 dans la souche positive. Enfin, l'ÉTUDE 4 a évalué la contribution de différentes propriétés de virulence dans un modèle *in vivo* en larves du papillon de nuit *Galleria mellonella*: Stx2d est le facteur le plus virulent, tandis que les deux types de plasmides pS88 ne contribuent pas de manière importante à la virulence dans ce modèle en insecte. Quant à Stx1a, son rôle, s'il existe n'a pas pu être évalué à ce stade.

SUMMARY

O80:H2 is an emerging Shigatoxigenic *Escherichia coli* (STEC) serotype since 2010 in humans in Western Europe. STEC O80:H2 was previously considered as a "minor" serotype, much less frequently associated with the haemolytic-uremic syndrome (HUS) than the classical "major" serotypes, such as O26:H11 and O157:H7. The primary virulence properties of STEC O80:H2 include (i) the production of Shiga toxins (Stx) encoded by phage-borne *stx* genes (e.g., *stx1a*, *stx2a*, *stx2c*, and *stx2d*...), and (ii) the ability to form attaching and effacing (A/E) lesions via a Type3 Secretion System (T3SS) and the intimin adhesin encoded by genes located on a pathogenicity island called "Locus of Enterocyte Effacement" (LEE). Since these STEC produce the A/E lesion, they will be named AE-STEC from now on. The *eae* gene coding for the intimin adhesin of AE-STEC O80:H2 belongs to the specific *eaeXI* (*eae*□) variant. However, unlike the major serotypes, STEC O80:H2 are distinct in their involvement not only in hemorrhagic colitis (HC) and HUS, but also in systemic infections, such as bacteremia and septicemia, due to additional invasiveness factors encoded by genes located on ColV pS88-like plasmids. Furthermore, not only AE-STEC O80:H2, but also enteropathogenic *E. coli* (EPEC) O80:H2, producing the A/E lesion but no Stx, have been isolated more frequently since 2009 from diarrheic, and occasionally septicemic, calves in Belgium, suggesting a possible animal reservoir. However, although asymptomatic cattle and other ruminants are indeed carriers of the major AE-STEC serotypes, the true reservoir of STEC O80:H2 remains unidentified, due to their limited or absent association with adult healthy cattle and with bovine-related foodstuffs.

The general purpose of this Ph.D. work was therefore to obtain more information on the structural and functional understanding of *E. coli* O80:H2 population by: (i) renewing attempts to isolate AE-STEC and/or EPEC from asymptomatic adult cattle at slaughterhouses and in farms (STUDIES 1 and 2); (ii) confirming the genome structure, the virulotypes and the phylogenesis of human and calf AE-STEC and EPEC O80:H2 populations (STUDY 3); and (iii) assessing the respective roles of different properties of AE-STEC and EPEC O80:H2 in an *in vivo* model in larvae of *Galleria mellonella* moth (STUDY 4).

STUDIES 1 and 2 focused on attempting to isolate AE-STEC or EPEC O80:H2 from healthy cattle using both non-specific (STUDY 1) and trait-based specific (STUDY 2: non-melibiose fermentation and potassium tellurite resistance) methods. Although some O80 PCR-positive fecal samples were recovered from healthy cattle at two slaughterhouses and from healthy cows in nine farms during STUDY 1, none of the isolates belonged to the serotype O80:H2. Whole genome sequencing confirmed that these *E. coli* O80:nonH2 belonged to serotypes O80:H6 and O80:H45, that are phylogenetically distant from serotype O80:H2, and were non-AE-STEC non-EPEC. In STUDY 2, all 52 human and calf AE-STEC and EPEC O80:H2 analysed did not harbour the *mel* operon and were non-melibiose fermentative (only calf isolates were phenotypically tested). Conversely, the resistance levels to potassium tellurite and the presence of the *ter* operon were different among the human and calf AE-STEC and EPEC. Most of the human and calf AE-STEC O80:H2 did not harbor the *ter* operon and had low Minimal Inhibitory Concentrations (MIC) to tellurite, in contrast to most calf EPEC (only calf isolates were phenotypically tested). Applying these two properties on faecal samples from one slaughterhouse, none of the isolates O80 belonged to the serotype O80:H2.

STUDY 3 aimed to clarify (i) the combination of virulence-associated genes (virulotypes) and the population phylogenetic structure by expanding the set of analyzed human and calf AE-STEC and EPEC O80:H2 from Belgium from 52 to 129 and (ii) the virulence gene localization by generating complete genomic sequences of two *stx2f*-positive calf AE-STEC O80:H2. Besides the detection of the *stx2f* gene in two calf AE-STEC in addition to the *stx1a*, *stx2a* and *stx2d* genes, the virulotypes of calf and human AE-STEC and EPEC were confirmed by the detection of (i) the *eaeξ* LEE-located gene coding for the intimin adhesin; (ii) different T3SS-encoding (non)-LEE-located genes (*espA*, *espB, espF*, *tir*/*nleA*, *nleB*, *nleC*); (iii) the pS88-located (*cia*, *cvaA*, *etsC*, *iucC*, *hlyF*, *ompTp*, *iss*, *iroN*, *sitA*) genes; (iv) several other plasmid- or chromosome-located virulence-associated genes (*cia*, *imm*, *espP*, *ehxA*, *efa1*...). The population structure was also confirmed with the presence of two lineages (L) in a Single Nucleotide Polymorphism (SNP) phylogenetic tree and with L1 further subdivided in four sub-lineages (SL), whom SL1.1 and SL1.2 grouped 96% of the human and calf AE-STEC and EPEC O80:H2. Moreover, two virulence-associated gene profiles differentiated the strains of SL1.1 and SL1.2 based on the presence / absence of genes or gene clusters located, in theory, on the pS88 plasmids (*ets*, *iuc*) or not (*cma, iha*).

Complete genomic sequences of the two calf *stx2f* AE-STEC (SES0057 belonging to SL1.1 and SES0108 belonging to SL1.2) was generated by long-read sequencing to clarify the genome structures of the LEE regions, of the Stx2f phages and of the pS88 plasmids, and to confirm the localization of the *ets*, *iuc, cma, iha* genes. At first, the LEE regions were identical. Two identical copies of the Stx2f were detected in either strain with one copy inserted within the *ssrA* tmRNA locus and the second one integrated into another prophage at the height of the *thrW* tRNA locus (prophage-in-prophage). The

Stx2f phages of strain SES0108 were >99% identical to the Stx2f phages of two *E. coli* strains belonging to other serotypes while the Stx2f phages of strain SES0057 were different. The pS88 plasmid of strain SES0057 belonging to SL1.1 did not carry the *ets* and *iuc* genes that had not been detected in this strain, but surprisingly carried the *cma* gene, while the pS88 plasmid of strain SES0108 belonging to SL1.2 carried the *ets* and *iuc* genes, but not the *cma* gene that had not been detected in this strain. Finally, the *iha* gene detected only in strain SES0057 was located on the chromosome-integrated "Sakai-prophage like element-1" (SpLE-1).

STUDY 4 assessed the functional contribution of different factors to the virulence of AE-STEC and EPEC O80:H2 by testing them in the larvae of the moth *Galleria mellonella*. Three *stx1a* AE-STEC, three *stx2d* AE-STEC and two EPEC strains harboring different pS88 plasmids, one K12 DH10B laboratory *E. coli* strain transduced with the Stx2d phage, one K12 DH10B laboratory *E. coli* strain conjugated with a pS88 plasmid carrying the *ets* and *iuc* genes, and three control strains were tested in this *in vivo* model. The *stx2d* AE-STEC and Stx2d-transduced K12 DH10B strains had the statistically significant highest lethality of all, but the positive control strains suggesting that the Stx2d is a major virulence factor in this model. The virulence of the Stx1a AE-STEC was not significantly higher than the virulence of the EPEC strains, leaving open the question of the actual role of Stx1a in this model. Similarly, the contribution of the different pS88 plasmids to the virulence of the different AE-STEC strains was statistically not significant, while the contribution of the pS88 plasmid carrying the *ets* and *iuc* genes, but not of the pS88 plasmid not carrying these two genes, to the virulence of the EPEC and of the transconjugated K12 DH10B strains was slightly significant. These results

suggest that the pS88 plasmids do not highly contribute to the virulence of AE-STEC and EPEC O80:H2 in the *Galleria mellonella* larva model.

In conclusion, STUDIES 1 and 2 about isolation and identification highlight that non-melibiose fermentation, but not resistance to tellurite could help to isolate AE-STEC and EPEC O80:H2 from different sources, especially when present in low numbers like probably in healthy cattle carriers. Therefore, several other methodologies, not only agar-dependent conventional or specific ones testing different antibiotics and antiseptics, but also agar-independent ones, should be developed and assessed in future surveys. In STUDY 3, the population structure of Belgian human and calf AE-STEC and EPEC O80:H2, including the phylogenetic classification (two lineages with one of them subdivided in four sub-lineages) and the virulotypes (presence of a LEE in all but one strain, of different *stx* genes in the STEC and of a pS88 plasmid), was confirmed when extending the number of strains studied from 52 to 129. The existence of two gene profiles according to the belonging to one of the sub-lineages based on the presence / absence of, in theory pS88-located (*iuc* and *ets*) or not (*cma* and *iha*) genes or gene clusters was also confirmed. Also the features and the genome structures of the LEE regions, Stx2f phages, and pS88 plasmids were clarified by generating and analyzing the whole genome sequences of two calf *stx2f* AE-STEC belonging to the sub-lineages with different gene profiles. Surprisingly, the *cma* gene was also found to be located on the pS88 plasmid in the positive strain. Lastly, STUDY 4 assessed the contribution of several virulence properties using larvae of the moth *Galleria mellonella* as an *in vivo* model: the Stx2d is the most virulent property, while the two different pS88 plasmids do not highly contribute to the virulence in this insect model. The contribution of Stx1a to virulence in this model, if any, was not possible to assess at this stage.

GENERAL INTRODUCTION

FOREWORD

Escherichia coli (*E. coli)* is a Gram-negative facultative anaerobe, fermentative rod bacterium described in 1885 by the German pediatrician, Theodor Escherich, that belongs to the intestinal microbiota of humans and warm-blooded animals (Escherich, 1885). The history of the pathogenicity of *E. coli* is closely related to advances in microbiology and infectious disease research. Although *E. coli* is now widely recognized as one of the main causative agents of gastro-intestinal diseases and an ‘essential cause of diarrhea’, the situation was totally different about 150 years ago. Hence, the discovery of the link between diarrhea and *E. coli* by Lesage in 1897 was undeniably an important event for the field (Lesage, 1897). Although at the time, the bacterial virulence criteria proposed by Robert Koch were widely used as a framework for identifying pathogens, *E. coli* was still regarded as a commensal and not as a specific pathogen (Koch, 1876 ; Koch, 1882). Thus, Lesage's hypothesis that *E. coli* could cause diarrhea was questioned at the time. Nevertheless, in 1893, the Danish veterinary surgeon Carl O. Jensen proposed that *E. coli* bacterial species contains both pathogenic and symbiotic strains (Jensen, 1893). However, the hypotheses proposed by Lesage and Jensen remained 'just hypotheses' for decades, as no suitable method existed at that time to distinguish between the putative pathogenic and symbiotic strains.

Various studies were conducted in the following years to establish a classification and an identification method for *E. coli* and to elucidate its pathogenicity. In 1947, a taxonomy of *E. coli* was proposed for the first time by Kauffmann in Denmark (Kauffmann, 1947). This was a classification and identification method based on the presence of three antigens at the surface of the bacterial cell: O (a part of LPS), K (a surface polysaccharide called capsule) and H (flagella which allows bacteria to move) antigens: The different combinations of these three antigens give the serotypes of *E. coli* strains. Serotyping is still routinely used today to identify *E. coli* strains. At that time, diarrhea in children was prevalent in the UK and, by using the Kauffmann classification method, the cause of diarrhea was identified as *E. coli* O111, which was the first recognition of the pathogenic potential of a specific *E. coli* serotype (Stock and Shuman,

1956).

The concept of enteropathogenic *E. coli* (EPEC) was at first introduced in the 1950s. All *E. coli* associated with diarrhea in young children were termed 'EPEC' (Neter et al., 1955). However, the definition of the term EPEC gradually evolved as research on the pathogenic mechanisms of *E. coli* progressed. In other words, EPEC was progressively used in a much narrow meaning, rather than a broad one, and a new term was introduced for the *E. coli* strains causing diarrhea: diarrheagenic *E. coli* or DEC. In the late 1950s and early 1960s, studies on the classification and pathogenicity of EPEC strains in humans and animals were intensively conducted and two types of 'DEC' were already recognized: those showing Shigella-like dysentery syndrome by invading the enterocytes (Hale et al., 1985) and those adhering to the enterocytes and producing toxins active on the enterocytes or enterotoxins (Smith and Halls, 1967). The former were named entero-invasive *E. coli* (EIEC) and the latter enterotoxigenic *E. coli* (ETEC).

In 1980, the WHO (World Health Organization) defined EPEC as pathogenic *E. coli* that are neither EIEC, nor ETEC and whose pathogenic mechanisms were still unknown, although they were proved to cause diarrhea in human volunteers (Levine et al., 1987; WHO, 1980). Thereafter, different pathogenic mechanisms were still unraveled in the 1980s and 1990s allowing to further identifying different types of pathogenic mechanisms of DEC and narrowing even more the definition of the term EPEC.

Today, DEC comprise at least six most important types on the basis of their pathogenic mechanisms: enteroinvasive (EIEC), enterotoxigenic (ETEC), enteropathogenic (EPEC), Shigatoxigenic (STEC), enterohemorrhagic (EHEC), and enteroaggregative (EAEC) (Russo and Johnson, 2000). The term EPEC designates DEC having a very specific pathogenic mechanism, that will be detailed in the following sections, as will be those of STEC and EHEC that are the subject of this work.

INTRODUCTION

1. *Escherichia coli*

Escherichia coli is a bacterial species described in children stool and named "*Bacillus coli commune*" by a German pediatrician, Theodor Escherich (1857-1911) (Escherich, 1885). In 1919, *Bacillus coli commune* was re-named and the official today well-known species name "*Escherichia coli*" was adopted (Castellani and Chalmers, 1919). *E. coli* is a representative bacterial species of the family *Enterobacteriacae*, a member of the intestinal microbiota of warm-blooded animals and an indicator of fecal contamination of foodstuffs in public health. *E. coli* forms shining, round shape colonies on ordinary agar after overnight growth at the optimal temperature of 37.5°C (Basavaraju and Gunashree, 2023).

1.1 The cellular structure

1.1.1 Membrane structure

E. coli is characterized as a “Gram-negative” bacterium with the membrane structure composed of three main constituents from inside to outside: the inner or cytoplasmic membrane, the peptidoglycan layer and the outer membrane (**Fig. 1a**).

The inner membrane is a structure that surround the cytoplasm that consists of phospholipid bilayers and proteins. It has selective permeability and is involved in mass transport, signal transduction and energy generation.

The peptidoglycan (PDG) layer is the major constituent of the cell wall of bacteria and is a macromolecule composed of carbon hydrates and amino acids forming a huge network surrounding the bacterial cells. In Gram-negative bacteria, the PDG is present in the periplasm that is the space between the inner and outer membranes. This reticulated structure maintains the shape of cell and is also involved in various physiological processes, such as intracellular and extracellular interactions, e.g. material transport for bacterial survival.

The outer membrane is a specific structure for Gram-negative bacteria located at the outer layer of bacterial cells. This membrane includes some constituents similar to the cytoplasmic membrane, like phospholipid bilayers and proteins, and some specific constituents, especially the lipopolysaccharide (LPS) with three constituents (**Fig. 1b**): the lipid A embedded in the outer membrane, the polysaccharidic core and the polysaccharidic enigen that is one of the three antigens forming the Kaufmann serotypes (Dieckmann et al., 2011; Schumann et al., 2024). The outer membrane protects the bacterial cells from external harmful stimulus, like toxic heavy metals or antibiotics, by prohibiting them to penetrate while allowing the passage of nutrients. The LPS is the one of the crucial molecules for Gram-negative bacteria and plays different roles: protection of the bacteria by its intricated structure and negative charge, triggering an immune response by the O antigen, endotoxic effect by the lipid A, and even for some of them, a role in the bacterial virulence (Mainil and Fairbrother, 2014; Mellata et al., 2003).

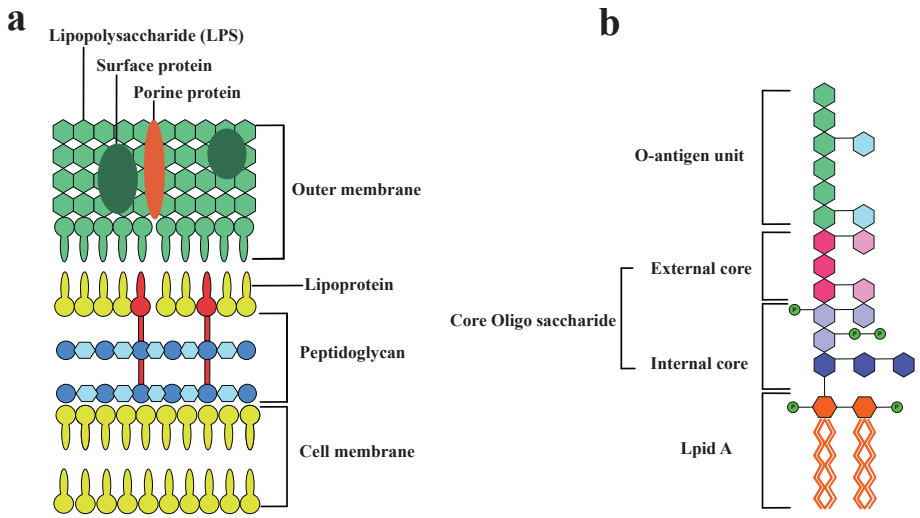

Figure 1a and 1b. Membrane structure of *Escherichia coli* (adapted from Major Difference, accessed on 21 May 2025; https://www.majordifferences.com/). (a) Three main constituents from inside to outside: the inner (cytoplasmic) membrane, the peptidoglycan layer, and the outer membrane. (b) Structure of the lipopolysaccharide (LPS) with three constituents: the O-antigen unit, the core oligosaccharide, and lipid A adapted from Steimle et al., 2016.

1.1.2 External structures

External to the bacterial cell, three primary structures can be described: flagella, pili and fimbriae (**Fig. 2**).

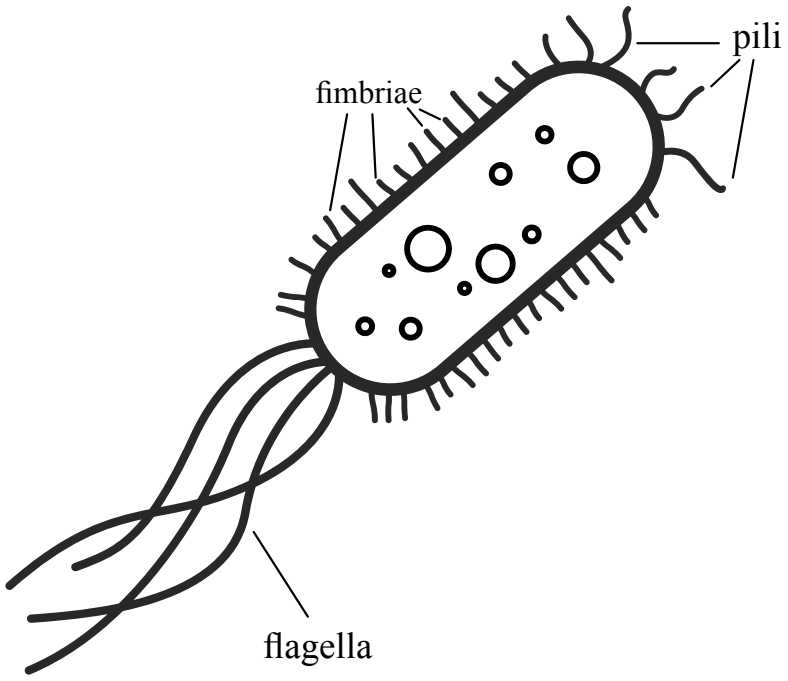

Figure 2. External structure of *Escherichia coli*: flagella, pili, and fimbriae. Based on MICROSCOPE MASTER, "Pili and Fimbriae Types, Function, and Difference" (https://www.microscopemaster.com/), accessed on 21 March 2025.

The flagella are also proteinaceous spiral fibers that allow *E. coli* to move. They represent the H surface antigen in the Kauffmann serotype, but are not produced by all *E. coli* strains. The fimbriae are short (0.1~1.5 μm) and thin (4~8 nm) fibrous structure

composed of different proteins (Barer, 2012). Fimbriae play an essential role in the virulence of pathogenic *E. coli* by mediating their adherence to the host cells and extra-cellular matrix. When produced, they represent the F surface antigen, that was added to the O:K:H antigens in the Kauffmann serotypes (Orskov and Orskov, 1983). The pili resemble the fimbriae, but are thicker and longer. Pili are encoded by plasmid-located genes and are involved in plasmid conjugation, or horizontal transfer of plasmids between bacterial cells. For this reason, they are also named sex pili. Different sex pili encoded by different plasmid types have been described: F-pili, I-pili …. (Willetts and Paranchych, 1974).

In addition to these three external structures, amorphous layers external to the outer membrane can be produced by *E. coli*. The most important one is the polysaccharide capsule that is an additional protective layer also known as the bacterial antigen K in the Kauffmann serotypes, but is not produced by all *E. coli* strains. In addition to their protective role, some capsular antigens are involved in the virulence of extra-intestinal *E. coli*, like the antigen K1 involved neonatal-meningitis in children by its anti-phagocytic role (Peigne et al., 2009).

1.2 Biochemical properties

Like all living organisms, *E. coli* must find inorganic and/or organic sources of carbon, nitrogen and other elements, and of energy, and catabolizes them using different pathways to produce energy that is necessary for their anabolism in order to survive and multiply. The use of inorganic and/or organic sources and of catabolic pathways can help to identify *in vitro* the different bacterial species. For instance, *E. coli* can ferment glucose with gas production, and lactose and production of indole by decomposing tryptophan. These three reactions are fundamental for identifying *E. coli* using the so-called TSI (Triple Sugar Iron: glucose, lactose, and white sugar) medium. Some agar plates selective for enterobacteria (BTB lactose agar, DHL, Gassner, MacConkey, SS, etc.) also take advantage of the lactose fermentation by *E. coli* and by a very few other bacterial species (*Klebsiella* sp., *Enterobacter* sp.) to differentiate them from non-lactose fermenting pathogenic species like *Salmonella*. Nevertheless, several others are also used forming what is called a “sugar set” (**Table 1**).

Biochemical properties of bacteria can be examined nowadays relatively easily using (semi-)automated tools. One of these tools are the commercialized API sugar sets that are still used in routine certain clinical laboratories. For *E. coli* and all *Enterobacteriaceae*, the specific set is called API20E (BioMérieux, https://www.biomerieux-industry.com/ja/products/api). Interestingly not all strains of *E. coli* give 100% of positive or negative reactions at all tests (**Table 1**). This can help to some extent to differentiate some pathogenic strains (see 1.3.1). Additional reactions studied *in vitro* are the production of (per)oxidases. In that respect, *E. coli* produces a catalase but does not produce any (per)oxidase.

Table 1. General API20E sugar sets reaction of *Escherichia coli* based on Internet-based APIWEB™ service by BioMérieux accessed on 7th December 2024 https://www.biomerieux.com/us/en/our-offer/clinical-products/api.html

Number	API20E	Identification	*Escherichia coli*	Positive rate (%)
1	ONPG	β-Galactosidase	+	90
2	ADH	Arginine Dihydrolase	−	1
3	LDC	Lysine Decarboxylase	+	74
4	ODC	Ornithine Decarboxylase	+	70
5	CIT	Citrate Utilization	−	0
6	H_2S	Hydrogen Sulfide Production	−	1
7	URE	Urease	−	3
8	TDA	Tryptophan Deaminase	−	0
9	IND	Indole Production	+	89
10	VP	Voges-Proskauer Test	−	1
11	GEL	Gelatin Hydrolysis	−	0
(*Sugar Fermentation*)				
12	GLN	Glucose	+	99
13	MAN	Mannitol	+	98
14	INO	Inositol	−	1
15	SOR	Sorbitol	+	91
16	RHA	Rhamnose	+	82
17	SAC	Sucrose	−	36
18	MEL	Melibiose	−	75
19	AMY	Amygdalin	+	3
20	ARA	Arabinose	+	99
-	(OX)	Oxidase	−	0

1.3 Identification and classification methods of pathogenic *Escherichia coli* strains

Today, pathogenic *Escherichia coli* strains can be identified and classified by different phenotypic and genetic methods. The following methods are detailed hereunder: biochemical properties (or biotyping), serotyping, virulotyping, phylogrouping, MultiLocus Sequence Typing, and Single Nucleotide Polymorphism.

1.3.1 Biotyping

Although most pathogenic *E. coli* gives biochemical profiles similar to all *E coli* strains (**Table 1**), some of them can have a relatively specific biochemical reaction that is shared by no or by a few of the *E. coli* strains.

For example, some EAEC and EIEC are reported as non-lactose ferment traits, and some ETEC is also found "atypical" because of urease production (Parreira et al., 2008; Sharma et al., 2023). Focusing on STEC, several sugars (rhamnose, sucrose, salicin and dulcitol) were not or delayed ferment by one of the HUS-associated serotypes O165:H25 was identified. In addition to it, like of a sort of ETEC, the urease production was also observed in STEC serotype O5:H- (Mercado et al., 2004; Nakamura et al., 2023).

Another example of interest in the study of EHEC is the non-sorbitol fermentation by serotype O157:H7, arguably the most notorious one worldwide while the great majority of (ca. more than 90%) *E. coli* strains do. Thus, the specific MacConkey agar "sorbitol MacConkey agar plate" containing some other selective agents was developed and is commercialized for rapid O157 detection (Karmali et al., 1989). Other specific biochemical properties of different EPEC and STEC will be described later (see section 2.2.5).

Today, characterization using biochemical properties is still an important first step in the routine isolation and identification of pathogenic *E. coli*, and the development of isolation agar plate is one of the most urgent tasks for researchers, but must be completed by more specific genetic methods.

1.3.2 Serotyping

The serotyping classification method developed by the Danish bacteriologist Philip Kauffmann, is based on the "Kauffmann-White method" to classify *Salmonella* species (Kauffmann, 1947). As already explained *E. coli* serotypes are determined by the combination of three specific surface antigens: O antigen, K antigen, and H antigen. Today a fourth one, the F antigen is also part of the serotyping of some pathogenic *E. coli* strains (Orskov and Orskov, 1983).

The O antigen (or somatic antigen) after the German" Ohne Kapsel", is the terminal variable polysaccharide moiety of the LPS attached to the outer membrane of Gram-negative bacteria. Today, up to 182 O antigens (O1 to O188, except O31, O47, O67, O72, O93, and O94) have been identified (Joensen et al., 2015; Lang et al., 2019). The enzymes involved in the O antigen synthesis are grouped in the *rfb* gene cluster. Of the several genes present in the gene clusters coding for the O antigens, the *wzx* (O antigen flipasse for transporter), *wzy* (O antigen polymerase for polymerisation of glycans) and *wzm*/*wzt* (for ABC-transporter) are specific. Thanks to the *rfb* gene cluster, the addition, binding and modification of specific sugars is possible leading to the diversity of O antigens (Schnaitman and Klena, 1993).

The capsular K antigen (after either the "Kauffman" family name or the German "Kapsel") is a surface polysaccharide forming an amorphous layer outside the outer membrane. Generally, the synthesis of the capsule polysaccharide is encoded by the *kps* gene cluster (Meredith and Woodard, 2006). Today, more than 80 K antigens have been identified (Orskov and Orskov, 1992; Willis and Whitfield, 2013). Nevertheless, some *E. coli* strains do not produce any K antigen. When present, the K-antigens prevent the detection of the O antigens and some also protect *E. coli* from host defenses, such as the phagocytosis, like the K1 antigen. The K1 antigen-producing *E. coli* actually cause severe bacterial infections, such as urinary tract infections and neonatal meningitis (Johnson, 1991).

The H antigen (after the German "Hauch) is the carried by the proteinaceous flagellar structure that allows bacteria to move. Up today, 53 H antigens (H1 to H56,

except H13, H22, and H50) have been identified (Joensen et al., 2015; Lang et al., 2019), but some *E. coli* strains do not produce any H antigen and are therefore non-motile strains. Five encoding genes are involved in the H antigen synthesis *fliC*, *flkA, fllA, flmA*, and *fln* (Banjo et al., 2018; Wang et al., 2003).

The serotype of *E. coli* is identified by the combinations of these three antigens (O: K:H) and by numerals, like O18:K1:H7. Since 182 O antigens, 80 K antigens and 53 H antigens have been described so far, thousands of serotypes have been progressively identified since the years 1940s (Furevi et al., 2020; Joensen et al., 2015; Orskov and Orskov, 1992). When a strain produces no K or H antigen, it is either noted K- or H-, or not mentioned: O157(:K-):H7, O101:K30(:H-), O80(:K-):H2, O26(:K-):H11. Serotypes can be identified serologically and genetically (Joensen et al., 2015). Indeed, since the O, K and H antigens are exposed at the bacterial surface, they represent molecular targets of the immune response. Moreover, their specific encoding genes can be detected by PCR or after whole genome sequencing (Banjo et al 2018; Iguchi et al., 2015).

Since recognized as antigen of bacterial surface, the F antigens generally indicated 'fimbriae' of *E. coli* (**Table 2**). F antigens are encoded by *pap* genes and often associated with UPEC strains. Of these, 11 known serological variants: F7-1, F7-2, and F8 to F16 (Johnson et al., 2000). The main role of F antigens is resistant to phagocytosis of immune cells, and some of them are cause hemagglutination (Orskov et al., 1982).

Table 2. Specific virulence factors associated with various *Escherichia coli* pathotypes, distinguishing between diarrhoeic *E. coli* (DEC) and extraintestinal pathogenic *E. coli* (ExPEC). Information includes representative primary and secondary hosts and major virulence factors, adapted from Mainil and Fairbrother, 2014.

Diarrhoeic E. coli (acronym)	Representative primay host (secondary host)	Virulence factors
Entero Toxigenic(ETEC)	Humans Ruminants Post weaning pigs Dogs	.Afimbrial adhesins (AIDA) .α haemolysin (α-Hly) .Fimbrial adherence (F2 to F6, F17a,F18, and F41..) .Heat stable (Sta, STb)/ Heat labile enterotoxins (LT1,LT2)
Entero Pathogenic(EPEC)	Humans Young calves Pigs Rabbits	.Attaching and Effacing (A/E) lesion .AF R1/R2 (Adhesive factor/Rabbit), Ral (Rabbits) .Paa Fimbriae (Porcine attaching- and effacing associated) .Type IV Fimbriae (tEPEC)
Attaching-Effacing and ShigaToxigenic (AE-STEC) **Entero Hemorragic (EHEC)** **Shiga Toxigenic (STEC)**	Humans (Ruminants, pet animals) Young calves Pigs Pet animals (dogs, cats..)	.Attaching and Effacing (A/E) lesion:AE-STEC / EHEC .F18 Fimbriae (Pigs) .Saa (STEC autoagglutinating adhesin) .ShigaToxin (No pathogenic to Adult Cattle)
Entero Invasive (EIEC)	Humans	Invasion and multiplication in the enterocytes
Entero Aggregative (EAEC)	Humans	.Fimbrial adhesins (Aaf, Had..) .Toxins (Pet, EAST, ShET1) .Transcriptional activator AggR
Diffsely Adherent (DAEC)	Humans Humans	.Afimbrial adhesins (AIDA) .Dr family of adhesins
***Extraintestinal E. coli* (acronym)**		
Avian Pahtogenic (APEC)	Chickens, ducks, turkeys(Other Poultry)	.Adhesins (Pap/prs, Sfa/F1C, F17b/c, AFA-VIII..) .Anti-phagocytosis (O/K antigen)
Septicaemic (SePEC)	Ruminants (Other mamals)	.Iron-uptake system (Aero/Yersiniabactins, Salmochelin) .Membrane protein for invasion to bloos vessels (IbeA..)
Uro Pathogenic (UPEC)	Humans and pet animals (Other mamals)	.Toxins (CNF1/2, CDT-III/VI, α-Hly..) .Resustance to immune response (Iss , TraT..)
Neonatal Meningitis (NMEC)	Newborn babies (Other mammals)	.Iron-uptake system (Yersiniabactins, Salmochelin) .K1 capsuler

With the establishment of this serotyping classification method, *E. coli* belonging to specific serotypes were beginning to be recognized as being associated with different diseases, including gastrointestinal diseases, both in humans and in animal species (Beutin, 1999; Ewing, 1956; Kaper et al., 2004). For this reason, phenotypic and/or genetic serotyping still represents a first basis of pathogenic *E. coli* classification today. Nevertheless, today, classification methods based on the identification of different virulence-associated properties or on whole genome information are proposed and used.

1.3.3 Pathotyping and Virulotyping

The classification of pathogenic *E. coli*, at first, in 'pathotypes' is determined by the location of their specific pathological interactions with the hosts and, then, in 'virulotypes' by the functions and modes of interactions with the host tissues of their specific virulence factors. As briefly introduced in the foreword, pathogenic *E. coli* are, at first, broadly classified into two pathotypes: intestinal or diarrheic *E. coli* (DEC) and extra-intestinal pathogenic (ExPEC). The former pathotype is today further divided into enteroinvasive (EIEC), enterotoxigenic (ETEC), enteropathogenic (EPEC), Shigatoxigenic (STEC), enterohemorragic (EHEC), enteroaggregative (EAEC) and diffusely adherent (DAEC) *E. coli*, and the latter pathotype into septicaemic (SePEC), avian pathogenic (APEC), uropathogenic (UPEC), and neonatal meningitis-associated (NMEC) *E. coli*. Other pathotypes have been described, but these 11 ones are the most frequent and important in terms of diseases.

Within each pathotype, different virulotypes can be present whose specific combinations of virulence factors can be identified by phenotypic (tests in animals and on cells in culture, immune assays…) and genetic assays (DNA-DNA colony hybridization, PCR, genome sequencing). The specific virulence factors of the different pathotypes are presented in Table 2 and will be detailed later for enteropathogenic and Shigatoxigenic pathotypes (see section 2).

Thus, there is a wide variety of pathogenic *E. coli*, which cause diseases in and outside the intestinal tracts of humans and animals. This diversity is caused by the acquisition and transfer of genes and mobile genetic factors between bacterial species, and also *E. coli* strains by conjugation, transformation or transduction (Kaper et al., 2004; Mainil and Fairbrother, 2014; Russo and Johnson, 2000; Tozzoli and Scheutz, 2014).

1.3.4 Phylogrouping

There are several methods of classification of *E. coli* based not only on their whole genetic information. One of them is the phylogroup-based method or phylogenetic grouping. In 1984, Ochman and Selander used polymorphic electrophoresis of 11 metabolic enzymes to classify about 2600 *E. coli* isolates from various countries and

regions into the following four phylogroups: A (commensal strains, non-pathogenic), B1 (origin from environment or animals, low pathogenic), B2 (including urinary tract infections and neonatal meningitis-causing strains, highly pathogenic), and D (pathogenic in some cases, basically members of the intestinal microbiota) (Ochman and Selander, 1984). Based on their classification, Goullet and Picard further added the phylogroups C (low pathogenic, including commensal strains) and E (pathogenic in some cases, associated to human infection strains) (Goullet and Picard, 1989) and eight phylogenetic groups are described, A, B1, B2, C, D, E, plus F (pathogenic in some case, resemnle to Group B2) and G (high pathogenic with antimicrobial resistance in majority, resemble Group B2 and D) (Clermont et al., 2013 ; Clermont et al., 2019). When Ochman and Selander reported their typing result, this method was considered phenotypic method since it analyzed the migration patterns of products encoded by genes. However, with advancements in molecular biology, including the emergence of Next-Generation Sequencers (NGS), current phylogrouping methods, such as those by Clermont and collaborators, now this method is recognized as genetic method using PCR and genome sequencing.

E. coli evolved from a common ancestor and diverged into diverse phylogroups during the evolutionary process and adaptation to different environments. The phylogrouping method of classifying is of great importance not only for tracing their evolutionary history in response to environment and host, but also for understanding their genetic diversity and their epidemiology.

This classification scheme can indeed be very useful from an epidemiological point of view, as the prevalence of strain groups varies according to the host species of interest. Thus, in humans, Group A, consisting of commensal strains, is the most frequent (approximately 40%), followed by the highly pathogenic Group B2 (approximately 25%), which includes extra-intestinal pathogenic strains. In other mammals, the situation differs slightly from humans, with Group B1, which is commonly identified in animals and the environment, predominating (41%), followed by Groups A (22%) and B2 (21%) (Beghain et al., 2018; Duriez, 2001; Touchon 2020).

1.3.5 Sequence typing (ST)

Similar to phylogroup-based classification, the Sequence Type (ST) classification method is based on genome information, more specifically on the sequences of seven different housekeeping genes that are conserved in the *E. coli* population. Another name of this method is MultiLocus Sequence Typing (MLST). These seven housekeeping genes are associated with enzymes essential for bacterial survival. Their genetic mutations accumulate gradually over long periods, making them useful for tracking bacterial evolution. These mutations can be detected by specific PCR followed by sequencing of the amplified DNA fragments. Today up to 16,943 *E. coli* strain lineages classified by their ST $_{\text{Achtman}}$ have been described (https://pubmlst.org/). When different STs are closely related (differing for instance by only one mutation on one of the seven genes), they are grouped in Clonal Complex (CC). For example, the CC32 which include the representative STEC O145:H28, is consisting of several ST such as ST32, ST137, ST6130, and ST8625 (Rodwell et al., 2022). In bacterial infections and epidemiological studies, specific clonal complexes can be responsible for outbreaks and spread of infection: the ST131 of *E. coli* is a known pathogenic clone that spreads worldwide causing urinary tract infections and bloodstream infections (Ben Zakour et al., 2016; Park et al., 2012; Petty et al., 2014).

The recent development of faster and cheaper Whole Genome Sequencing (WGS) technology and analysis led to the introduction of the cgMLST (core-genome MLST) approach. cgMLST uses a multigene approach that targets the entire core genome, allowing more detailed relationships between strains to be revealed (Quainoo et al., 2017). On the other hand, if more extensive genomic information is required, pangenome MLST, which includes both core genome and accessory genes, such as virulence or antibiotic resistance genes can be performed (Do et al., 2024).

1.3.6 Single Nucleotide Polymorphism (SNP)

SNP-based methods are one of the most resolution genomic methods and can be divided into those that use a reference genome or do not (kSNP) (Gardner et al., 2015). Since focusing on single nucleotide differences between strains, these methods are

particularly useful for elucidating the evolutionary background of closely related lineages and for detecting outbreaks (Den Bakker et al., 2011; Octavia and Lan, 2010). In addition, MLST does not include intergenic regions, which prevents it from detecting host-specific SNPs located in these regions. In contrast to MLST that does not include intergenic regions, SNP-based methods can identify host-specific SNPs located in these intergenic regions as well (White et al., 2011). However, unlike MLST, SNP-based methods lack an internationally standardized database, with diverse algorithms further making them less suitable for cross-laboratory comparisons (Pearce et al., 2018). Single reference genomes are commonly used to detect SNPs, enabling the identification of SNPs in genes, loci, and intergenic regions within a query genome (Harris et al., 2010; Turabelidze et al., 2013). This approach relies on algorithms that align raw sequence reads to a reference genome, allowing for a certain degree of variation between the reads and the reference. However, it has inherent limitations, particularly the potential exclusion of lineage-specific regions absent in the reference genome, which can restrict both the results and their interpretation. As a result, SNP-based methods are most effective when a high-quality reference genome is available (Quainoo et al., 2017).

2. Enteropathogenic, Shigatoxigenic and Enterohemorrhagic *Escherichia coli*

2.1 Enteropathogenic *Escherichia coli* (EPEC)

2.1.1 Definitions

As explained in the foreword, the acronym EPEC was at first used in a broad meaning, including all *E. coli* responsible for diarrhea and was therefore a synonym of DEC. For instance, EPEC were reported in England in 1945 as a cause of infantile watery diarrhea without blood in the stools, but without of course any pathotype identification (Bray, 1945). Nevertheless, the meaning of EPEC progressively narrowed with the description of different virulence factors and the definition of more and more pathotypes and virulotypes. Today, EPEC is only one of the many pathotypes of DEC and includes *E. coli* strains whose main pathogenicity property is the production of the histological lesion on intestinal epithelial cells, called Attaching and effacing (A/E) lesion that can be reproduced on epithelial cell lines in culture (Frankel, 1998). The A/E lesion is

characterized by the disappearance of the brush border microvilli and by the intimate attachment of the EPEC to the bare cytoplasmic membrane of the small intestine enterocytes. The A/E lesion formation involves genes located on a Pathogenicity island (Pai), the Locus of Enterocyte Effacement (LEE), an exogenous genomic region acquired by horizontal-transfer and coding a.o. for a Type III Secretion System (T3SS). The LEE will be described in more details on section 2.3.

2.1.2 EPEC infections in humans and animals

EPEC infects humans, especially infants in developing countries, and causes clinical symptoms such as abdominal pain and non-bloody diarrhea accompanied by vomiting, while immune-competent adults rarely show the above symptoms when infected (Fischer Walker et al., 2012; Ochoa and Contreras, 2011). EPEC is capable of infecting not only humans but also a variety of mammals. For example, it has been reported to cause diarrhea in calves (< 3 months old), lambs, post-weaning piglets, poultry, rabbits, and even pet animals such as dogs and cats, resembling infections seen in humans (Dutta et al., 2011; Mainil and Fairbrother, 2014; Puño-Sarmiento et al., 2013; Renzhammer et al., 2024; Wani et al., 2004). Some EPEC strains have host specificity, such as serotype O127:H- in humans and serotype, O15:H- and O103:H2 in rabbits, O26:H11, and O111:H- particularly in young calves (Mainil and Fairbrother, 2014; Newton et al., 2004; Tozzoli and Scheutz, 2014). On the other hand, most other EPEC strains are non-host specific serotypes (see next section), and some have been reported to belong to O serotypes (H antigen is described in some cases), such as O26:H11, O103:H2, O111:H8, O113, O145:H28/H34, and O157:H45/NM which are known to be frequent serotypes of EHEC (Habets et al., 2020; Makino et al., 1999; Kinnula et al., 2018; Rodwell et al., 2022; Tóth et al., 2009; van Hoek et al., 2019). Although it might not be possible to delineated all of them even today but represent the abundance of pathogenic types in EPEC.

2.1.3 Typical and atypical EPEC

In 1995, EPEC were divided into 'typical EPEC (t EPEC)' and 'atypical EPEC (aEPEC)' on the basis of presence or absence of the 'EPEC Adherence Factor' plasmid (pEAF) (Nataro et al., 1987), that is present in tEPEC, but not in aEPEC (Kaper, 1996). pEAF carries different genes involved in the early steps of the pathogenesis of tEPEC, including the *bfp* gene cluster encoding a type IV Bundle-Forming Pili (BFP) (Girón et al., 1991). Therefore, aEPEC can be defined as EPEC that form the A/E lesion, but do not express BFP. Several strains have been identified belonging to both typical and atypical EPEC. The most studied EPEC belong to the following 12 O serotypes (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158) with more or less specific associated H antigens (Campos et al., 2004; Deborah Chen and Frankel, 2005; Trabulsi et al., 2002). (**Table 3**)

Table 3. Frequent O:H serotypes of *Enteropathogenic Escherichia coli* (EPEC) and the presence or absence of the *bfp* gene cluster encoding type IV Bundle-Forming Pili (BFP), distinguishing typical and atypical EPEC, along with primary hosts. Adapted from Trabulsi et al., 2002.

Pathotype	*eae*/*bfp*	Frequent O:H serotypes	Primary host
Typical EPEC	+/+	**O55**:[H6], **O86**:H34, **O111**:[H2], **O114**:H2,**O119**:[H6],**O127**:H6, **O142**:H6,	Only Humans
Atypical EPEC	+/-	**O26**:H[11], **O55**:[H7],**O86**:H8, **O111**ac:[H8],**O111**:H25, **O119**:H2, **O125**ac:H6, **O128**ab:H2	Humans and mammals .O26,O111: young calves .O111 : Monkeys .O128 : Rabbits, dogs

*__Bold__: EPEC O serotypes recpgnized by WHO, 1987.

As far as the host range is concerned, tEPEC are mainly hosted by human, including both adult and infants and are very rarely isolated from animal hosts (Mainil and Fairbrother, 2014; Nataro and Kaper, 1998; Trabulsi et al., 2002). In contrast, aEPEC have been isolated from a wide range of diseased mammals, including not only humans, but also livestock such as cattle (calves), swine, sheep, buffaloes (including calves), rabbits, monkeys, pets (dogs and cats) and some wildlife (Awad et al., 2020; Beraldo et al., 2023;

Moura et al., 2009; Watson et al., 2017). The wide host range of aEPEC suggests that they represent a potential zoonosis, with the main route of transmission to humans being fecal-oral via water and foodstuffs contaminated with animal feces (Fairbrother and Nadeau, 2006).

Since tEPEC are not the topic of this work, EPEC will be used synonymously to aEPEC in this manuscript for the sake of clarity.

2.2 Shigatoxigenic and Enterohemorrhagic *Escherichia coli*

2.2.1 Definitions

Shigatoxigenic, or Shiga-toxin producing *Escherichia coli* (STEC) produce a potent and specific cytotoxin that destroys the vascular endothelium of small arteries and capillary vessels in some organs, first identified in *Shigella dysenteriae* type I: the Shiga toxin named after Dr. Kiyoshi Shiga (Lampel et al., 2018). Shiga toxins will be described in details in section 2.3, along with the other virulence factors of EPEC and STEC.

Different virulotypes of the STEC pathotypes have been described in the course of time. Some STEC form A/E lesions on enterocytes along with the production of a Shiga toxin and are associated with more severe gastrointestinal diseases, such as hemorrhagic colitis (HC), hence the term EnteroHemorrhagic *Escherichia coli* (EHEC), with hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) as sequelae. The name EHEC is, today regarded as obsolete by the European Food Safety Agency (EFSA BIOHAZARD Panel Koutsoumanis et al., 2020). Therefore, the name Attaching-Effacing Shigatoxigenic *E. coli* or AE-STEC was proposed (Piérard et al., 2012) for these hybrid STEC and will be used in this manuscript.

2.2.2 (AE-)STEC infections in humans

While EPEC are mainly associated with diarrhea in infants in developing countries, STEC are one of the major causes of bacterial food poisoning in humans, involving both in infants and adults in developed countries. Actually, AE-STEC is today one of the most serious global burden of diseases and was first identified in 1982 in a patient with hemorrhagic enteritis (Riley et al., 1983). Estimated total infections exceeded ONE

MILLION in the 2000s (Havelaar et al. 2015), often resulting in outbreaks in many countries worldwide. The largest outbreaks of AE-STEC were caused by serotype O157:H7, which spread in 1993 from undercooked beef served in hamburger restaurants in the USA and in 1996 from school lunches at elementary schools in Sakai City, Osaka, Japan, resulting in many patients and fatalities. Both two strains (the strain name: 'EDL933' in USA, 'O157 Sakai' in Japan, respectively) were identified by whole-genome sequencing in 2001 and are still used in many studies as the main O157:H7 reference strains (Hayashi et al 2001: Perna et al 2001). The latest and large outbreaks caused by O157:H7 were reported in USA, in 2024. Epidemiologic and traceback data revealed that resh, slivered onions served at McDonald's were the likely source of this outbreak (https://www.cdc.gov/ecoli/outbreaks/investigation-update-e-coli-o157-2024.html). Due to the outbreaks, total 104 people with 14 states were infected and onset HUS in 4 patients and one died.

In European countries e.g. in Belgium and in United Kingdom, outbreaks due to O157:H7 sometimes have been reported (Cunningham et al., 2024; Gobin et al., 2018; Robaeys et al., 1987), as were the cases in Japan and the USA, while cases of AE-STEC (EHEC) infections due to other O:H serotypes are reported annually (EFSA, 2022). The most frequent seven AE-STEC serotypes detected and implicated in serious diseases worldwide are O26:H11, O45:H2, O103:H2, O111:H-, O121:H9, O145:H-, O157:H7, and sometimes O165:H25 (Auvray et al., 2023; Beutin and Fach, 2014; Karmali et al., 2003; Tozzoli and Scheutz, 2014). Although their frequency of isolation varies between countries and regions, they are known as the 'Top 7 serotypes' (‘gang of seven’) and are under worldwide vigilance (CDC, 2018; EFSA, 2021). Other AE-STEC serotypes are qualified as minor serotypes (Beutin et al., 2004; Miko et al., 2009).

The most frequent symptoms caused by AE-STEC infections in humans are gastrointestinal disorders with diarrhea. However, much more severe symptoms can be often observed, such as HC characterized by abdominal pain and bloody diarrhea, as seen in patients infected with O157:H7. Serious sequelae have of AE-STEC infections have also been described: HUS in ca. 10 % of the patients, characterized by the triad of renal failure, hemolytic anemia and thrombocytopenia, is particularly prevalent in groups with incomplete or impaired immune functions, like infants, elderlies and

immunocompromised individuals. HUS itself was first described in 1955 (Gasser et al., 1995), but it was not until the early 1980s that Shiga toxin-producing *E. coli* were found to cause HUS (Karmali et al., 1985). HUS onset is most often associated with major AE-STEC serotypes of the 'gang of seven', mainly O157:H7 and O26:H11.

However, vice versa, the 'gang of seven ' serotypes are certainly not the only threat. Different minor AE-STEC serotypes (e.g. O118:H6, O128:H2, O146:H21, and O178:H19.. ; Beutin et al., 2004; Buvens et al., 2010) and other STEC strains (i.e. non-O157 STEC, like the O91:H21, O113:H2 STEC serotypes; Beutin et al., 2007; Bielaszewska et al., 2009) can also cause HUS, though less frequently (Karmali et al., 2003).

In addition, non-AE-STEC can also produce HUS and sometimes diarrhea depending on their actual virulotype. This was dramatically confirmed in 2011 by a major outbreak in Germany caused by a largely unrecognized Agg-STEC serotype, O104:H4. Since this serotype did not possess *eae* gene, which is the marker of EPEC, O104:H4 cannot describe as AE-STEC or EHEC (Piérard et al., 2012). In this outbreak, nearly 4,000 people were infected of whom 900 developed HUS and 54 died (Frank et al., 2011; Ibarra et al., 2013). Still other STEC like the Saa-STEC can also cause HUS in humans. This type of STEC has been identified several STEC serotypes, such as O91:H21, O113:H21(Paton et al., 2001; Krause et al., 2018). Thus, regardless of their isolation frequency and virulotypes, all STEC can cause HUS or other severe symptoms, and their monitoring is of the upmost importance in world public health.

2.2.3 (AE-)STEC as a zoonosis

If EPEC human infections are potential zoonoses, (AE-)STEC infections are well recognized zoonoses. The routes of transmission of (AE-)STEC to humans can be diverse, with the most important being fecal-oral transmission via meat, fruit, vegetables, fresh food and water contaminated with feces of healthy animals carrying the (AE-)STEC in their intestines. Of all animal species, domestic ruminants, especially cattle, are well known as the major asymptomatic carriers of (AE-)STEC (Tozzoli and Sheultz, 2014) that are associated with the lymph follicles and squamous cells of the gastrointestinal tract,

particularly of the colon, rectum, and recto-anal junction, but can also be found in segments part of the small intestine, like the ileum, and occasionally in the gallbladder (Menge, 2020b). The prevalence of (AE-)STEC serotype in each ruminant have been studied, O157 (AE-)STEC is isolated from cattle, while non-O157 (AE-)STEC serotypes are also associated with cattle including diarrheic calves. For example, serotype O26 and O111 are well-known ones (Bettelheim, 2000; Girard et al., 2007; van Diemen et al., 2005). Ruminants other than cattle, such as sheep or goat, can also excrete different (AE-)STEC serotypes in their feces (Ndegwa et al., 2022; Vu-Khac and Cornick, 2008). Furthermore, non-ruminants have also been reported as potential reservoirs of (AE-)STEC. For example, (AE-)STEC serotypes including O157, non-O157 and other emerging have been isolated from poultry and pig feces (Al-Marri et al., 2021; Elsayed et al., 2021; Meng et al., 2014; Wieczorek and Osek, 2020)

Moreover, wildlife, more especially wild ruminants, can also be carriers of (AE)-STEC, although the number of reports are fewer than for cattle and other domestic animals due to differences in the study populations, difficulty to tracking, attention levels and frequency of human contacts. These reports include terrestrial wildlife such as red deer, red fox and wild boar, and aquatic animals such as otters (Nüesch-Inderbinen et al., 2024; Ray and Singh, 2022). However, since deer was associated with several outbreaks caused by both O157 and non-O157 (AE-)STEC, they are also considered as one of the important reservoirs of (AE-)STEC (Laidler et al., 2013; Rounds et al., 2012; Singh et al., 2015).

2.2.4. (AE-)STEC infections in animals

In addition to the carrier state in different animal species, (AE-)STEC is also a cause of infection in some of them. For example, the edema disease in weaned pigs is caused by non-AE F18-STEC (García et al., 2020). Moreover, just like EPEC, (AE-)STEC can cause diarrhea in young calves and lambs (Mainil and Fairbrother, 2014; Mainil and Daube, 2005; Mokhbatly et al., 2022). Conversely, (AE-)STEC in association with disease in other animal species have not been frequently reported, but recent study announced the relation between dogs and acute colitis (Nagao et al., 2024). Porcine F18-

STEC belong to host-specific O serogroup are such as O139, O147(Helgerson et al., 2006 ; Perrat et al., 2022), while the most frequent (AE-)STEC causing diarrhea in <three months of age calves can belong to major serotypes like O26:H11, O111:H-, and to minor serotypes, such as O5:H-, O118:H16 and have also been reported (Fakih et al., 2017).

2.2.5. Biochemical properties of EPEC and (AE-)STEC

EPEC and AE-STEC can of course be identified as *E. coli* species on the basis of their biochemical characters. Nevertheless, as explained earlier, some of them show a relatively specific profile in their sugar fermentation properties. For instance, the majority of (AE-)STEC serotype O157:H7, arguably the most notorious one worldwide, are unable to ferment sorbitol, in contrast to the other *Escherichia coli* strains. Thus, the specific "Sorbitol MacConkey agar" containing sorbitol in place of lactose and two selective agents (cefixim and tellurite; see sections 3) was developed and commercialized for their detection (Fujisawa et al., 2000; Karmali, 1989). Other more or less specific sugar fermentation profiles of (AE-)STEC non-O157 have been identified and specific growth media tested although not commercialized yet are concerned (Hiramatsu et al., 2002; Nakamura et al., 2023). Since most (AE-)STEC represent a public health hazard, the development of specific selective isolation media is one of the most urgent tasks for researchers.

2.3 Virulence properties of EPEC and AE-STEC

2.3.1 Initial Adherence

The first step in bacterial infection is a physical contact between the bacteria and the host cells or tissues, which in the case of EPEC and AE-STEC is adherence to the surface of the intestinal epithelial cells, the enterocytes. This requires specific or not adherence factors.

In the case of tEPEC, adherence occurs in part via the type IV BFP (Ramboarina et al., 2005), that are fimbriae forming typical braids. The BFP ensure not only adherence of the bacteria to the enterocyte surface, but also between bacteria allowing tEPEC to

form microcolonies at the gut surface. The BFPs are encoded by the *bfp* gene cluster, including *bfpA* gene coding the fimbrial major subunit, that is located on the EAF plasmid (Lee et al., 2022; McDougall et al., 2023). While pEAF is indeed a marker for tEPEC, it does not roll all aspects of initial adherence. Other factors, such as intimin and Tir, might involve in this process (Frankel et al., 1998).

Table 4. Initial adherence factors of pathogenic *Escherichia coli*, including typical/atypical enteropathogenic E. coli (tEPEC/aEPEC) and attaching–effacing Shiga toxin-producing *E. coli* (AE-STEC), showing factor names, abbreviations, associated pathotypes, and primary hosts. Adapted from Buvens et al., 2010

Adherence factors	Unabbreviated name	*E. coli* pathotype	Host
Bfp	Bundle forming pili	**tEPEC**	**Humans**
AF/R1, AF/R2	Adhesive factor for Rabbit	**aEPEC**	**Rabbits**
Ral	Rabbit EPEC adherence locus		
F18-Fimbriae		**ETEC**	**Pigs**
Saa	STEC autoagglutinating adhesin	**STEC**	**Humans Cattle/Pigs**
Spf	Sorbitol fermenting protein	**EPEC**	**Humans Cattle**
Efa/lifA	EHEC factor for adherence/lymphocyte inhibitory factor A		
ToxB			
Lpf	Long polar fimbriae	**AE-STEC (EHEC)**	
F9-Fimbriae			
Paa	Porcine attaching effacing associated adehesin		**Humans Pigs**
AIDA	Adhesin involved in diffused adherence		

Since the lack of pEAF unlike tEPEC, the marker for initial adherence of aEPEC and AE-STEC have not been clearly defined. Several putative adherence factors have been proposed in previous studies, including AF/R1 and R2 (Adhesive factor/Rabbit), Efa1/lifA (the EHEC factor for adherence 1/lymphocyte inhibitory factor A), LdaG (The locus of diffuse adherence), LpfO113 (long polar fimbriae), Paa (porcine attaching and effacing-associated fimbriae), Saa (STEC autoagglutinating adhesin), and ToxB (Afset et al., 2006; Bardiau et al., 2010; Hernandes et al., 2009; Paton et al., 2001; Slinger et al.,

2017) (**Table 4**).

Among these factors, the Iha (IrgA Homologue Adhesin) protein is noteworthy. This adherence factor, encoded by a chromosomal gene located on the Sakai Prophage-Like Element-1 (SpLE-1), resembles the Iron Regulatory Gene A (IrgA) adhesin of Vibrio cholerae. SpLE-1, an approximately 90 kbp prophage-like element initially identified in the AE-STEC O157:H7 Sakai strain, also harbors various other genes, as described in section 2.3.4 (Tarr et al., 2000; Hayashi et al., 2001). In addition, different adherence patterns have also been identified *in vitro*, to epithelial cells in culture (Knutton et al., 1989; Trabulsi et al., 2002). For instance, BFP confer a Localized Adherence (LA) patterns to tEPEC (Scaletsky et al., 1984). On the other hand, aEPEC and AE-STEC likely to show more diversed patterns, Diffuse Adherence (DA) or an LA-like (LAL) (Rodrigues et al., 1996; Scaltsky et al., 1984; Trabulsi et al., 2002; Vieira et al., 2001).

2.3.2 The Locus of Enterocyte Effacement (LEE)

2.3.2.1 Description

As briefly introduced earlier, the genes required for the generation of the A/E lesion by tEPEC O127:H6 E2348/69 are located on a 35.6 kbp (35,6296 bp) exogenous genomic region, a Pathogenicity Island (Pai), known as the Locus of Enterocyte Effacement (LEE) (Elliott et al., 1998; McDaniel et al., 1995). The LEE consists of 41 ORFs, distributed in five functional polycistronic operons: LEE1, LEE2, LEE3, LEE5, and LEE4 (Deng et al., 2004; Ruano-Gallego et al., 2015; Schmidt and Hensel, 2004; Stevens and Frankel, 2014) (**Fig. 3**).

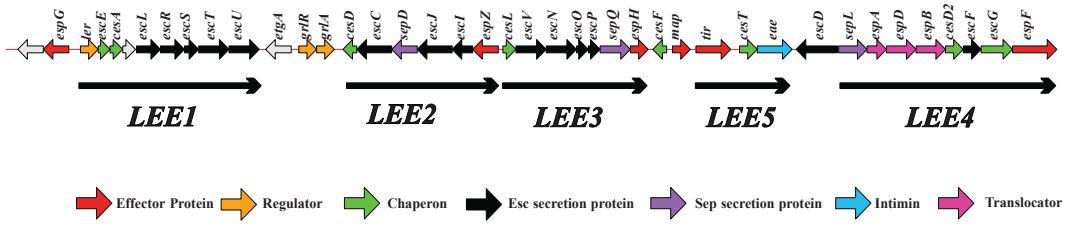

Figure 3. General organization of the *Locus of Enterocyte Effacement* (LEE) pathogenicity island, showing five operons (LEE1–LEE5) and their encoded components, including regulators, chaperones, effector proteins, secretion system proteins, intimin, and translocators. Adapted from Deng et al., 2014; Ruano-Gallego et al., 2015; Stevens and Frankel, 2014.

LEE1, LEE2, and LEE3 contain a group of *esc* genes encoding key components of the type III secretion system, and a group of *sep* genes encoding secretion of *E. coli* protein; LEE1 also possesses the LEE regulatory gene (*ler*), which acts as the main regulator of the LEE2, LEE3, LEE5, and LEE4 region (Franzin and Sircili, 2015; Mellies et al., 1999); LEE 5 contains the *eae* gene and a translocated intimin receptor (*tir*) gene, encoding the bacterial outer membrane INTIMIN adhesin and its translocated receptor, Tir, respectively (Jerse et al., 1990; Kenny et al., 1997). The Intimin protein is not Type3- but Type2-secreted. Finally, LEE4 contains the *espA*, *espB*, *espD*, *espF*, and *sepL* genes, encoding the translocator proteins (EspA, EspB, EspD), *E. coli* secreted protein (EspF), and secretion proteins (SepL) respectively. These 'effector proteins' are involved in the translocation of virulence factors into host cells through the Type III secretion system (T3SS) (Deng et al., 2015; Sekiya et al., 2001; Franzin and Sircili, 2015).

Not only EPEC but also AE-STEC possess LEE, which was first detected and sequenced in AE-STEC O157:H7 strain EDL933 (Perna et al., 2001). This LEE locus is inserted at the same site (the *selC* tRNA loci) as the LEE region of EPEC strain E2348/69, and the 41 ORFs shown earlier are common to these two pathogenic types. Nevertheless, the LEE region of the AE-STEC strain EDL933 contains a further 13 ORFs. Some

proteins encoded by LEE-located genes of these two tEPEC and AE-STEC have high sequence amino-acid identity (>90%), although others (e.g. EspB, EspD, EspA, Intimin, Tir) have sequence identity ranging from 66% to 84%, indicating sequence diversity between these two different strains and pathotypes (Schmidt and Hensel, 2004). Moreover, the characteristics of the LEE regions which have been found in other EPEC and AE-STEC strains can also be different according to the strains and pathotypes (**Table 5**).

Table 5. Characteristics of the *Locus of Enterocyte Effacement* (LEE) region identified in representative Enteropathogenic *E. coli* (EPEC) and Attaching-Effacing Shiga Toxin-producing *E. coli* (AE-STEC) strains, including O:H serotypes, insertion sites, and number of predicted open reading frames (ORFs). Adapted from Ogura et al., 2009; Zhu et al., 2001.

Strain name	*E. coli* pathotype	O:H serotype	Insertion site	Number of ORF
EDL933	AE-STEC	O157:H7	*SelC*	54
E2348/69	EPEC	O126:H7	*SelC*	41
RDEC-1	EPEC	O15:H-	Between IS2 and lifA gene	40
413189-1/O26-11368	EPEC/AE-STEC	O26:H11	*PheU*	Unknown
B171/O111-11128		O111:H-	*PheV*	
RW1374/1372		O103:H2	*PheV*	

Sequence variation is particularly notable in the Intimin protein and *eae* gene. As said above, the Intimin is a bacterial outer membrane protein that interacts with the Translocated Intimin Receptor (Tir) effector protein to promote the adherence of the bacteria to the cytoplasmic membrane of the host intestinal epithelial cells after destruction of the enterocyte microvilli (Paton et al., 1998). These intimin variants are characterized by amino acid sequence variations in the C-terminal region called the D3 domain. The intimin protein and the *eae* gene variants are named with Greek letters. The following *eae* gene variants have been described and named: α, β, γ, ε, ξ, z ,η, θ, τ, ι, κ, λ, μ, ν, υ, ο, π, ρ and σ, but some of these variants have nearly identical sequences (e.g. *eae* α and *eae* ξ, *eae* β2 and *eae* δ, *eae* γ2 and *eae* θ) and considered to be of the same type of *eae* in some cases (Adu-Bobie et al., 1998; Blanco et al., 2004; Zhang et al., 2002).. To

some extent they are associated with the pathotype (EPEC or AE-STEC) and to the serotypes (Bibbal et al., 2014; Blanco et al., 2005; Wang et al., 2023; Yang et al., 2020). The sequence variations in the other genes will be described in the next section about the Type III secretion system.

2.3.2.2 The Type III Secretion System.

The Type III Secretion System (T3SS) is a needle-like secretion system that allows Gram-negative bacteria to transfer effector proteins directly into the host eukaryotic cells. The T3SS structure is a key element for bacteria to evade host infection defense mechanisms and facilitate the infection process (Kubori et al., 1998). In EPEC and AE-STEC, the T3SS itself is encoded by genes located on the LEE, while T3-secreted effectors are encoded by genes located also on the LEE or on the non-LEE chromosomal regions (Dean and Kenny, 2009) (**Table 6**).

Table 6. Virulence genes associated with the Type III secretion system (T3SS), located in the LEE region, non-LEE regions, or other genomic locations in *Escherichia coli*, with their localization and main cellular functions adapted from Dean and Kenny, 2009; Dziva et al., 2007.

Gene name	Unabbreviated name	Localization	General cellular functions
espA	*E. coli* secreted protein	Choromosome LEE region	Signal transduction to host cell
espB			Translocation of Tir
espD			Induced apoptosis
			Microvilli effacement
espF			Tight junction disruption
map	Mitochondria associated protein		Mitochondoria dysfunction Microvilli effacement Tight junction disruption
tir	Tranclocated intimin receptor		Formation of A/E lesion, pedestal Receptor for Intimin
eae	Intimin		Formation of A/E lesion, pedestal Intimin adhesin protein
espH	*E. coli* secreted protein		Cytoskeleton disruption Modulating actin dynamics
espZ			Preventing protein translocation
espG			Micotubule disruption
nleA	Non LEE-located effector	Choromosome Not LEE region	Inhibition of secretion pathway
nleB			Inhibition of immune response
nleC			
cif	Cycle inhibition factor		Preventing cell cycle progression Induced apoptosis
espJ	*E. coli* secreted protein		Anti-phagocytosis
espC	*E. coli* secreted protein(EPEC)		Cleavin host protein
espP	Serin protease	plasmid(pO157)	Epitherial jucation disruption

The Esc (*E. coli* secretion proteins) encoded by LEE1-2-3-located genes form three main components of the T3SS: (i) the 'needle complex' (EscC, EscD, EscF) located in the outer membrane; (ii) the 'transport apparatus' (EscR, EscS, EscT) located in the inner membrane; and (iii) the 'sorting platform' (EscA, EscK, EscL) present in the cytoplasm. These three components allow bacteria to efficiently transfer effector proteins into the host cell (Gaytán et al., 2016).

The Esp (*E. coli* secreted proteins) proteins also encoded by LEE1-2-3-located genes are the first T3S-secreted proteins, but do not enter into the host cells. Nevertheless, they play important complementary roles in the T3SS : (i) the EspA forms part of the needle complex and aids the transport of effector proteins (Kenny et al., 1996); (ii) the EspB and EspD form pores in the host cell membrane, through which the effector proteins enter into the cell (Abe et al., 1997; Chatterjee et al., 2015); (iii) the EspB also has a role in evading the host immune response by inhibiting macrophage phagocytosis (Iizumi et al., 2007).

LEE-encoded effector proteins injected into host cells include Tir (Translocated intimin receptor), EspF (*E. coli* secreted protein), EspG and Map (Mitochondria associated protein). The genes encoding these effectors are located within also the different LEE region: *tir* is located in LEE5, *espF* is in LEE4 while *espG* (rorf2) is located 5' of LEE1, and *map* (orf19) is 5' of LEE5 (between LEE 3 and LEE5) (Elliot et al., 1998; Kenny and Jepson, 2000; Stevens and Frankel, 2014). Their role is to re-organise the actin fiber network of the host cell cytoskeleton leading to the effacement of the enterocytes microvilli and, sometimes the formation of a pedestal structure. Tir also integrates into the host cell cytoplasmic membrane and interacts with the outer membrane bacterial Intimin adhesin promoting bacterial to adhere tightly to the enterocytes cytoplasmic membrane at the height of the pedestal when present. EPEC and AE-STEC can multiply on site forming microcolonies and later allowing the infection to spread to other sites (Devinney et al., 2001; Goosney et al., 1999).

At the molecular level, different biochemical pathways resulting from different

interactions between bacterial effectors and host cell cytoskeleton have been described in different serotypes of EPEC and AE-STEC, but are beyond the scope of this work. Ultimately all pathways result in the huge reorganization of the actin fiber network of the host cell cytoskeleton leading to the effacement of the microvilli and to the formation of actin-rich pedestals under the intimately attached bacteria.

Finally, other effector proteins such as NleA (non-LEE-encoded), NleB, NleC, EspP (*E. coli* secreted protein), EspJ and Cif (Cycle inhibiting factor), which are encoded by non-LEE located genes also contribute to the formation of the AE lesion and spread of infection, such as pedestal formation, actin re-arrangement, microtubule and epithelial barrier disruption, and suppression of immune response (Gao et al., 2013 ; Sham et al., 2011). However, they are not produced by all EPEC and AE-STEC (Loukiadis et al., 2008).

This chain of interactions of the T3SS of EPEC and AE-STEC with enterocytes cause intestinal inflammation and non-bloody diarrhea (Dean et al. 2006; Iizumi et al. 2007). Bloody diarrhea can be observed in humans, but is the results of interactions of Shiga toxins with the intestinal tract. Let us now describe the *E. coli* Shiga toxins.

2.3.3 Shiga toxins

A number of virulence factors possessed by *E. coli* and other pathogenic bacteria are encoded in genes located on bacteriophages and most Shiga toxins, the main virulence factor of STEC, are encoded by such phage-located genes (Wagner and Waldor, 2002).

2.3.3.1 Early description

In 1977, Konowalchuk and collaborators from Canada observed the production of a cytotoxin causing death of African green monkey kidney-derived Vero cells (Vero Toxin: VT) (Konowalchuk et al., 1977), but this did not attract much attention at the time. In 1982, O'Brien and collaborators from the USA further analyzed the cytotoxin produced by *E. coli* H30 strain (O26:H11) using HeLa cell, already studied by Konowalchuk and collaborators: this H30 cytotoxin has structure and biological activity similar to the Shiga

toxin produced by *Shigella dysenteriae* type I. O'Bien and collaborators named this *E. coli* cytotoxin, Shiga-Like Toxin (SLT) (O'Brien and Holmes, 1987). For many years, there were disputes about how to name this cytotoxin (Verotoxin or Shiga-like toxin), until a consensus reached to keep the name Shiga toxin (Stx) or *E. coli* Shiga toxin. At the same time, Scotland and collaborators from United Kingdom reported that the *stx* genes encoding the *E. coli* H19 strain (O26:H11) Stx, but not the *S. dysenteriae* Stx, could be horizontally transmitted by bacteriophages (Scotland et al., 1983).

2.3.3.2 The E. coli Stx families

A few years after the description of the *E. coli* H30 strain Stx, another immunologically unrelated Stx type was described: the former was renamed Stx1, and the latter was named Stx2 (Calderwood et al., 1996). Even later, different antigenic and genetic subtypes were identified within these two types that are today named 'families' and will be described hereunder.

All four *E. coli* Stx1 subtypes described so far (Stx1a, Stx1c-e) are genetically almost identical to the *S. dysenteriae* Stx. The amino acid sequence differences between the Stx and these four Stx1 subtypes are negligible and are not thought to affect antigenicity and cytotoxicity (Paton and Paton, 1998). Of the four Stx1 subtypes, Stx1a and Stx1c are often identified in STEC from human clinical and animal samples, including ruminants, commonly sheep or wild deer (Boer et al., 2015; Brett et al., 2003; Buven et al., 2012 ; Hofer et al., 2012 ; Fierz et al., 2017), while Stx1d or Stx1e are rarely reported in human diseases. If any, the symptoms would be mild (Melton-Celasa, 2014). Stx1d have also been identified in STEC from flour and wild deer in some European Countries (Hofer et al., 2012; Kindle et al., 2019a; Projahn et al., 2021). Apart from other subtypes, Stx1e was first detected in an *Enterobacter cloacae* isolated from an HUS patient in 2014 (Probert et al., 2014) and there are still very few case reports of Stx1e-positive *E. coli* from any examined samples (McMahon et al., 2024).

Conversely, Stx2 are relatively highly different from Stx and from the *E. coli* Stx1 family. The original *stx2* gene for instance has only about 60% sequence identity to the original *stx1* gene (**Table 7**). Another main difference between the Stx1 and Stx2 families

is that up-to-day 14 Stx2 subtypes have been described, some quite highly genetically related, but others much lowlier. For many years there was a lot of confusion in the names given to these subtypes, with no actual rules. But today the consensus nomenclature identified 15 Stx2 subtypes with latin small letters: Stx2a to Stx2o (Lindsey et al., 2023; Scheutz et al., 2012).

Table 7. The primary molecular differences between Stx1 and Stx2: DNA sequence identity, primary host, contanimation soure, cross-protecitve, and receptor adapted from Melton-Celsa, 2014 and Menge, 2020a.

	STX1	**STX2**
DNA homology vs STX of *S.dysenteriae*	Almost identical	50~60% identity
Primary host	Mammals including humans ***Stx2e subtype is specific to swine**	
Primary contamination source	Ruminants (especially cattle)	
Cross-protective	Partially occur, but Not between STX1 and STX2	
Receptor	Gb3/CD77 ***Stx2e subtype:Gb4**	

At the beginning, the Stx2 was recognized as the most common toxin among (AE-)STEC with the Stx2a, Stx2c and Stx2d subtypes being more frequently isolated from STEC-infected humans especially suffering of HUS (EFSA BIOHAZARD Panel Koutsoumanis et al., 2020: Alotaibi and Khan, 2023). In contrast, some subtypes such as Stx2b, Stx2e, Stx2f and Stx2g are relatively rare in humans and are more frequently associated with diseased or healthy animals (Farooq et al., 2009: Luna-Gierke et al., 2014: Hughes et al., 2019; Schmidt et al., 2000).

Of them, the Stx2b is often isolated from animals, and sometimes found in flour, like Stx1e subtype (Kindle et al., 2019a; Nüesch-Inderbinen et al., 2024). The Stx2e is most well-known, since it is associated with the edema disease in pigs (Hamabata et al.,

2021). Actually, this subtype was already described in the 1950s under the name ‘Edema Disease Principle’ (Timoney, 1950; Dobrescu, 1983) but was rediscovered by O’Brien and collaborators in the 1980s and renamed Stx2e (Weinstein et al., 1988). Stx2e has also been described in a few STEC isolated from human, but the gene localization and the *E. coli* serotype are different (Beutin et al., 2004; De Rauw et al., 2018; Piérard et al., 2012). Stx2f is often isolated from pigeons and other birds (Friesema et al., 2014: Schmidt et al., 2000; Yang et al., 2024). Before, this Stx2f subtype was not associated with serious diseases in humans and was therefore considered to have a less serious impact on humans. However, recently it has been increasingly isolated from HUS patients, mainly in Europe, alongside Stx2a, Stx2c and Stx2d, and could also become a public health concern in the future (Friesema et al., 2015; Cointe et al., 2020b). The Stx2h, Stx2i, and Stx2j were identified in STEC strains isolated from Tibetan marmots, crustaceans and also human patient, respectively, confirming the diversity of sources of Stx2 isolates (Bai et al., 2018: Gill et al., 2022; Lacher et al., 2016) The Stx2k in domestic animals such as goats and pigs, or meat derived from them. Moreover, Stx2k was also reported in human patients with diarrhea (Hughes et al., 2019). As well as Stx2k, the rest of four subtypes which recently identified and reported: Stx2l, Stx2m, Stx2n and Stx2o are also more associated with human patients (EFSA BIOHAZARD Panel Koutsoumanis et al., 2020; Lindsey et al., 2023; Yang et al., 2022)

Stx1 subtype-encoding genes can be identified by a generic PCR followed by sequencing of the amplified DNA fragments, while more or less specific PCR assays have been developed to recognized most of the different subtypes of Stx2-encoding genes (Johnson et al., 1990; Scheutz et al., 2012). Today of course whole genome sequencing renders this subtype identification much easier.

2.3.3.3 Molecular structure and in vitro and in vivo activity

Similarly, to the cholera toxin, Stx belong to the two-subunit A1-B5 family toxins, and the encoding operon has a common structure consisting of a single transcription unit of the A- and B-subunit encoding gene (*stxA* and *stxB*). The A-subunit is responsible for N-glycosidase-type activity, hydrolyzing and releasing an adenine residue from the 28

ribosomal RNA. As a result, binding of EF1-dependent aminoacyl-tRNA to the 60S ribosomal sub-particles is inhibited and the cellular protein synthesis is stopped. The five B subunits are involved in the binding of the toxin to its target receptor (Melton-Celsa, 2014; Paton and Paton,1998). (**Fig. 4**)

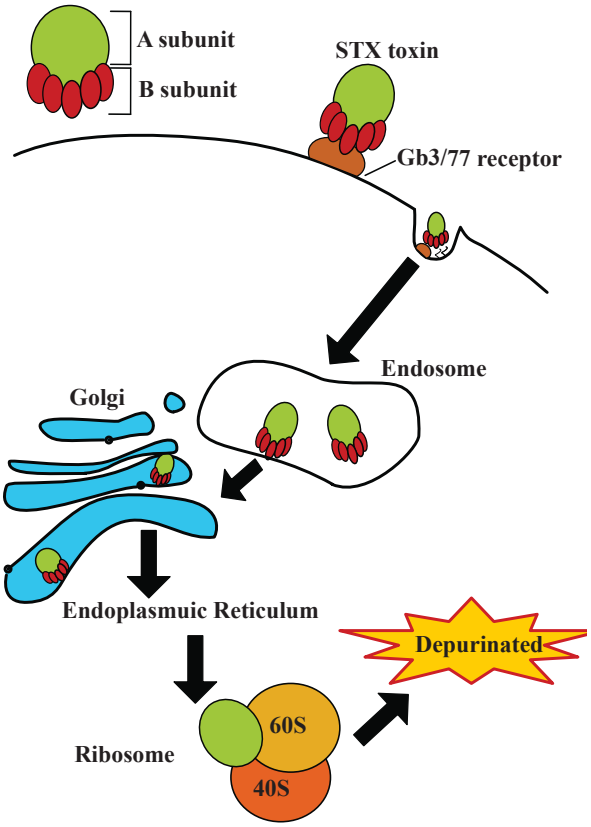

Figure 4. Schematic representation of Shiga toxin internalization and retrograde trafficking. The toxin binds to the Gb3/CD77 receptor via its B subunit, is internalized into endosomes, and traffics retrogradely through the Golgi apparatus to the endoplasmic reticulum. The A subunit is then translocated into the cytosol, where it cleaves a specific adenine from the 28S rRNA of the 60S ribosomal subunit. Adapted from Hall et al., 2017

The receptor of Stx1 and Stx2 is Gb3/CD77 <Globotriaosylceramide (Gb3) a listed as the CD77 leukocyte antigen>. (Melton-Celsa, 2014; Menge, 2020a), though their binding affinity is thought to be slightly different (Chark et al., 2004; Gallegos et al., 2012). Moreover, there is at least one exception: the Stx2e associated with the edema disease in swine does not bind to Gb3/CD77 but to Gb4 (Steil et al., 2016). Although the main receptors of other subtypes, such as Stx2a and Stx2c, which are associated with human infections, are recognized as Gb3/CD77, it has been reported that Stx2a exhibits weak interactions with Gb4 (Ching et al., 2002; Nakajima et al., 2001). However, performing precise studies remains challenging because these Stx receptors are not soluble (Melton-Celsa, 2014). In human body, the expression of the Gb3/CD77 receptor concentrates in vascular endothelial cells in intestinal tract or kidney (especially in cortical region), whereas in animals, the location of expression depends on species (Menge, 2020a) (**Table 8**).

Table 8. Comparative expression patterns of the Gb3/CD77 receptor in epithelial and endothelial cells from humans, cattle, and pigs. "+/+" indicates strong expression in both examined areas, "+/–" partial or absent expression in one area, and "Inconclusive" or "Unknown" indicates insufficient or unavailable data. Adapted from Menge et al., 2020a.

Organ / Host	Epithelial		Endothelial			
	Intestinal/Panet	Renal tubular/Glomeruli	Intestinal	Renal glomeruli	Blood vassels	Micro vascular
Humans	+/+	+/+	+	+	+	+
Cattle	Inconclusive	+/–	–	–	Inconclusive	
Pigs	Unknown	+/–	Unknown	+	Unknown	

At first Shiga toxin is produced in the intestinal tract, passing through the intestinal epithelium and epithelial cells, and is taken up into capillaries by transcytosis (Bauwens et al., 2013; Sandvig and van Deurs, 1996). Subsequently, it binds to the Gb3/CD77 receptor distributed in vascular endothelial cells, and endosomal vesicles containing the Shiga toxin are retrogradely transported to the endoplasmic reticulum via the Golgi. Finally, translocated to the cytoplasm, they induce the cell death by stopping the protein synthesis as described earlier (Lingwood, 1996; Obrig, 2010, Paton and Paton, 1998). This process damages the vascular wall, triggering an inflammatory response that promotes microvascular thrombosis (formation of small clots in the blood vessels), leading to ischemia and organ dysfunction. In the case of hemolytic uremic syndrome (HUS), Shiga toxins (Stx) enter the bloodstream, bind to receptors on endothelial cells, and induce systemic thrombotic microangiopathy (TMA), which particularly affects the kidneys (Kaname et al., 2024). While hemorrhagic colitis (HC) primarily results from localized effects of Stx on intestinal epithelial cells. These toxins cause epithelial damage, inflammation, and microvascular injury within the intestinal wall, leading to bloody diarrhea and abdominal pain (Griffin et al., 1990). Ruminants, particularly cattle, are the main reservoir of (AE)-STEC. However, clinical symptoms such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) have not been described in these animals. Indeed, previous studies have reported that Stx receptors are absent on bovine endothelial cells, and that Stx toxins do not perform transcytosis in enterocytes, thereby failing to reach the bloodstream (Pruimboom-Brees et al., 2000). Nevertheless, the presence and distribution of *stx* receptors in cattle have been frequently discussed, with some suggestions of species- or tissue-specific variations in receptor distribution (Hoey et al., 2002; Naylor et al., 2003; Van Diemen et al., 2005). For instance, Gb3/CD77 expression in the bovine intestinal epithelium has been reported to be associated only with crypt cells and to be lost during during their migration and differentiation towards the apical surface. Therefore, stable Gb3/CD77 receptor presence at sites relevant for mucosal and vascular injury is unlikely, which contributes to the absence of hemorrhagic colitis in cattle (Hoey et al., 2002). In addition, the same authors also reported that Gb3 isoforms differing from those found in humans are present in the bovine kidney, resulting in weaker Stx binding and an inability of the toxin to exert its full cytotoxicity. Therefore, the presence or the absence of Stx receptor of cattle to Shiga toxin has not been formerly concluded yet.

2.3.3.4 The STX phages

The different Stx-converting phages (STX phages) carrying the *stx* genes have similar features to lambda phage (Herold et al., 2004). Lambda phages are a type of bacteriophage known for being lysogenic, i.e. capable of incorporating their genomes into the chromosome and entering a temperate cycle by replicating along with the bacterial chromosome (Van Duyne and Landy, 2024). Under stress conditions (lack of nutrients, UV light, antibiotics, radiations…), lambda phage genome is excised from the chromosome and enter the lytic cycle leading to phage multiplication, bacterial cell destruction and phage release (Filipiak et al., 2020).

The expression of the genome of STX lambdoid phages can be divided into an early and a late genome region (Krüger and Lucchesi, 2015; Rodríguez-Rubio et al., 2021). The early region contains genes that are first expressed after phage infection and mainly encode proteins involved in phage self-replication in case of lytic cycle and recombination with the host bacterium in case of temperate cycle, like the recombination enzymes (integrase and excises) required for phage DNA integration into the host chromosome at specific sites (*attP* and *attB*) (De Greve et al., 2002; Landy and Ross, 1977). The late region contains genes that are not expressed when the phage genome is stably integrated into the host bacterial genome. In STX phages, the *stx* genes and their promoters are located in the late region and their expression is therefore repressed (Campbell, 1994; Krüger and Lucchesi, 2015; Smith et al., 2012).

After excision, the genes of the late region, including the *stx* genes are expressed and the production of Stx is initiated (Calderwood and Mekalanos, 1990; Wagner et al., 2001a). Thus, the shared roles of the early and late regions of STX phages are important for the regulation not only of phage replication, but also of *stx* gene expression. These sequential gene expressions explain why Stx are rapidly produced when the host bacterium is exposed to external stress, a.o. exposure to antibiotics used empirically to treat the diarrhea observed (Shaikh and Tarr, 2003; Wagner et al., 2001b). The consequence can be dramatic with possible development of an HUS syndrome in susceptible hosts (Freedman et al., 2016).

In 1971, Smith and Linggood reported that an enterotoxigenicity, which later became known as 'phage H-19B', could transfer *E. coli* K12 from *E. coli* O26:H11 in vitro (Smith and Linggood, 1971). Since then, many researches about transferring STX phage both i*n vitro* and *in vivo* have been conducted (Acheson et al., 1998; Bielaszewska et al., 2007; Cornick et al., 2006; Habets et al., 2022), and among these studies, the hypothesis that AE-STEC emerge from EPEC by horizontal transfer of *stx* genes also have been demonstrated (Tóth et al., 2003).

2.3.4 Other putative virulence factors and markers

The first main virulence factors of EPEC and of AE-STEC is the A/E lesion on intestinal epithelial cells and a second one for AE-STEC is the Shiga cytotoxins, as mentioned earlier. However, other putative virulence factors have been identified in EPEC and/or AE-STEC, that can be located on mobile genetic elements (Donnenberg and Whittam, 2001; Schmidt and Hensel, 2004).

For instance, in 2001, Hayashi and collaborators identified 18 prophages and 6 prophage-like elements in the genome of AE-STEC O157:H7 strain Sakai, which caused a major outbreak in Sakai, Osaka, Japan, and named the latter 'Sakai prophage-Like Elements' or SpLEs (Hayashi et al., 2001). Among these SpLEs, the SpLE-1 is approximately 90 kbp long and carries the tellurite resistance operon (*ter* operon) and the *iha* gene encoding the Iha adhesin (IrgA Homologue Adhesin) involved in adhesion to the host cells (see also section 2.3.1) and is integrated on *serX*-tRNA. SpLE-1 is also sometimes referred to as a Pathogenicity Island (Pai) or the Tellurite resistance and Adherence-conferring Island (TAI), and the terms ‘TAI’, ‘Pai’ and ‘SpLE-1’ are often treated almost synonymously (Schmidt et al., 2004). SpLE-1 is therefore an essential prophage-like element for resistance and virulence-conferring and has been identified in the genomes of AE-STEC of not only serotype O157:H7, but also of other major serotypes (O26, O103, O111 for instance) (Ogura et al., 2009). In EPEC strains, no clear studies have been reported, that SpLE-1-like element exist as in AE-STEC so far. However, several studies have shown that EPEC possess the *iha* gene, one of the SpLE-1 associated-gene, that confer adherence property similar to those of AE-STEC (Bardiau

et al., 2009; Gomes et al., 2011).

Another putative virulence factor of especially AE-STEC and some EPEC is the Enterohemolysin (Ehly) whose encoding genes (named *ehx* or sometimes still *hly*) was first described by Schmidt and collaborators on the plasmid pO157 in AE-STEC O157:H7 strain EDL933 (Beutin et al., 1988; Magalhães et al., 2011; Schmidt et al., 1995). The structural enterohemolysin-encoding *ehxA* gene is similar to the structural *hlyA* gene, which encodes the α-hemolysin (Hlyα). Ehly is involved in lymphocyte and erythrocyte cytolysis causing hemolysis on sheep blood agar after washing of the erythrocytes but is inactive on normal sheep blood agar in opposition to Hlyα. Specific blood agar for the detection of the production of an Ehly are commercially available. This hemolytic effect might contribute to the development of HUS (Aldick et al., 2007; Bielaszewska et al., 2014).

3. Resistance profile determinants in EPEC and (AE-)STEC

To survive in the host body and in the environment, pathogenic bacteria need to develop resistance mechanisms against harmful physico-chemical conditions and biological threats. The following sections will describe the resistance mechanisms that are an important part of EPEC and (AE-)STEC survival strategy against antimicrobials, like heavy metals and classical antibiotics that were and are respectively, used in bacterial infection chemotherapy.

3.1 Heavy metals

3.1.1 Heavy metal use and bacterial resistance

In the period before the development of antibiotics, heavy metals were used in chemotherapy to treat a variety of infectious diseases (Briffa et al., 2020; Frippiat et al., 2021). Unfortunately, heavy metals are chemically stable elements that contaminate and accumulate in the environment leading to pollution of soils and water. Heavy metals can be, moreover, harmful to humans and animals, like for instance mercury and arsenic. Therefore, the use of heavy metals is highly restricted or banned in different countries and regions (Tchounwou et al., 2012). Nevertheless, some are still used as fertilizers, preservatives or fungicides in others (Jaishankar et al., 2014; Balali-Mood et al., 2021).

To protect themselves from heavy metal bactericidal action, bacteria in the environment or in the host, including of course different pathogenic species, such as *E. coli*, have acquired mechanisms (e.g. efflux pumps) conferring resistance against a.o. mercury, lead, cadmium, arsenic, silver and tellurium. The resistance encoding genes can be located on mobile genetic elements, like bacterial plasmids that can be horizontally transmitted (Silver and Phung, 1996; Cervantes et al., 1994). Therefore, resistance mechanisms to heavy metals are likely to spread horizontally between bacterial strains or even species, including pathogenic ones, as consequence of the selection pressure due to the presence of heavy metals more especially in the environment and less in the host today, at least in developed countries that banned their use (Seiler and Berendonk, 2012).

Heavy metals exert their antibacterial multifaceted effects on bacterial physiology.

They indeed bind to intracellular proteins and enzymes, disrupting their function and impairing cellular metabolism (Nies, 1999). Furthermore, heavy metals promote the generation of reactive oxygen species (ROS), which cause damage to cellular membranes and DNA, contributing to their antibacterial activity and toxic effects on the host (Imlay, 2003).

3.1.2 Tellurium resistance of Escherichia coli

With regard to tellurium resistance, particular attention has been paid to the mechanism of resistance of *E. coli* to tellurite ($TeO3^{2-}$), an oxo-anionic form of tellurium, and to potassium tellurite compound (K_2TeO_3). Tellurite has been used since the early 1930 years and is known to be highly toxic to micro-organisms, especially Gram-negative bacteria, due to the production of ROS damaging cellular components (Molina-Quiroz et al., 2013; Sandoval et al., 2015; Turner et al., 2001).

In the early 2000 years, the whole genomes of two AE-STEC O157:H7 were sequenced and analyzed. In these AE-STEC O157:H7 strains, the genes encoding resistance to tellurite (*ter* genes) are located on the duplicated O-Island: OI-43 and OI-48 (strain EDL933) and the SpLE-1 element (strain Sakai O157) that also harbor the *iha* gene (see section 2.3.4 (Penal et al., 2001; Hayashi et al., 2001). Since then, according to several studies, not only (AE-)STEC O157:H7 but also other (AE)-STEC serotypes, and EPEC shown the presence of the *ter* resistance genes (Nakamura et al., 2023; Nguyen et al., 2021).

The *ter* genes are present not only on the chromosome, but also on plasmids, e.g. pMER610 identified in *Alcaligenes* strain, and R478 from *Serratia marcescens* (Gilmour et al., 2004; Jobling and Ritchie, 1987; Jobling and Ritchie, 1988; Whelan et al., 1995). They confer high levels of resistance to *E. coli* and other bacterial species such as *Pseudomonas aeruginosa*, and *Yersinia pestis*, allowing them to reach minimal inhibitory concentration (MIC) of 1,024 μg/mL (Peng et al., 2022; Taylor, 1999).

The *ter* resistance gene cluster consists of seven genes (*terZABCDEF*) that code for a series of proteins, that confer resistance to tellurite by different mechanisms. These

proteins are involved in the reduction of reactive oxygen species (ROS), minimizing the oxidative stress caused by tellurite exposure. Nevertheless, the actual interaction between these genes have not been cleared (Taylor, 1999; Vávrová et al., 2024). Additionally, the *terZABCDE* genes have been implicated in promoting normal cell growth under stress conditions, as *terA* and *terZ* genes can recover cell growth inhibited by the core *terC, D* genes. This operon might contribute not only to ROS reduction, but also to maintaining cellular homeostasis during tellurite stress (Peng et al., 2022).

The *ter* gene cluster is considered to represent a major contribution to resistance to tellurite, but different other mechanisms of resistance, specific or not (including co-resistance with other heavy metals), are considered to be also involved in tellurite resistance directly or indirectly: e.g. arsenic efflux gene (*arsA,B,C.*.), cystine metabolism gene (*cysK, M*…), nitrate reduction genes (*narG,H,I,J*...), superoxide dismutation genes (*sodA, sodB,* and *soxS*), hydrogen peroxide detoxification (*katG*), tellurite resistant proteins (*tehA,B, KilA*…) (Calderón et al., 2006; Chasteen et al., 2009; Pérez et al., 2007; Nguyen et al., 2021). However, their actual mechanisms of action are not known yet.

Although not allowed in treatment of bacterial infections, potassium tellurite is still widely used today *in vitro* for the selective growth of different EPEC and (AE-)STEC, with the development and commercialization of different growth media, like the Cefixim-Tellurite-Sorbitol-MacConkey (CT-SMAC) medium (Karmali, 1989; Kerangart et al., 2016).

3.2 Antibiotics

3.2.1 Treatment of EPEC and (AE)-STEC infection

Although antibiotics are considered as a classical treatment of bacterial infections, including EPEC infections, most antibiotics are essentially not recommended for the treatment of AE-STEC infections. On the other hand, some studies have suggested that certain antibiotics, such as macrolides or fosfomycin may be beneficial when administered at an appropriate timing (Myojin et al., 2022). In Japan, fosfomycin has been used in patients with STEC infections and several reports have indicated a potential

protective effect of this molecule (Ikeda et al., 1999; Okubo et al., 2019; Takeda, 1998). However, due to the methodological limitations, partly because fosfomycin is not available in most other countries, these results are still controversial. At the present stage, antibiotic therapy with for instance macrolides, like azithromycin, which are believed to not induce the release of Stx, are being evaluated. This is likely related to the facts that macrolides inhibit (i) bacterial protein synthesis without inducing DNA damage or activation of the bacterial SOS response; (ii) the lytic cycle of Stx-converting bacteriophages; (iii) the host inflammatory response induced by Stx, including the production of pro-inflammatory cytokines (Ohara et al., 2002). However, azithromycin-resistant strains of *Klebsiella pneumoniae* and other Gram-negative bacteria, even Shiga toxin-producing *Escherichia coli* are already emerging (Bizot et al., 2022a; Hambali et al., 2024). Beside these positive aspects, antibiotic treatment also contains negative ones. Indeed, not only is the overuse of antibiotics fraught with the possibility of selecting new resistant strains, but also there is a high-risk that the destruction of the bacteria by antibiotics leads to the release of high quantity Stx which subsequently enters the bloodstream, spreads throughout the host body, and binds to Gb3/CD77 receptors on target cells of each organs, causing severe cellular damage (**Fig. 4**). This process may in turn lead to a worsening of symptoms.

Besides possible antibiotic administration, two strategies are used in the treatment of HUS: supportive and specific therapies aimed at protecting and restoring renal function, normalizing blood-supplying, managing infection and preventing severe complications. Although much time and man-power have been devoted to the development of therapeutic or preventive treatments for AE-STEC-induced disease, few trials have progressed beyond phase II. Several therapeutic approaches have been tested, including complement inhibitors such as 'eculizumab' and 'avacopan', and monoclonal antibodies for antigen neutralization. Unfortunately, however, no fully satisfactory curative solution has yet been found to date (Ardissino et al., 2022; Duneton et al., 2024; Mühlen and Dersch, 2020).

3.2.2 Antibiotic resistance mechanisms of EPEC and (AE-)STEC

In 1940, Howard Florey and Ernst Chain established a production method of penicillin G, ushering in the era of antibiotic use by humans who now had a weapon to fight infections of bacterial origin (Chhabra et al., 2024). Since the years 1940s, antibiotics have progressively replaced heavy metals in the fight against bacterial infections. However, today, the emergence and spread of drug-resistant bacteria that do not respond to antibiotic administration is a major problem in medical and veterinary practice around the world. For example, β-lactams, including penicillins, cephalosporins and carbapenems, have long been used as one of the most effective groups of antibiotics for treating bacterial infections and have a mechanism of action that inhibits the bacterial cell wall synthesis pathway by binding to penicillin-binding protein (PBP) (Bush and Bradford, 2016; Tooke et al., 2019). However, their massive use lead to the progressive emergence of Gram-negative bacteria producing different classes of β-lactamases, (narrow spectrum β-lactamases, broad-spectrum β-lactamases and carbapenemases), that hydrolyze different groups of penicillins, cephalosporins and/or carbapenems, and to the rapid spread of their encoding genes that are present on mobile and transferable genetic structures (Blair et al., 2015; Vasoo et al., 2015).

In some cases, the resistance profiles of EPEC and (AE-)STEC help to develop selective growth media, like the Cefixim-Tellurite-Sorbitol-McConkey (CT-SMAC) medium for (AE-)STEC O157:H7 (Karmali, 1989; Kerangart et al., 2016). This is not the case yet for EPEC and for (AE-)STEC non-O157 (Hiramatsu et al., 2002; Verhaegen et al., 2015).

4. Enteropathogenic and Attaching-Effacing Shigatoxigenic *Escherichia coli* O80:H2

4.1 Emergence of AE-STEC O80:H2 in humans

In the early 2010, a minor AE-STEC serotype, O80:H2 started to emerge in France in humans suffering from not only (bloody) diarrhea and HUS, especially in children and elderlies, but also from bacteremia and internal organ infections.

According to the studies conducted by a French research team, AE-STEC O80:H2 were the second most common HUS-causing STEC especially in children and often caused life-threating HUS-associated complications (Soysal, et al., 2016: Bruyand et al., 2019). Moreover, in the recent annual reports of the European Food Safety Agency (EFSA), AE-STEC O80:H2 were also the second or third leading HUS-causing STEC along with serotypes O26:H11 and O157:H7(EFSA BIOHAZARD PanelKoutsoumanis et al., 2020; EFSA, 2021, 2022, 2023, 2024). In the latest ECDC and EFSA report about STEC infections based on the data from 2023, the most frequent STEC serotypes associated with HUS were the followings in decreasing frequency: O26:H11, O157:H7, and O145:H28 followed by O80:H2 (EFSA, 2024). Besides France, AE-STEC O80:H2 have also been confirmed in human clinical cases in other Western and Southern European countries, such as Belgium, Germany, Switzerland, the Netherlands and Italy (Cointe et al. 2018; De Rauw et al., 2019; Fierz et al., 2017; Fruth et al., 2024; Gigliucciet al., 2021, Habets et al., 2021; Nüesch-Inderbinen et al., 2018).

However, contrary to major AE-STEC serotypes, like O157:H7 and to the non-AE-STEC serotype O104:H4, AE-STEC serotypes O80:H2 has gradually emerged causing sporadic cases, but never caused any large outbreaks so far (Bruyand et al. 2019; EFSA BIOHAZARD Panel Koutsoumanis et al., 2020; Ingelbeen et al., 2018).

4.2 AE-STEC and EPEC O80:H2 in animals and environment

The O80:H2 serotype has also been isolated from young calves (<3 months of age) in Belgium since 2009. The majority were exclusively EPEC during the earlier years and

are still frequent later (about 70% of the total), although AE-STEC (30%) started to be isolated in 2016 (Thiry et al., 2017; Habets et al., 2021). These young calves suffered from diarrhea and enteritis, but systemic infections such as septicemia were very rare (Habets et al., 2021). Like in humans, EPEC and AE-STEC O80:H2 cause sporadic cases in young calves in farms (Thiry et al., 2017; Habets et al., 2021).

Moreover, some EPEC and AE-STEC O80:H2 have been isolated from healthy adult cattle, from healthy piglet and from the environment (water, pig farm environment), although very rarely so far (Cointe et al., 2018; Cointe et al., 2021; Soleau et al., 2024; Vu-Khac et al., 2007).

4.3 Phylogenomics

According to different publications, human and calf AE-STEC and EPEC O80:H2 belong to the MultiLocus Sequence Type 301 (ST301), a member of the clonal complex (CC) 165 (Cointe et al., 2018; Cointe et al., 2021; Mariani-Kurkdjian et al., 2014; Rodwell et al., 2021). Moreover, human AE-STEC and calf EPEC and AE-STEC are very closely related and even mingle in SNP-based phylogenetic trees, forming different lineages (L) and sub-lineages (SL) (Habets et al., 2021; Soleau et al., 2024).

4.4 Virulence properties

AE-STEC and EPEC O80:H2 are characterized by harboring the *eaeξ* gene, regardless of pathotype and host (human, diarrheic calf, health cattle, piglets, environment..), and about 80% of AE-STEC strains have the *stx2d* gene. Apart from *stx2d*, the *stx* subtypes *stx1a*, stx*2a or stx2c* have also been identified so far (Cointe et al., 2018: De Rauw, et al., 2019; EFSA Koutsoumanis et al., 2020; Habets et al., 2021; Mariani-Kurkdjian et al., 2014; Soysal et al., 2016)

EPEC and AE-STEC O80:H2 also possess a specific plasmid called pS88-like which is commonly found in extra-intestinal pathogenic *E. coli* (ExPEC) strains, such as avian pathogenic *E. coli* (APEC) and neonatal meningitis *E. coli* (NMEC) and is associated with severe systemic infections (Mariani-Kurkdjian et al . , 2014; Peigne et al.,

2009; Soysal et al., 2021; Habets et al., 2021). For these reasons, AE-STEC O80:H2 is a triple hybrid and could be named AE-STEC-ExPEC, while EPEC O80:H2 are a double hybrid and could be named EPEC-ExPEC.

The plasmid pS88, including those of EPEC and AE-STEC O80:H2 carries (putative) extra-intestinal virulence-associated genes for entry and survival of the bacteria in the bloodstream and internal organs, including the following genes (Peigne et al. 2009; Cointe et al 2018; Habets et al., 2021):

(i) Iron uptake systems: aerobactin siderophore (*iucABCD, iutA*), salmochelin siderophore (*iroN*), putative iron acquisition system (*sitABCD*);

(ii) Resistance to the bactericidal activity of the complement: "increased serum resistance" (*iss*) gene and an "outer membrane protein" (*ompTp*)-encoding gene;

(iii) Presumptive type I secretion system: *etsABC* genes;

(iv) Avian hemolysin: *hlyF* gene;

(v) Colicin-encoding genes: c*vaA,B*,C, *cia*, *cvi*... genes.

However, not all pS88 plasmids of EPEC and AE-STEC O80:H2 possess the complete set of all genes (Cointe et al., 2021; Rodwell et al., 2021; Soysal et al., 2016) and, in addition, some can also carry antibiotic resistance genes, like the pR444_A plasmid : tetracycline (*tetA*), trimethoprim (*dfrA5*), sulfonamide (*sul2*), β-lactam (blaTEM-IB), kanamycin (*aph[3]-Ia*), and streptomycin (*strA* and *strB*). (Cointe et al., 2018; Cointe et al., 2020a). Furthermore, a number of other non-pS88-located putative virulence genes have been detected in all or several EPEC and AE-STEC O80:H2, like for instance the Ehly- and the Iha-encoding genes (see section 2.3.4) (other references if they exist; Habets et al., 2021).

Actually, the majority of EPEC and AE-STEC O80:H2 have different gene profiles with regard to not only two pS88-located genes (*iuc/iut* and *ets* genes), but also two genes located either on the SpLE-1 (*iha*) and on another plasmid (*cma* coding for colicin M). Habets and collaborators observed that the majority of *stx2d* AE-STEC harbor the former

(*iuc/iut* and *ets*) but not the latter (*iha* and *cma*) genes, whereas the majority of EPEC and of *stx1a* or *stx2a* AE-STEC have the opposite gene profile (Habets et al., 2021).

4.5 Source of contamination

Different epidemiological surveys of pediatric HUS cases caused by AE-STEC O80:H2 in France failed to identify any common exposures, like consumption of ground beef or of raw dairy products and to confirm that cattle is at the origin of human contamination (ANSES, 2023; Bruyand et al., 2019; Ingelbeen et al., 2018). Moreover, until very recently, the few surveys performed failed to isolate AE-STEC or EPEC O80:H2 from healthy cattle at slaughterhouse or in farms in which they had been isolated from diarrheic calves in Belgium (Habets et al., 2020; Thiry et al., 2018). Nevertheless, a recent survey was successful, but in only one of the farms sampled during a longitudinal survey investigating the "top 5" STEC serotypes in cattle, emphasizing the low prevalence of STEC O80:H2 in healthy cattle (Soleau et al., 2024).

One of the reasons to explain the failure of more frequently isolating AE-STEC and EPEC O80:H2 from healthy cattle is the absence of a specific selective isolation procedure (Gally et al., 2003; Naylor et al., 2003). Recently however, a specific selective methodology based on non-melibiose fermentation (a very uncommon trait of *E. coli*) by and piperacillin antibiotic resistance of AE-STEC O80:H2 was successfully applied to isolate AE-STEC O80:H2 from human patients (Bizot et al., 2022b), but has not been tested to isolate AE-STEC or EPEC from diarrheic calves. This methodology should also be tested on fecal materials of healthy cattle with much lower concentration of AE-STEC and/or EPEC O80:H2.

The applicability of selective media for human (AE-)STEC diagnostics, including those containing antibiotics such as CT-SMAC, to fecal samples from healthy animals deserves careful consideration. Generally, feces from healthy cattle are characterized by a complex and dense intestinal microbiota. Under these conditions, the antibiotic resistance profiles of fecal *E. coli* are more difficult to predict (Garcia-Graells, 2024; Soleau et al., 2024), compared to human and calf isolates (Cointe et al., 2018; Cointe et al., 2020a; Fruth et al., 2024; Lang et al., 2019). Therefore, the addition of broad-spectrum

antibiotics, such as cefixim or piperacillin, may impose an unforeseen selective pressure on enteric bacterial populations (Minnig et al., 2025; Steinberger et al., 2025). Hence, alternative selective approaches that limit excessive antibiotic-driven effects should be considered when adapting (AE-)STEC screening to feces from healthy cettle.

5. Insect models for *in vivo* experiments: the *Galleria mellonella* larva model

In the field of biomedical sciences and public health, including veterinary medicine and medicine, conducting experiments not only *in vitro* but also *in vivo* is necessary and even essential when studying bacterial pathogens and their putative virulence-associated properties, to fulfill the Koch' and molecular Koch's postulates (Koch, 1876; Koch, 1882; Falkow et al., 1988). The use of mammals and birds for disease modelling, especially infection modelling with bacterial pathogens, is always fraught with problems of obtaining statistically reliable data, cost issues such as animal breeding and maintenance, and, above all, ethical issues. British scientists William Russell and Rex Birch considered these problems associated with the use of laboratory animals and proposed the basic principle of the 3Rs (Replacement, Reduction, and Refinement) to protect them as much as possible (Lewis, 2019).

In the Replacement part of the 3Rs, insects are increasingly being used as a useful model to study infectious diseases (Ahlawat and Sharma, 2023). Indeed, because of easy handling, low cost, short life cycle, potential to test multiple larvae in groups in a short time, relative ease of determining death and, last but not least, absence of ethical issues, *in vivo* experiments using insect larvae have recently attracted attention as an alternative to mammalian and avian models. Among others, larvae of the *Galleria* (*G.*) *mellonella* moth (**Fig.5**) are frequently used to study bacterial pathogens, including different pathogenic strains of *E. coli* and the respective role of their putative virulence properties (Cutuli et al., 2019: Pereira et al., 2020: Antoine et al., 2021). In addition, *G. mellonella* larvae have an innate immune system similar to that of mammals and can be maintained at 37°C similarly to the growth temperature range of mammalian bacterial pathogens.

The bacterial culture is injected in the pseudopods of the larvae (**Fig. 5**). Progress of the infection is followed by a scoring system based on the reduction of the activity and the change in body color, with progressive blackening, or melanization of the larvae, signing the progress of the infection with the larvae (**Fig. 6**). Melanization consists in the synthesis and deposit of melanin to encapsulate the pathogen at the infection site, followed by hemolymph coagulation and opsonization. Complete blackening signs the death of the larvae (Tsai et al., 2016).

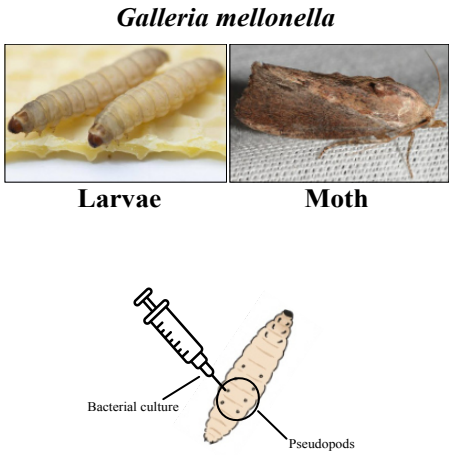

Figure 5. Larvae and adult forms of the *Galleria mellonella* moth and schematic overview of the experimental infection method, showing injection of bacterial culture into the larval proleg region. Larvae and moth images adapted from British Lepidoptera (https://britishlepidoptera.weebly.com/), accessed on 21st March 2025. Schematic illustration of larvae injection method created by Mare ADACHI, internship student in our laboratory.

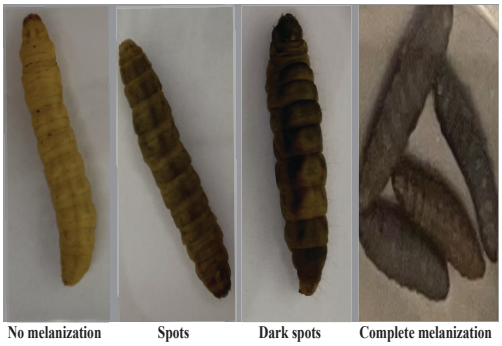

Figure 6. Scoring system based on the reduction of the activity and the change in body color, with progressive blackening, or melanization of the larvae, signing the progress of the infection with the larvae adapted from Tsai et al., 2016.

OBJECTIVES

OBJECTIVES

Of all Shigatoxigenic *E. coli* (STEC) serotypes, the seven major Attaching-Effacing-STEC (AE-STEC) serotypes (O157:H7, O26:H11, O103:H2, O111:H-, O121:H19, O145:H-), which are known to be highly pathogenic to humans, are one of the most common causes of food-borne infections (Karmali et al., 2003 ; Tozzoli and Scheutz, 2014. However, scores of other "minor" serotypes also exist and emerge from time to time either "explosively" causing short-live sporadic outbreaks, like Aggregative-STEC (Agg-STEC) serotype O104:H4 or gradually causing long-lasting clinical cases, like AE-STEC serotype O80:H2.

AE-STEC serotype O80:H2 has gradually emerged in Western European countries, particularly in France since ca. year 2010 and has been linked not only to (bloody) diarrhea and Hemolytic-Uremic Syndrome (HUS), but also to invasive infections (bacteremia and internal organ infection). Today AE-STEC O80:H2 represent the top cause of HUS in children and elderlies in Europe along with serotypes: these serotypes are O157:H7, O26:H11, and O145:H28 (EFSA, 2024). This serotype is indeed characterized by its peculiar virulotype. Besides the Locus of Enterocyte Effacement (LEE) pathogenicity island carrying a.o. the *eae* gene coding for the intimin adhesin and the Shiga toxin (Stx)-encoding genes (*stx*), AE-STEC O80:H2 harbor a specific plasmid called pS88-like carrying genes coding for putative virulence properties of extra-intestinal *E. coli* (ExPEC) (Mariani-Kurkdjian et al., 2014; Soysal et al., 2016). Furthermore, AE-STEC and, more especially enteropathogenic *E. coli* (EPEC) have been associated with diarrhea and enteritis, and though very rarely septicemia in young calves (<3 months of age) in farms (Thiry et al., 2017; Habets et al., 2021). Human and calf AE-STEC and EPEC are highly related by their virulotypes and phylogenetically (Thiry et al., 2017; Habets et al., 2021).

Although AE-STEC O80:H2 is considered a public health hazard, there still exist relatively few studies on AE-STEC and EPEC O80:H2 compared to the major AE-STEC serotypes. Therefore, different problems and questions still remain unresolved and unanswered, such as the identification of the actual source of infection, the way of

transmission, the host specificity if any, and the respective roles of the LEE, the Stx and the pS88-plasmids in their pathogenicity. In conclusion, more epidemiological, bacteriological, molecular and (phylo)-genetic studies are needed in order to more efficiently prevent and handle human infections.

Therefore, the general objective of this thesis work on AE-STEC and EPEC O80:H2 from both bovine and human origin was three-fold: (i) Attempt to isolation AE-STEC and EPEC O80:H2 (ii) Population structure including (Phylo)genetic analysis of O80:H2 isolates, and complete genome study (iii) Assessment the role of virulence factors of AE-STEC and EPEC O80:H2 *in vivo*.

To meet these three general objectives, five specific objectives were defined:

Specific objective #1: To identify healthy adult cattle at slaughterhouses and in farms as the contamination source of AE-STEC and EPEC O80:H2 using different non-specific and specific methodologies of isolation and identification;

Specific objective #2: To confirm virulotype: the identity and distribution of virulence-associated genes and the differences in pS88-located gene profiles in all those same human and calf AE-STEC and EPEC O80:H2;

Specific objective #3: To confirm the SNP-based (phylo)genomic structure by genomic analysis the distribution and intermixing of extended dataset of human and calf AE-STEC and EPEC O80:H2 than previously (Habets et al., 2021)

Specific objective #4: To study the genome structure of virulence properties: LEE region, Stx-converting phage, plasmids and localization of different genes identified in two calf *stx2f* AE-STEC O80:H2 belonging to two different sub-lineages after completed by long-reads;

Specific objective #5: To assess the respective roles of Stx-encoding genes and pS88 plasmids in the pathogenicity of AE-STEC and EPEC O80:H2 in *G. mellonella* larvae model.

RESULTS

RESULTS

PART 1: Identification of healthy adult cattle at slaughterhouses and in farms as the source of AE-STEC and EPEC O80:H2 using different non-selective and alternative selective methodologies of isolation.

FOREWORD

Ruminants, especially cattle, are the most frequent asymptomatic carriers of (AE-)STEC or EPEC, thus their feces are considered as main contamination source for humans. Serious clinical diseases in humans including HC and HUS, are associated with not only the frequent-major AE-STEC serotypes, but also the less-frequent-minor serotypes. The emerging AE-STEC serotype O80:H2 is one of the latter. This AE-STEC serotype is involved not only in HC and HUS, but also in bacteremia and internal organ infections in humans. Moreover, AE-STEC and EPEC are associated with diarrhea and sometimes septicemia in young calves (EFSA BIOHAZARD Panel Koutsoumanis et al., 2020; Habets et al., 2021; Soysal et al., 2016).

Identification of the contamination source of humans and of the way of transmission is of the upmost importance to prevent human infection. However, so far, there is no evidence that ruminants and cattle are the actual source of O80:H2 (ANSES, 2023; Bruyand et al., 2019; Ingelbeen et al., 2018; Habets et al., 2020; Thiry et al., 2018). One of the reasons is the lack of serotype-specific and selective medium.

Therefore, the objective of this study was to attempt to isolate AE-STEC or EPEC O80:H2 from healthy adult cattle using either a conventional non-selective methodology or a selective methodology based on putative specific biochemical and resistance properties: (i) non-melibiose fermentation like previously reported for human AE-STEC O80:H2, as the consequence of a deletion of the *mel* operon (Bizot et al., 2022b); (ii) resistance to potassium tellurite that is widely used, as a mean to isolate different major AE-STEC, including O157:H7 (Kerangart et al., 2016).

In the first approach, 53 of 343 fecal samples from one slaughterhouse and nine

farms tested positive with a PCR for the O80 serogroup after overnight growth in enrichment broths. Sixteen of a total of 835 colonies from these 53 fecal samples were identified as *E. coli* O80 with the same PCR, but genome sequencing identified them as belonging to serotypes O80:H45 and O80:H6. Moreover, they were phylogenetically distant from AE-STEC and EPEC O80:H2 and were non-AE-STEC non-EPEC.

In the second approach, all 52 human and calf AE-STEC and EPEC O80:H2 analyzed by Habets and collaborators (Habets et al., 2021) were non-melibiose fermentative and did not harbor the *mel* operon. Conversely, the resistance levels to potassium tellurite and the presence of the *ter* operon were different among the humans and calves strains. Most of the human and calf AE-STEC O80:H2 had lower MIC values and did not harbor the *ter* operon, in contrast to most calf EPEC. During this latter study, 96 fecal samples from one slaughterhouse were subjected to isolation testing again after overnight growth in enrichment broths containing or not tellurite. A total of 42 colonies were recovered from 11 O80-positive fecal samples of which 13 were non-melibiose fermentative. However, none of them was identified as belonging to serotype O80:H2.

Since AE-STEC and EPEC O80:H2 were not isolated from cattle fecal samples via the two methodologies, the contamination source remains unidentified even if some were isolated from dairy products years ago (InVS, 2007) or more recently from cattle feces in one farm (Soleau et al., 2024). Hence, this study highlights the need for establishment of more reliable, serotype-specific isolation methods for AE-STEC and EPEC O80:H2.

STUDY 1. Non-specific method for isolation of AE-STEC and EPEC O80:H2 from healthy adult cattle.

PUBLICATION 1

***Escherichia coli* O80 in Cattle: Absence of Shigatoxigenic and Enteropathogenic *E. coli* O80:H2 and (Phylo) Genomics of Non-Clonal Complex 165 *E. coli* O80**

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Escherichia coli O80 in Healthy Cattle: Absence of Shigatoxigenic and Enteropathogenic *E. coli* O80:H2 and (Phylo) Genomics of Non-Clonal Complex 165 *E. coli* O80

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Abstract: The origin of human and calf infections by Shigatoxigenic (STEC) and enteropathogenic (EPEC) *Escherichia coli* O80:H2 is still unknown. The aim of this study was to identify *E. coli* O80 in healthy cattle with an emphasis on melibiose non-fermenting *E. coli* O80:H2. Faecal materials collected from 149 bulls at 1 slaughterhouse and 194 cows on 9 farms were tested with O80 antigen-encoding gene PCR after overnight growth in enrichment broths. The 53 O80 PCR-positive broths were streaked on different (semi-)selective agar plates. Five *E. coli* colonies from 3 bulls and 11 from 2 cows tested positive with the O80 PCR, but no melibiose non-fermenting *E. coli* was isolated. However, these 16 *E. coli* O80 were negative with PCR targeting the *fliC*$_{H2}$, *eae*, *stx1*, *stx2* and *hlyF* genes and were identified by WGS to serotypes and sequence types O80:H6/ST8619 and O80:H45/ST4175. They were phylogenetically related to *E. coli* O80:H6 and O80:H45 isolated from different animal species in different countries, respectively, but neither to STEC and EPEC O80:H2/ST301, nor to other serotypes of the clonal complex 165. As a conclusion, healthy adult cattle were not identified as a source of contamination of humans and calves by STEC or EPEC O80:H2.

Keywords: healthy cattle; Shigatoxigenic *Escherichia coli*; enteropathogenic *Escherichia coli*; O80:H2; O80:H6; O80:H45; CC165; phylogenomics

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1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are a hybrid pathotype producing the Shiga toxins (Stx) of Shigatoxigenic *E. coli* (STEC) and the attaching–effacing (A/E) lesion of enteropathogenic *E. coli* (EPEC) [1]. Since the EHEC nomenclature is considered obsolete by EFSA [2], they will be named "Attaching-Effacing STEC" (AE-STEC) [3] in this manuscript.

The most frequent and pathogenic AE-STEC in humans belong to the following 6 serotypes: O26:H11, O103:H2, O111:H-, O121:H19, O145:H- and O157:H7 [1,4]. Nevertheless, other serotypes can emerge from time to time, either causing dramatic short-lived outbreaks, such as STEC O104:H4 in 2011 [5], or establishing themselves for longer periods, such as AE-STEC O80:H2 in France since ca. 2010 [6,7]. AE-STEC O80:H2 have also been reported in neighbouring countries (Belgium, Switzerland and the Netherlands), although not at the same frequency as in France [8–10]. Today, they represent the second- or third-leading cause of haemolytic uremic syndrome (HUS) in Europe [11,12]. In addition to haemorrhagic colitis and HUS, AE-STEC O80:H2 are responsible for systemic infections.

Indeed, they harbour a pS88-like ColV plasmid carrying genes encoding virulence properties of extra-intestinal *E. coli* [6,13–16]. AE-STEC and EPEC O80:H2 belong to the sequence type (ST) 301 that is member of the clonal complex (CC) 165 along with other *E. coli* O80 and non-O80 serotypes, and different STs [17,18].

AE-STEC and EPEC O80:H2 have also been frequently identified in young diarrheic and, more rarely, septicemic calves in Belgium since 2009 [19,20]. They are highly related to human AE-STEC O80:H2 phylogenetically and by their virulotypes, including the presence of a pS88-related plasmid [8,19].

Ruminants, especially cattle, are considered the most frequent source of human infection by the classical AE-STEC serotypes via foodstuffs contaminated by their faecal materials, since they can be asymptomatic carriers in their intestines [1]. However, the European Food Safety Agency reports no detection of AE-STEC O80:H2 in food in 2019 and 2020 [11,12], although they have been sporadically isolated from healthy cattle and dairy products in Spain and France, but not as yet in Belgium, in the past [6,13,17,21,22]. One possible reason is that AE-STEC O80:H2 were present under the detection limits of the different methodologies applied during those surveys. The rate of isolation could be increased using the recently described melibiose-MacConkey agar, since no human AE-STEC O80:H2 ferment melibiose, in contrast to most other *E. coli*. This is due to the deletion of the melibiose operon (*mel*) associated with the insertion of a 70 bp long DNA fragment (*70mel*) [23].

The purpose of the present study was, therefore, to (i) isolate *E. coli* O80 from healthy bulls at the slaughterhouse and healthy cows in farms, with emphasis on AE-STEC and EPEC O80:H2; (ii) identify the newly isolated *E. coli* O80 by PCR; and (iii) understand their phylogenomic relationships within the *E. coli* species after whole genome sequencing (WGS).

2. Materials and Methods

2.1. Identification of O80 PCR-Positive Faecal Samples

In November and December 2020, 149 slaughterhouse faecal samples were collected from young bulls during 3 visits to 1 slaughterhouse in the province of Liège, Belgium. The bulls originated from 43 different herds in the provinces of Liège, Limburg, Luxemburg and Namur, Belgium. Between October 2021 and February 2022, 194 faecal samples were collected from the rectum of healthy cows in late pregnancy, or maximum 1 month after calving, on 9 farms located in the provinces of Liège and Luxemburg, Belgium. One gram of each faecal sample was added to 9 mL of lauryl sulphate broth (VWR Chemicals, Leuven, Belgium) and incubated overnight at 37 °C with shaking, for enterobacterial enrichment.

After centrifugation of 2 mL of each enrichment broth for 2 min at 13,000 RPM, the bacterial pellets were suspended in 100 µL of DNase free water (VWR Life Science, Leuven, Belgium) and total DNA was extracted by boiling for 10 min. In parallel, 2 mL of the enrichment broths was transferred into 2 CRYO tubes (Greiner Bio-One, Frickhausen, Germany) with 2 mL of sterile 80% glycerol and stored at −20 °C and −80 °C, respectively. The DNA samples obtained were subjected to PCR targeting the *wzy* gene in the O80 antigen-encoding gene cluster (referred to as O80 PCR; Table 1) using the FASTGENE2x Optima Hotstart kit (Nippon Genetics, Filter service, Eupen, Belgium). The amplification condition employed was as follows [24]: initial denaturation at 94 °C for 1 min, 30 cycles of annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and denaturation at 94 °C for 30 s, and final extension at 72 °C for 2 min. The 285 bp-long amplicons were detected by electrophoresis in 1.5% agarose gel (VWR Life Science, Leuven, Belgium) in TAE buffer (Bio-Rad, Temse, Belgium) after staining with Midori Green (Nippon Genetics Europe, Düren, Germany).

Table 1. Target genes, primer sequences and amplified fragment lengths of the PCR.

PCR	Target Genes	Primer Sequences	Amplified Fragments	Reference
O80	wzy_{O80}	Og80-F: 5′-TGGTGTTGATTCCACTAGCGT-3′ Og80-R: 5′-CGAGAGTACCTGGTTCCCAAA-3′	285 bp	[24]
H2	$fliC_{H2}$	Hg2-F: 5′-TGATCCGACACTTCCTGATG-3 Hg2-R: 5′-CCGTCATCACCAATCAACGC-3′	228 bp	[25]
Intimin	*eae*	SK2-F: 5′-CCCGGATCCGTCTCGCCAGTATTCG-3′ SK1-R: 5′-CCCGAAATCGGCACAAGCATAAGC-3′	881 bp	[24]
Stx1	*stx1*	LP44-F: 5′-CACCAGACAATGTAACCGCTG-3′ LP43-R: 5′-CAGTTAATGTGGTGGCGAAGG-3′	348 bp	[24]
Stx2	*stx2a* to *stx2d*	LP31-F: 5′-GCGTCATCGTATACACAGGAGC-3′ LP30-R: 5′-ATCCTATTCCCGGGAGTTTACG-3′	584 bp	[24]
Avian hemolysin	*hlyF*	HlyF-F: 5′-GGCGATTTAGGCATTCCGATACTC-3′ HlyF-R: 5′-ACGGGGTCGCTAGTTAAGGAG-3′	599 bp	[16]

2.2. Identification of O80 PCR-Positive *E. coli*

O80 PCR-positive enrichment broths were streaked on 5 (semi-)selective agar plates: Chromocult Coliform ES agar, Chromocult Coliform ES agar complemented with 2.5 mg/mL of potassium tellurite (TeK) and Chromagar STEC agar (CHROMagar, Paris, France) for both slaughterhouse and farm samples, and either Rapid *E. coli* 2/agar (Bio-Rad, Temse, Belgium) for slaughterhouse samples or MacConkey agar (VWR Chemicals, Leuven, Belgium) for farm samples. According to the manufacturers, Chromocult Coliform ES, Rapid *E. coli* 2/and MacConkey are selective for enterobacteria and coliforms in general, while TeK Chromocult Coliform ES and Chromagar STEC are selective for Te^{++}-resistant coliforms, including a majority of STEC and EPEC.

All samples were also streaked on EnteroHemolysin (EHly) blood agar plates (Oxoid Deutschland, Wesel, Germany) to detect the production of enterohemolysin. After overnight incubation at 37 °C, up to 5 *E. coli*-like colonies were randomly picked up from the (semi-)selective agar plates. From the EHly blood agar plates, up to 10 colonies were picked up: 5 enterohemolyin-non-producing and 5 enterohemolysin-producing (if detected) colonies. In parallel, the O80 PCR-positive enrichment broths from farms were also streaked on melibiose-MacConkey agar plates. Melibiose-non-fermenting colonies (if detected) were picked up and subjected to species identification by API20E® (BioMérieux, Craponne, France), following the manufacturer's instructions.

O80 PCR was performed on all picked-up colonies as described above, using the DNA samples obtained from 2 mL of an overnight culture in Luria–Bertani (LB) broth (VWR Chemicals, Leuven, Belgium) at 37 °C with shaking.

2.3. PCR Typing

O80 PCR-positive *E. coli* isolates were further tested by PCR using the FASTGENE2x Optima Hotstart kit (Nippon Genetics, Filter service, Eupen, Belgium) to detect the $fliC_{H2}$ gene encoding the H2 antigen and the following virulence-associated genes: *eae* encoding the intimin adhesin involved in the A/E lesion, *stx1* and *stx2* encoding Stx1 and Stx2a to Stx2d toxins, and pS88-located *hlyF* encoding the avian haemolysin (Table 1). The amplification condition employed was as follows: initial denaturation at 95 °C for 15 min, 30 cycles of annealing at 55 °C for 90 s, extension at 72 °C for 90 s, denaturation at 94 °C for 30 s and final extension at 72 °C for 10 min [16,24,25]. The amplicons were detected by agarose gel electrophoresis, as described above.

2.4. Genome Analysis

The bovine O80 PCR-positive *E. coli* isolated during the abovementioned screening and 1 O80 PCR-positive isolate obtained from duck faecal material in 2009 were genome sequenced. Genomic DNA was purified from bacterial cells grown overnight in LB at 37 °C using NucleoSpin® Microbial DNA (Macherey-Nagel, Düren, Germany). Libraries

for Illumina sequencing were prepared using NEBNext UltraII FS DNA Library Prep Kit for Illumina (NEW ENGLAND BioLabs, Tokyo, Japan) and sequenced on the Illumina Miseq platform (Illumina) to generate 300 bp paired-end reads. Assembly of the Illumina sequence reads was performed using the SPAdes (v3.13.0) assembler [26]. Sequencing statistics of each isolate are shown in Table S1. Raw read sequences obtained in this study were deposited to GenBank/EMBL/DDBJ under the BioProject PRJNA906740.

Gene annotation and in silico H antigen-genotyping were conducted by using DFAST [27] and SeroTypeFinder 2.0 [28], respectively. The ST was determined by MLST 2.0 based on Achtman's scheme of multi-locus sequence typing (MLST) [29]. Plasmid replicons were identified by using PlasmidFinder v2.1.6 [30]. Virulence-associated and antimicrobial resistance (AMR) genes were searched by VirulenceFinder v2.0.4 and ResFinder v.4.1.11, respectively [31,32]. pS88 plasmid-located genes were detected by BLASTN search of each genome in the DNA sequence dataset for the pS88-located genes associated with bacteriocin production and immunity (*cia*, *imm*, *cvaABC* and *cvi*), iron acquisition (*iucABCD*, *iutA*, *shiF* and *sitABCD*) and virulence (*iss*, *etsABC*, *ompT* and *hlyF*) [16] (Table S2). All searches were performed at a threshold of >90% identity and >60% coverage.

2.5. Database Search for Genomes of E. coli O80 Identified Serotypes

The NCBI and EnteroBase databases (final access: 4 October 2022) were searched for the genome sequences of *E. coli* belonging to the O80 serotypes identified during the abovementioned survey (Table 2). Their Illumina reads were downloaded and assembled using SPAdes as described above (Table S1). Using assembled genomes, ST determination and analyses of plasmid replicons, virulence-associated genes, AMR genes and pS88-encoded genes were performed as described above.

2.6. Phylogenomics

To understand the phylogenetic positions of the *E. coli* O80 identified in healthy cattle, closed chromosome sequences of 104 *E. coli* strains representing each of 104 serotypes were selected and downloaded from the NCBI database and annotated by DFAST (Tables S1 and S3). The chromosome sequence of *Escherichia* cryptic clade I strain TW10509 (No. AEKA00000000) was also downloaded and annotated to be used as an outgroup. The core genes (n = 2560) of all those *E. coli* were identified, and their concatenated sequence alignments were generated by Roary [33]. Based on the 97,551 SNP sites extracted from the alignment using SNP-sites [34], a maximum likelihood (ML) tree was constructed using RAxML [35]. Strains were deduplicated if the core sequences were identical. The phylogroup of each strain was determined by EzClermont [36] and the ML tree was displayed using iTOL [37].

3. Results

3.1. Identification of E. coli O80 in Faecal Samples from Slaughterhouse

After overnight enrichment growth in lauryl sulphate broths, 35 out of the 149 faecal samples (23%) from young bulls at 1 slaughterhouse were positive with the O80 PCR. Using the non-specific methodology, 450 colonies were picked up, with the majority from Chromocult Coliform ES (40%) and Chromagar STEC (30%) agar plates (Figure 1a). After performing the O80 PCR twice, 5 isolates (1%) from 3 faecal samples (2%) were confirmed as *E. coli* O80. Three of the 5 isolates were isolated from 2 bulls on the Chromocult Coliform ES agar and the remaining 2 from another bull on EHly agar.

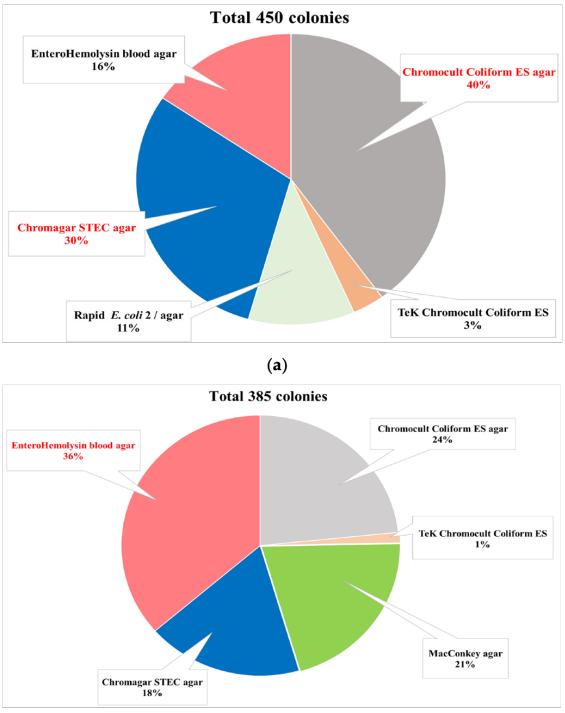

Figure 1. Proportions of the collected colonies from the different agar plates inoculated with the 35 O80 PCR-positive enrichment broths from slaughterhouse faecal samples (**a**) and with the 18 O80 PCR-positive enrichment broths from farm faecal samples (**b**).

3.2. Identification of E. coli O80 in Faecal Samples from Farms

After overnight enrichment growth in Lauryl Sulfate broths, 18 out of the 194 faecal samples (9%) from cows in 5 out of the 9 farms (55%) were positive with the O80-antigen PCR. Using the non-specific methodology, 385 colonies were collected, with the majority from EHly (36%) and Chromocult Coliform ES (24%) agar plates (Figure 1b). After performing the O80 PCR twice, 11 isolates (3%) from 2 cow faecal samples (1%) from 2 different farms (22%) were confirmed as *E. coli* O80. Ten of the 11 isolates from farm samples were isolated from 1 cow on Chromocult Coliform ES (5 isolates) and MacConkey (5 isolates) agar, and the remaining 1 from a second cow in another farm on EHly agar.

The 18 positive enrichment broths of faecal samples from cows in farms were also streaked on melibiose-MacConkey agar plates. Although melibiose non-fermenting colonies could be isolated after overnight growth, none of them was identified to *E. coli*.

3.3. Genomic Identification and Characterization

To further identify the O80 PCR-positive isolates to AE-STEC or EPEC O80:H2, PCR and WGS analysis were performed to detect the presence of the $fliC_{H2}$, *eae*, *stx1*, *stx2* and *hlyF* genes. However, none of these 5 genes was detected in any of the 16 O80 PCR-positive isolates.

In silico analysis of the genome sequences of these 16 O80 PCR-positive isolates revealed that their H-serotypes were H6 (n = 10) or H45 (n = 6) (Table 2). All 10 *E. coli* O80:H6 were obtained from the same cow in the screening of farm samples. Of the 6 *E. coli* O80:H45, 1 was obtained from the second cow in the screening of farm samples and 5 were obtained from the 3 bulls in the screening of slaughterhouse samples. The O80:H6 and O80:H45 isolates belonged to ST8619 and ST4175, respectively (Table 2).

Table 2. *E. coli* O80:H6/ST8619 and O80:H45/ST4175 analysed in this study.

O:H/ST Genotype (Nr Isolates)	Isolation			BioSample No. (Bioproject PRJNA906740)	Data Source
	Source (Nr Isolates)	Country	Year		
O80:H6/ ST8619 (18)	Cows (10)	Belgium	2022	SAMN32092024–SAMN32092033	This study
	Turkeys (8) [1]	USA	2018–2021	SAMN11372876, SAMN12913176, SAMN1299068, SAMN18586312, SAMN20862110, SAMN23100074, SAMN25980720, SAMN26027222	NCBI
O80:H45/ ST4175 (15)	Cow (1)	Belgium	2022	SAMN32092034	This study
	Bulls (5)	Belgium	2020	SAMN32092019–SAMN32092023	This study
	Duck (1)	Belgium	2009	SAMN32092035	This study
	Cow (1) [2]	Canada	2007	SAMN14379539	NCBI
	Cow (1)	France	2010	SAMEA5619080	NCBI
	Cow (1) [2]	Germany	2004	SAMEA5619042	NCBI
	Unknown (1)	Poland	2016	ESC_TA7527AA [3]	EnteroBase
	Cattle (1)	UK	2017	ESC_BB1134AA [3]	EnteroBase
	Pig (1)	UK	2015	SAMEA4645274	NCBI
	Pig farm soil (1)	UK	2017	SAMN15488558	NCBI
	Cow manure (1)	USA	2020	SAMN17058957	NCBI

[1] One isolate belonged to ST12217, which is a single locus variant of ST8619 (Table S2). [2] These 2 isolates belonged to ST1301, which is a single locus variant of ST4175. [3] IDs in EnteroBase.

The core gene sequence analysis also revealed that the 10 *E. coli* O80:H6 obtained from the same cow in 1 farm were identical, as were the 4 *E. coli* O80:H45 obtained from 2 bulls at the slaughterhouse. Therefore, only 1 *E. coli* O80:H6 from this cow and 3 *E. coli* O80:H45 from 2 bulls and 1 cow were included in the phylogenomic and other genome sequence-based analyses.

3.4. Genetic Features and Phylogenomics of the E. coli O80:H6 and O80:H45

The genome sequences of these 4 bovine Belgian *E. coli* O80:H6 and O80:H45 were compared with the genome sequences of 8 O80:H6 and 8 O80:H45 strains obtained from the NCBI and EnteroBase databases, and of 1 Belgian *E. coli* O80:H45 previously isolated from duck faecal material (Table 2). The position of these 2 *E. coli* serotypes in the entire *E. coli* phylogeny was compared to the position of *E. coli* O80:H2.

While the 9 *E. coli* O80:H6 were either Belgian bovine (n = 1) or US turkey (n = 8) isolates, the *E. coli* O80:H45 (n = 12) were isolated from cattle, duck, pig and the environment, in different countries (Belgium, Canada, France, Poland, UK and USA). Like the Belgian bovine isolates, all but 1 US turkey *E. coli* O80:H6 belonged to ST8619, and the remaining 1 belonged to ST12217, a single locus variant (SLV) of ST8619 (Tables 2 and S2). Similarly, 7 of the additional 9 *E. coli* O80:H45, including the Belgian duck isolate, belonged to ST4175, like the 3 Belgian bovine isolates, and the remaining 2 belonged to an SLV of ST4175, ST1301 (Tables 2 and S2).

The core gene-based phylogenetic analysis of the *E. coli* O80:H6 and O80:H45 and of the 104 *E. coli* whose chromosome sequences were downloaded from the NCBI database (Table S3) revealed that all 21 *E. coli* O80:H6 and O80:H45 belonged to phylogroup E and formed 2 single clusters, far distantly related to the AE-STEC and EPEC O80:H2 and to the clonal complex CC165 (Figure 2a).

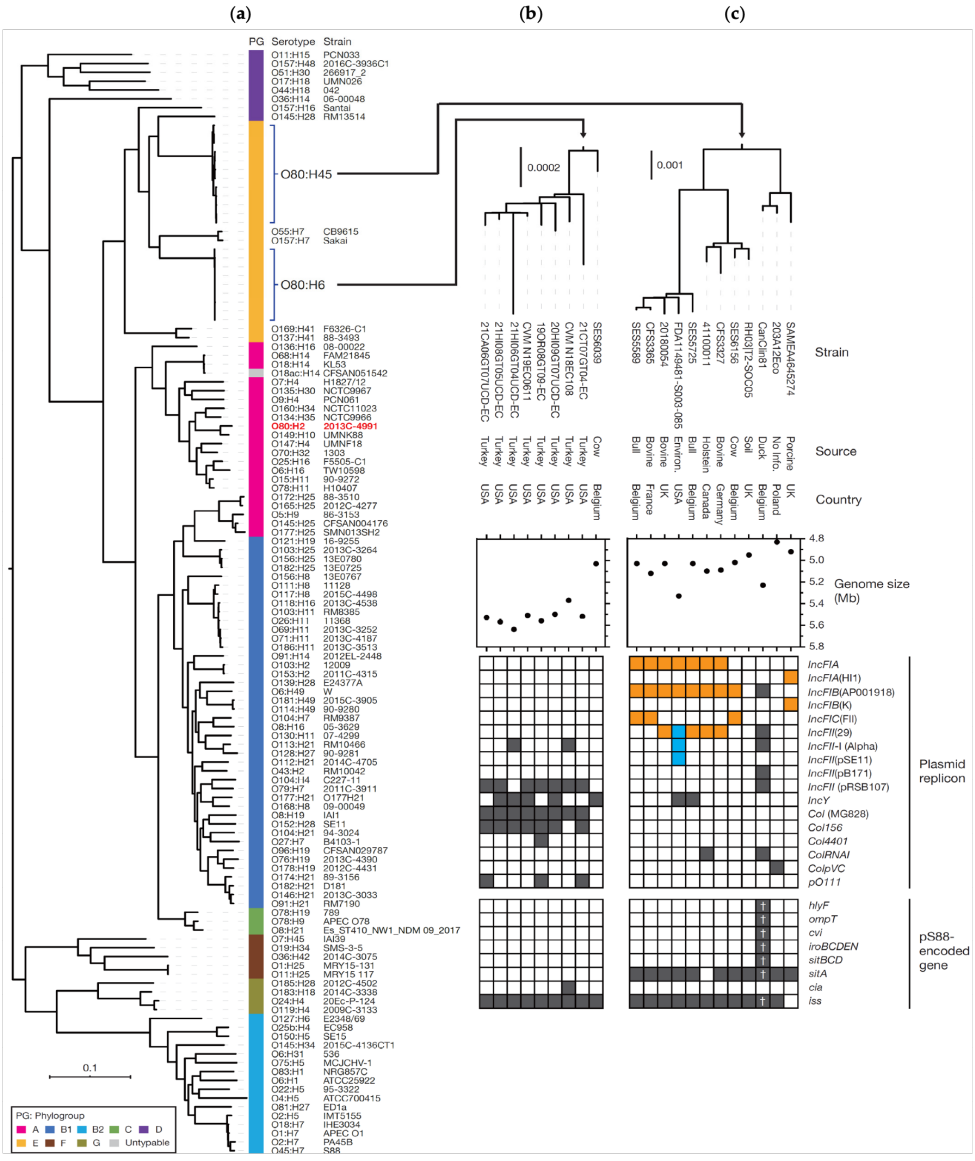

Figure 2. The phylogenetic positions and genetic features of the analysed *E. coli* O80:H6 and O80:H45, including genome sizes and distribution of plasmid replicons and pS88-located genes. In the left panel

(**a**), a core gene-based ML tree of 104 chromosome-closed *E. coli* strains with an *Escherichia* cryptic clade I strain TW10509 (No. AEKA00000000) as an outgroup is shown along with names and serotypes of each strain. The tree was constructed based on the 97,551 SNPs identified in 2560 core genes. Phylogroups of each strain are also indicated. The right panels show the fine phylogenetic relationships of the 9 O80:H6 (**b**) or of the 12 O80:H45 (**c**) strains along with strain information. The genome sizes and the results of plasmid replicon and pS88-encoded gene search are also shown. The presence/absence of each replicon and gene is indicated by a filled/open box. The replicons found in the same contig in each strain are indicated by orange and cyan boxes. Genes nearly identical to those of pS88 (threshold: >99% identity and 100% coverage) are shown by daggers. Bar: the mean number of nucleotide substitutions per site.

3.4.1. Genetic Features of *E. coli* O80:H6 Isolates

Phylogenetically, the Belgian bovine isolate (SES6039) formed a distinct branch from the 8 US turkey isolates (Figure 2b). Moreover, its genome size was significantly smaller (5033 kb vs. 5367 kb–5566 kb) and genome analysis by PlasmidFinder revealed the presence of only 1 plasmid replicon (*IncY*) in the Belgian bovine isolate, whereas the US isolates contained 3 to 5 replicons.

A search of virulence-associated genes by VirulenceFinder (Figure S1) revealed the presence of a range of potentially virulence-related genes in all 9 *E. coli* O80:H6. In addition, the *cib* gene encoding the Colicin Ib and the genes for the biosynthesis of pyelonephritis-associated pili (PAP) were detected in 1 and 2 US isolates, respectively. Conversely, no horizontally acquired AMR gene was detected in the Belgian bovine isolate by ResFinder (Figure S1), while 1 to 5 AMR genes were detected in the US turkey isolates.

3.4.2. Genetic Features of *E. coli* O80:H45 Isolates

The 12 O80:H45 isolates (3 Belgian bovine, 1 Belgian duck and 8 other isolates) formed 3 distinct sub-clusters (Figure 2c) that are highly heterogeneous in terms of regions and sources of isolation. Of the 3 Belgian bovine isolates, 2 belonged to the same sub-cluster, although they are not closely related, and the third one to another sub-cluster. The duck isolate belongs to the third sub-cluster. Notable variation in genome size was also observed, even in the same sub-cluster (ranging from 4832 kb to 5331 kb) (Figure 2c). Plasmid replicon search revealed that the isolates containing the largest and second largest genomes (FDA1149481-S003-085S isolated in the US and the Belgian duck isolate, respectively) contained more replicons (7 and 6 replicons, respectively) than the other isolates (Figure 2c). Contigs carrying multiple replicons were present in 9 isolates (Figure 2c). In 8 of them, including the 3 bovine, but not the duck Belgian isolates, similar sets of 2 or 3 of the *IncFIA*, *IncFIB*(AP001918), *IncFIC*(FII) and/or *IncFII*(29) replicons were detected and, in 1 of these 8 isolates (FDA1149481-S003-085S with the largest genome), an additional contig containing 3 replicons was detected.

Search of virulence-related genes by VirulenceFinder (Figure S1) revealed that, in addition to a set of potentially virulence-related genes in all 12 *E. coli* O80:H45, some isolates contained additional virulence-related genes: *afaAB* (regulator and chaperone for afimbrial adhesins) in a French isolate, *cdtB* (B subunit of Cytolethal distending toxin) in a Canadian isolate, and different pS88-located genes in addition to *sitA* and *iss* (*hlyF*, *iroBCDEN*, *cma*, *ompT*, *cvi*, *sitBCD* and *cva*C) in the Belgian duck isolate (Figures 2c and S1). In the search of horizontally acquired AMR genes by ResFinder, AMR genes were only detected in the UK porcine isolate, which contained 9 AMR genes (Figure S1) conferring resistance to 8 antibiotic families.

4. Discussion

Although AE-STEC and EPEC O80:H2/ST301 emerged in humans and in calves more than a decade ago, there is still a lack of knowledge about their epidemiology. In comparison with several other AE-STEC serotypes, cattle are highly suspected as the source of contamination, since they can be asymptomatic carriers in their intestines [1]. However,

surveys to isolate AE-STEC or EPEC O80:H2 from healthy adult cattle or young calves have been so far unfruitful, with a very few sporadic exceptions [6,13,17,21,22].

Using the same non-selective methodology as previously [22], a majority of AE-STEC and EPEC are expected to grow on the TeK Chromocult Coliform ES and Chromagar STEC agar media at the opposite of the majority of non-STEC non-EPEC strains that are Te^{++}-sensitive [38]. Surprisingly, however, very few colonies from the 53 O80 PCR-positive enrichment broths grow on the TeK Chromocult Coliform ES agar plates compared to Chromagar STEC plates (Figure 1). The authors have no explanation for these different results between the 2 agar media, results that were not observed at such a scale in the previous study [22].

A total of 16 *E. coli* O80 were isolated from 5 of the 53 O80 PCR-positive enrichment broths (9%), although many more colonies from farm (36%) and slaughterhouse (16%) samples produce an enterohemolysin on the EHly agar (Figure 1), like AE-STEC and EPEC O80:H2 [6,15,19,20]. However, these 16 *E. coli* O80 belong to serotypes O80:H6 and O80:H45, and to ST8619 and ST4175, or their SLVs (Tables 2 and S2), respectively. The most probable reason for this negative result is that *E. coli* O80:H2 was present, if at all, under the detection limits of this methodology. Testing more colonies is one alternative to increase the probability of isolating AE-STEC and EPEC O80:H2 but would be time- and labour-consuming. Another alternative is the use of a specific agar medium.

Therefore, melibiose-MacConkey agar plates [23] were streaked with the O80 PCR-positive enrichment broths of the faecal samples from the cows in farms, but no melibiose non-fermenting *E. coli* could be isolated. The reasons for this recurring negative result can be: (i) the human stool samples tested by Bizot and collaborators were clinical samples, probably with high numbers of AE-STEC O80:H2, while the bovine faeces tested in this survey were sampled from healthy animals, most probably with much lower numbers of *E. coli* O80:H2, if any, and (ii) the melibiose-MacConkey agar tested with the human stool samples also contains piperacillin, which was not used during our survey. Identifying the antibiotic/heavy metal, including to Te^{++}, resistance profiles of the bovine AE-STEC and EPEC O80:H2 sequenced to date [17,19,39] will help to design more selective enrichment broths and agar plates to increase the rate of successful isolation. The rate of isolation of *E. coli* O80 could also be increased by designing an O80 antigen-specific capture method, like for other highly pathogenic AE-STEC serotypes [40,41].

E. coli O80:non-H2 from humans and animals have already been reported and can belong to one of the numerous *E. coli* pathotypes [1,17,42,43]. Like AE-STEC and EPEC O80:H2, some of them (O80:H19 and O80:H26) belong to phylogroup A and CC165 [17,18]. Nevertheless, neither the bovine *E. coli* O80:H6 and O80:H45 isolated during this survey, nor the 16 *E. coli* O80:H6 and O80:H45 whose genome sequences were downloaded from NCBI and EnteroBase databases, nor the additional Belgian duck *E. coli* O80:H45, are closely related to CC165 and members of phylogroup A (Figure 2).

Within serotype O80:H6, the only Belgian bovine isolate is placed in a distinct branch, but the other O80:H6 isolates were all isolated from turkeys in the USA (Figure 2). Within serotype O80:H45, the 3 Belgian bovine isolates are present in 2 different sub-clusters, along with other European and American bovine-related isolates (Figure 2), while the duck isolate is located in a third sub-cluster. Regarding the differences of the genome size within either serotype, the presence of more plasmid replicons in the isolates with the larger genomes suggests that the differences in plasmid content are partly contributing to the differences in genome size (Figure 2). Moreover, the presence of similar sets of replicons in 7 of the *E. coli* O80:H45 suggests that similar plasmids may be distributed in these strains, even though these 7 isolates are not related geographically, nor by their origins, nor by the year of isolation (Figure 2; Tables 2 and S2). Clearly, more isolates of both serotypes are needed to refine their phylogenomic analysis, including the determination of complete genome sequences, before discussing further these results and hypotheses.

Search of virulence-associated genes by VirulenceFinder (Figure S1) revealed that no *E. coli* O80:H6 or O80:H45 are either (AE-)STEC, or EPEC, or belong to any other classical

E. coli pathotype in humans or animals, with the possible exception of the duck *E. coli* O80:H45. Indeed, with the exceptions of the *afa* and *cdtB* genes in 2 O80:H45 isolates, the majority of the potentially virulence-related genes detected in the other *E. coli* O80:H6 and O80:H45 are widely distributed in *E. coli*, including in the laboratory strain K-12. Therefore, their importance in their pathogenicity, if any of these *E. coli* O80:H6 and O80:H45 is unclear at this stage, although some of them (*sitA* and *iss*) are involved in the survival of ExPEC in blood stream and internal organs and are plasmid-located [16,43].

Conversely, the duck *E. coli* O80:H45 harbours several pS88-located genes, including the *hlyF* gene (Figure 2) which is a marker of the virulence plasmids of ExPEC, avian pathogenic *E. coli* (APEC), AE-STEC and EPEC O80:H2 [16,17,19,39,44]. A more detailed examination of the putative pS88-located genes detected in the *E. coli* O80:H6 and O80:H45 of this study (Figure 2 and Table S1) was therefore performed. The *sitA* gene detected in most *E. coli* O80:H45 (11/12) has 100% identity to the *sitA* gene of pS88 in the duck isolate vs. 97.9–98.0% identity in the other isolates. Similarly, the *iss* gene detected in all *E. coli* O80:H6 (9/9) and most *E. coli* O80:H45 (11/12) also possesses 100% identity to the pS88-located *iss* gene in the duck isolate vs. 90.4–95.9% identity in the other isolates. In addition, nearly half (13/27) of the pS88-located genes are present in the duck isolate, with >99% sequence identity and 100% coverage to the pS88-located genes, and not in the other isolates. Finally, this duck isolate contains 1 of the 2 replicons of pS88 (*IncFIB*(AP001918)). These different data strongly suggest that the duck isolate, in contrast to the other *E.coli* O80:H6 and O80:H45, including the Belgian bovine ones, harbour a pS88-like plasmid. pS88 plasmids have already been detected in APEC O80:H26 [17], but to the authors' knowledge, this is the first description in *E. coli* O80 outside of the CC165. Nevertheless, this duck *E. coli* O80:H45 was isolated from the faecal material and its actual virulence potential is not known at this stage.

Regarding horizontally acquired AMR genes, searching by ResFinder was negative for the Belgian bovine *E. coli* O80:H6 and O80:H45 (Figure S1). A few AMR genes are present in the US turkey *E. coli* O80:H6, but only the porcine *E. coli* O80:H45 can be genetically defined as a multidrug-resistant strain, with 9 AMR genes conferring resistance to 8 classes of antimicrobials (Figure S1). These results do not, however, exclude the existence of other non-horizontally acquired antimicrobial resistance mechanisms [45].

5. Conclusions

As general and specific conclusions, neither AE-STEC nor EPEC O80:H2 were isolated during this survey, and healthy adult cattle were not identified as the source of contamination of calves and humans. Moreover, the bovine *E. coli* O80:H6 and O80:H45 isolated during this survey are neither AE-STEC nor EPEC and phylogenetically, are only distantly related to the AE-STEC and EPEC O80:H2 or to the other *E. coli* O80 serotypes of the clonal complex CC165. More surveys targeting other putative sources of contaminations, such as the environment and wildlife, should be performed using selective methodologies to identify the source of contamination of humans and calves by AE-STEC or EPEC O80:H2. Further experiments should also be conducted to refine the (phylo)genomics and to assess the virulence potential, if any, of the Belgian bovine *E. coli* O80:H6 and O80:H45, and of the Belgian duck *E. coli* O80:H45.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11020230/s1; Figure S1: Virulence-associated and antimicrobial resistance (AMR) genes detected in *E. coli* O80:H6 and O80:H45; Table S1: *E. coli* O80:H45 and O80:H6 strains used in WGS analyses; Table S2: The pS88-located genes used for repertoire analysis as references; Table S3: *E. coli* strains used for the construction of a core gene-based phylogenetic tree.

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References

1. Tozzoli, R.; Scheutz, F. Diarrheagenic *Escherichia coli* infections in humans. In *Pathogenic Escherichia coli: Molecular and Cellular Microbiology*; Morabito, S., Ed.; Caister Academic Press: Norfolk, UK, 2014; pp. 1–18, ISBN 978-1-908230-37-9.
2. Koutsoumanis, K.; Allende, A.; Alvarez-Ordonez, A.; Bover-Cid, S.; Chemaly, M.; Davies, R.; Cesare, A.D.; Herman, L.; Hilbert, F.; Lindqvist, R.; et al. Pathogenicity assessment of Shiga toxin-producing *Escherichia coli* (STEC) and the public health risk posed by contamination of food with STEC. *EFSA J.* **2020**, *17*, e05898. [CrossRef]
3. Piérard, D.; De Greve, H.; Haesebrouck, F.; Mainil, J. O157:H7 and O104:H4 Vero/Shiga toxin-producing *Escherichia coli* outbreaks: Respective role of cattle and humans. *Vet. Res.* **2012**, *43*, 13. [CrossRef] [PubMed]
4. Karmali, M.A.; Mascarenhas, M.; Shen, S.; Ziebell, K.; Johnson, S.; Reid-Smith, R.; Isaac-Renton, J.; Clark, C.; Rahn, K.; Kaper, J.B. Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J. Clin. Microbiol.* **2003**, *41*, 4930–4940. [CrossRef] [PubMed]
5. Navarro-Garcia, F. *Escherichia coli* O104:H4 Pathogenesis: An enteroaggregative *E. coli*/Shiga toxin-producing *E. coli* explosive cocktail of high virulence. *Microbiol. Spectr.* **2014**, 2, EHEC-0008-2013. [CrossRef] [PubMed]
6. Soysal, N.; Mariani-Kurkdjian, P.; Smail, Y.; Liguori, S.; Gouali, M.; Loukiadis, E.; Fach, P.; Bruyand, M.; Blanco, J.; Bidet, P.; et al. Enterohemorrhagic *Escherichia coli* hybrid pathotype O80:H2 as a new therapeutic challenge. *Emerg. Infect. Dis.* **2016**, *22*, 1604–1612. [CrossRef]
7. Bruyand, M.; Mariani-Kurkdjian, P.; Le Hello, S.; King, L.-A.; Van Cauteren, D.; Lefevre, S.; Gouali, M.; Jourdan-da Silva, N.; Mailles, A.; Donguy, M.-P.; et al. Réseau Français Hospitalier de Surveillance du SHU Pédiatrique. Paediatric haemolytic uraemic syndrome related to Shiga toxin-producing *Escherichia coli*, an overview of 10 years of surveillance in France, 2007 to 2016. *Euro Surveill.* **2019**, *24*, 1800068. [CrossRef]
8. De Rauw, K.; Thiry, D.; Caljon, B.; Saulmont, M.; Mainil, J.; Piérard, D. Characteristics of Shiga toxin-producing and enteropathogenic *Escherichia coli* of the emerging serotype O80:H2 isolated from humans and diarrhoeic calves in Belgium. *Clin. Microbiol. Infect.* **2019**, *25*, e5–e111. [CrossRef]
9. Nüesch-Inderbinen, M.; Cernela, N.; Wüthrich, D.; Egli, A.; Stephan, R. Genetic characterization of Shiga toxin producing *Escherichia coli* belonging to the emerging hybrid pathotype O80:H2 isolated from humans 2010–2017 in Switzerland. *Int. J. Med. Microbiol.* **2018**, *308*, 534–538. [CrossRef]
10. Wijnsma, K.L.; Schijvens, A.M.; Rossen, J.W.A.; Kooistra-Smid, A.M.D.M.; Schreuder, M.F.; van de Kar, N.C.A.J. Unusual severe case of hemolytic uremic syndrome due to Shiga toxin 2d-producing *E. coli* O80:H2. *Pediatr. Nephrol.* **2017**, *32*, 1263–1268. [CrossRef]
11. European Food Safety Authority; European Centre for Disease Prevention and Control. The European Union One Health 2019 zoonoses report. *EFSA J.* **2021**, *19*, 6406. [CrossRef]
12. European Food Safety Authority; European Centre for Disease Prevention and Control. The European Union One Health 2020 zoonoses report. *EFSA J.* **2021**, *19*, 6971. [CrossRef]
13. Blanco, M.; Blanco, J.E.; Mora, A.; Dahbi, G.; Alonso, M.P.; González, E.A.; Bernárdez, M.I.; Blanco, J. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (eae-xi). *J. Clin. Microbiol.* **2004**, *42*, 645–651. [CrossRef] [PubMed]

14. Cookson, A.L.; Bennett, J.; Thomson-Carter, F.; Attwood, G.T. Molecular subtyping and genetic analysis of the enterohemolysin gene (*ehxA*) from Shiga toxin-producing *Escherichia coli* and atypical enteropathogenic *E. coli*. *Appl. Environ. Microbiol.* **2007**, *73*, 6360–6369. [CrossRef] [PubMed]
15. Ingelbeen, B.; Bruyand, M.; Mariani-Kurkjian, P.; Le Hello, S.; Danis, K.; Sommen, C.; Bonacorsi, S.; de Valk, H. Emerging Shiga-toxin-producing *Escherichia coli* serogroup O80 associated with hemolytic and uremic syndrome in France, 2013–2016: Differences with other serogroups. *PLoS ONE* **2018**, *13*, e0207492. [CrossRef] [PubMed]
16. Peigne, C.; Bidet, P.; Mahjoub-Messai, F.; Plainvert, C.; Barbe, V.; Médigue, C.; Frapy, E.; Nassif, X.; Denamur, E.; Bingen, E.; et al. The plasmid of *Escherichia coli* strain S88 (O45:K1:H7) that causes neonatal meningitis is closely related to avian pathogenic *E. coli* plasmids and is associated with high-level bacteremia in a neonatal rat meningitis model. *Infect Immun.* **2009**, *77*, 2272–2284. [CrossRef] [PubMed]
17. Cointe, A.; Birgy, A.; Mariani-Kurkdjian, P.; Liguori, S.; Courroux, C.; Blanco, J.; Delannoy, S.; Fach, P.; Loukiadis, E.; Bidet, P.; et al. Emerging multidrug-resistant hybrid pathotype Shiga toxin-producing *Escherichia coli* O80 and related strains of Clonal Complex 165, Europe. *Emerg. Infect. Dis.* **2018**, *24*, 2262–2269. [CrossRef]
18. Zhou, Z.; Alikhan, N.-F.; Mohamed, K.; Fan, Y.; the Agama Study Group; Achtman, M. The EnteroBase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. *Genome Res.* **2020**, *30*, 138–152. [CrossRef]
19. Habets, A.; Crombé, F.; Nakamura, K.; Guérin, V.; De Rauw, K.; Piérard, D.; Saulmont, M.; Hayashi, T.; Mainil, J.G.; Thiry, D. Genetic characterization of Shigatoxigenic and enteropathogenic *Escherichia coli* O80:H2 from diarrhoeic and septicaemic calves and relatedness to human Shigatoxigenic *E. coli* O80:H2. *J. Appl. Microbiol.* **2021**, *130*, 258–264. [CrossRef]
20. Thiry, D.; Saulmont, M.; Takaki, S.; De Rauw, K.; Duprez, J.-N.; Iguchi, A.; Piérard, D.; Mainil, J.G. Enteropathogenic *Escherichia coli* O80:H2 in young calves with diarrhea, Belgium. *Emerg. Infect. Dis.* **2017**, *23*, 2093–2095. [CrossRef]
21. Habets, A.; Engelen, F.; Duprez, J.-N.; Devleesschauwer, B.; Heyndrickx, M.; De Zutter, L.; Thiry, D.; Cox, E.; Mainil, J. Identification of Shigatoxigenic and enteropathogenic *Escherichia coli* serotypes in healthy young dairy calves in Belgium by recto-anal mucosal swabbing. *Vet. Sci.* **2020**, *7*, 167. [CrossRef]
22. Thiry, D.; De Rauw, K.; Takaki, S.; Duprez, J.-N.; Iguchi, A.; Piérard, D.; Korsak, N.; Mainil, J.G. Low prevalence of the "gang of seven" and absence of the O80:H2 serotypes among Shigatoxigenic and enteropathogenic *Escherichia coli* (STEC and EPEC) in intestinal contents of healthy cattle at two slaughterhouses in Belgium in 2014. *J. Appl. Microbiol.* **2018**, *124*, 867–873. [CrossRef] [PubMed]
23. Bizot, E.; Cointe, A.; Smadja, N.; Sergentet, D.; Lefèvre, S.; Weill, F.-X.; Levy, C.; Cohen, R.; Mariani-Kurkdjian, P.; Bonacorsi, S. Improved molecular diagnosis and culture of the emerging heteropathotype enterohemorrhagic *Escherichia coli* O80:H2 using its non-melibiose-fermenting and antibiotic-resistance properties. *J. Clin. Microbiol.* **2022**, *60*, e0153021. [CrossRef] [PubMed]
24. Iguchi, A.; Iyoda, S.; Seto, K.; Morita-Ishihara, T.; Scheutz, F.; Ohnishi, M. Pathogenic *E. coli* Working Group in Japan. *Escherichia coli* O-genotyping PCR: A comprehensive and practical platform for molecular O serogrouping. *J. Clin. Microbiol.* **2015**, *53*, 2427–2432. [CrossRef] [PubMed]
25. Banjo, M.; Iguchi, A.; Seto, K.; Kikuchi, T.; Harada, T.; Scheutz, F.; Iyoda, S. *Escherichia coli* H-genotyping PCR: A complete and practical platform for molecular H typing. *J. Clin. Microbiol.* **2018**, *56*, e00190-18. [CrossRef]
26. Prjibelski, A.; Antipov, D.; Meleshko, D.; Lapidus, A.; Korobeynikov, A. Using SPAdes *de novo* assembler. *Curr. Protoc. Bioinform.* **2020**, *70*, e102. [CrossRef] [PubMed]
27. Tanizawa, Y.; Fujisawa, T.; Nakamura, Y. DFAST: A flexible prokaryotic genome annotation pipeline for faster genome publication. *Bioinformatics* **2018**, *34*, 1037–1039. [CrossRef] [PubMed]
28. Joensen, K.G.; Tetzschner, A.M.; Iguchi, A.; Aarestrup, F.M.; Scheutz, F. Rapid and easy in silico serotyping of *Escherichia coli* using whole genome sequencing (WGS) data. *J. Clin. Microbiol.* **2015**, *53*, 2410–2426. [CrossRef]
29. Wirth, T.; Falush, D.; Lan, R.; Colles, F.; Mensa, P.; Wieler, L.H.; Karch, H.; Reeves, P.R.; Maiden, M.C.J.; Ochman, H.; et al. Sex and virulence in *Escherichia coli*: An evolutionary perspective. *Mol. Microbiol.* **2006**, *60*, 1136–1151. [CrossRef]
30. Carattoli, A.; Zankari, E.; Garcia-Fernandez, A.; Voldby Larsen, M.; Lund, O.; Villa, L.; Aarestrup, F.M.; Hasman, H. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* **2014**, *58*, 3895–3903. [CrossRef]
31. Joensen, K.G.; Scheutz, F.; Lund, O.; Hasman, H.; Kaas, R.S.; Nielsen, E.M.; Aarestrup, F.M. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* **2014**, *52*, 1501–1510. [CrossRef]
32. Bortolaia, V.; Kaas, R.F.; Ruppe, E.; Roberts, M.C.; Schwarz, S.; Cattoir, V.; Philippon, A.; Allesoe, R.L.; Rebelo, A.R.; Florensa, A.R.; et al. ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* **2020**, *75*, 3491–3500. [CrossRef] [PubMed]
33. Page, A.J.; Cummins, C.A.; Hunt, M.; Wong, V.K.; Reuter, S.; Holden, M.T.G.; Fookes, M.; Falush, D.; Keane, J.A.; Parkhill, J. Roary: Rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **2015**, *31*, 3691–3693. [CrossRef] [PubMed]
34. Page, A.J.; Taylor, B.; Delaney, A.J.; Soares, J.; Seemann, T.; Keane, J.A.; Harris, S.R. SNP-sites: Rapid efficient extraction of SNPs from multi-FASTA alignments. *Microb. Genom.* **2016**, *2*, e000056. [CrossRef]
35. Stamatakis, A. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **2006**, *22*, 2688–2690. [CrossRef]

36. Waters, N.R.; Abram, F.; Brennan, F.; Holmes, A.; Pritchard, L. Easy phylotyping of *Escherichia coli* via the EzClermont web app and command-line tool. *Access Microbiol.* **2020**, *2*, acmi000143. [CrossRef]
37. Letunic, I.; Bork, P. Interactive Tree Of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* **2021**, *49*, W293–W296. [CrossRef]
38. Verhaegen, B.; De Reu, K.; Heyndrickx, M.; De Zutter, L. Comparison of six chromogenic agar media for the isolation of a boroa variety of non-O157 Shigatoxin-producing *Escherichia coli* (STEC) serogroups. *Int. J. Environ. Res. Public Health* **2015**, *12*, 6965–6978. [CrossRef] [PubMed]
39. Gigliucci, F.; van Hoek, A.H.A.M.; Chiani, P.; Knijn, A.; Minelli, F.; Scavia, G.; Franz, E.; Morabito, S.; Michelacci, V. Genomic characterization of *hlyF*-positive Shiga toxin-producing *Escherichia coli*, Italy and the Netherlands, 2000–2019. *Emerg. Infect. Dis.* **2021**, *27*, 853–861. [CrossRef]
40. Zhu, F.; Rogelj, S.; Kieft, T. Rapid detection of *Escherichia coli* O157:H7 by immunomagnetic separation and real-time PCR. *Int. J. Food Microbiol.* **2005**, *99*, 47–57. [CrossRef]
41. Fratamico, P.M.; Bagi, L.K.; Cray, W.J., Jr.; Narang, N.; Yan, X.; Medina, M.; Liu, Y. Detection by multiplex real-time polymerase chain reaction assays and isolation of Shiga toxin-producing Escherichia coli serogroups O26, O45, O103, O111, O121, and O145 in ground beef. *Foodb. Pathog. Dis.* **2011**, *8*, 601–607. [CrossRef]
42. Penaranda, M.E.; Evans, D.G.; Murray, B.E.; Evans, D.J., Jr. ST:LT:CFA/II plasmids in enterotoxigenic *Escherichia coli* belonging to serogroups 06, 08, 080, 085, and 0139. *J. Bacteriol.* **1983**, *154*, 980–983. [CrossRef] [PubMed]
43. Mainil, J.G.; Fairbrother, J.M. Pathogenic *Escherichia coli* in domestic mammals and birds. In *Pathogenic Escherichia coli: Molecular and Cellular Microbiology*; Morabito, S., Ed.; Caister Academic Press: Norfolk, UK, 2014; pp. 19–43, ISBN 978-1-908230-37-9.
44. Murase, K.; Martin, P.; Porcheron, G.; Houle, S.; Helloin, E.; Pénary, M.; Nougayrède, J.-P.; Dozois, C.M.; Hayashi, T.; Oswald, E. HlyF produced by extraintestinal pathogenic *Escherichia coli* is a virulence factor that regulates outer membrane vesicle biogenesis. *J. Infect. Dis.* **2016**, *213*, 856–865. [CrossRef] [PubMed]
45. Kakoullis, L.; Papachristodoulou, E.; Chra, P.; Panos, G. Mechanisms of antibiotic resistance in important Gram-positive and Gram-negative pathogens and novel antibiotic solutions. *Antibiotics* **2021**, *10*, 415. [CrossRef] [PubMed]

STUDY 2. Alternative specific method for isolation of AE-STEC and EPEC O80:H2 from healthy adult cattle

PUBLICATION 2

Non-Melibiose Fermentation and Tellurite Resistance by Enteropathogenic and Shigatoxigenic *Escherichia coli* O80:H2 from Diseased Calves: Comparison with Human Shigatoxigenic *E. coli* O80:H2

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Article

Non-Melibiose Fermentation and Tellurite Resistance by Shigatoxigenic and Enteropathogenic *Escherichia coli* O80:H2 from Diseased Calves: Comparison with Human Shigatoxigenic *E. coli* O80:H2

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Simple Summary: Although healthy cattle is the main reservoir of Attaching-Effacing Shigatoxigenic *Escherichia coli* (AE-STEC), the source of contamination of humans by AE-STEC O80:H2 remains unidentified, due in part to the absence of specific selective growth methodology. The aim of this study was to assess a procedure based on non-melibiose fermentation and resistance to tellurite to isolate AE-STEC and enteropathogenic (EPEC) O80:H2 from healthy cattle. If the 40 calf and human AE-STEC and EPEC O80:H2 did not harbor the *mel* operon, only 16 *stx1a/stx2a* AE-STEC and EPEC O80:H harbored one *ter*-type 1 operon. The 21 calf strains were further tested phenotypically: none fermented melibiose while 10 of the 11 *ter*-type 1-positive strains had Minimal Inhibitory Concentrations (MIC) $\geq$ 128 µg/mL. In contrast, the 10 *ter*-negative strains had MIC of 2 µg/mL. Accordingly, enrichment broths containing two µg/mL of potassium tellurite and inoculated with one high MIC ($\geq$256 µg/mL) AE-STEC tested positive with the O80 PCR after overnight growth, but not the enrichment broths inoculated with one low MIC (two µg/mL) EPEC. As a conclusion, this procedure may help to isolate most *stx1a/stx2a* AE-STEC and EPEC O80:H2, but not *stx2d* AE-STEC that are not resistant to tellurite.

Abstract: Despite their prevalence in Europe, the source of contamination of humans by Attaching-Effacing Shigatoxigenic *Escherichia coli* (AE-STEC) O80:H2 remains unidentified. This study aimed to assess a procedure based on non-melibiose fermentation and resistance to tellurite to isolate AE-STEC and enteropathogenic (EPEC) O80:H2 from healthy cattle. The genome sequences of 40 calf and human AE-STEC and EPEC O80:H2 were analyzed: (i) none harbored the *mel* operon, but the *70mel* DNA sequence instead; (ii) the *ter*-type 1 operon was detected in 16 EPEC and *stx1a* or *stx2a* AE-STEC, while no *ter*-type 1 operon was detected in the remaining 24 EPEC and *stx2d* AE-STEC. The 21 calf AE-STEC and EPEC O80:H2 were tested phenotypically: (i) none fermented melibiose on melibiose-MacConkey agar plates; (ii) ten of the 11 *ter*-type 1-positive strains had Minimal Inhibitory Concentrations (MIC) $\geq$ 128 µg/mL to potassium tellurite; (iii) conversely, the ten *ter*-negative strains had MIC of two µg/mL. Accordingly, enrichment broths containing two µg/mL of potassium tellurite and inoculated with one high MIC ($\geq$256 µg/mL) *stx1a*

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AE-STEC O80:H2 tested positive with the O80 PCR after overnight growth, but not the enrichment broths inoculated with one low MIC (two µg/mL) EPEC. Nevertheless, neither AE-STEC nor EPEC O80:H2 were recovered from 96 rectal fecal samples collected from healthy cattle at one slaughterhouse after overnight growth under the same conditions. In conclusion, this procedure may help to isolate *stx1a* and *stx2a* AE-STEC and EPEC O80:H2, but not *stx2d* AE-STEC that are tellurite sensitive, and new surveys using different procedures are necessary to identify their animal source, if any.

Keywords: Shigatoxigenic *E. coli*; enteropathogenic *E. coli*; serotype O80:H2; melibiose fermentation; tellurite resistance; healthy cattle; zoonosis

1. Introduction

Enterohemorrhagic *Escherichia* (*E.*) *coli* (EHEC) are a major cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans and are also associated with diarrhea in young calves [1,2]. Since EHEC produce Shiga toxins (Stx1 and/or Stx2) and the Attaching and Effacing (A/E) lesion of enteropathogenic *E. coli* (EPEC), they are named "Attaching-Effacing Shigatoxigenic *E. coli*" or "AE-STEC" in this manuscript after these two pathogenic traits, as previously proposed [3].

The most frequent and pathogenic AE-STEC in humans belong to the following major O:H serotypes: O26:H11, O103:H2, O111:H-, O121:H19, O145:H-, and O157:H7 [1,4]. The main source of infection in humans are foodstuffs contaminated by intestinal contents of ruminants, such as cattle, that can be asymptomatic carriers in their intestines [1]. Serotypes O26:H11 and O111:H- are also most frequent amongst calf AE-STEC [2,5]. Besides these major AE-STEC O:H serotypes, minor ones can emerge from time to time and cause short-lived dramatic outbreaks [6] or long-lasting clinical rates [7].

Since 2010, AE-STEC serotype O80:H2 has been emerging in France in humans suffering not only from HC and HUS, but also from systemic infection [8–10]. In 2022, AE-STEC O80:H2 was the third leading cause of HUS in Europe, especially in young children and the elderly, behind AE-STEC O157:H7 and O26:H11 [11]. In parallel, AE-STEC and EPEC O80:H2 have also been increasingly isolated in Belgium from young calves (<3 months of age) with diarrhea or sepsis [12,13]. Calf AE-STEC and EPEC O80:H2 are related to human AE-STEC O80:H2 not only by their Sequence Type (ST301) and virulotypes but also in whole genome sequence-based phylogenetic analysis [13,14], showing the importance of this serotype as a putative agent of a serious zoonosis.

Although AE-STEC and EPEC O80:H2 have been sporadically isolated from healthy cattle and dairy products in some European countries [8,9,14], the actual source of contamination of humans is yet to be identified [10]. The lack of appropriate selective growth media is among the possible reasons. Recently, a piperacillin-supplemented melibiose-MacConkey (mel-MAC) agar was developed to isolate AE-STEC O80:H2 from diseased humans, as they do not ferment melibiose following the deletion of the *mel* operon, along with the insertion of a 70 bp DNA fragment (*70mel*) of unknown origin and function [15]. Since ureidopenicillins are not permitted in veterinary medicine in European Union (Implementing regulation—2022/1255—EN—EUR-Lex (europa.eu)) and the antibiotic resistance profile of *E. coli* from healthy cattle is difficult to predict, the general aim of this study was to assess non-melibiose fermentation (non-MF), as published by others [15], and tellurite resistance (TeR) to increase the isolation rate of AE-STEC and EPEC O80:H2 from healthy cattle. Although the resistance of AE-STEC and EPEC O80:H2 to tellurite is unknown, several other STEC and EPEC serotypes are indeed highly resistant to tellurite with Minimal

Inhibitory Concentrations (MIC) > 16 µg/mL [16,17]. Therefore, different Te-supplemented growth media exist to specifically isolate the major O:H serotypes, like the cefixime-tellurite sorbitol MacConkey (CT-SMAC) agar for AE-STEC O157:H7 [18]. High levels of TeR of AE-STEC is linked to the presence of a *ter* operon that comprises six genes (*terA*, *terB*, *terC*, *terD*, *terE*, and *terZ*) and whose four variants (type 1–4) have been described [18,19].

The specific aims of this study were to: (i) detect the presence of the *70mel* fragment and of an intact *ter* operon in Belgian calf and human AE-STEC and EPEC O80:H2; (ii) confirm the non-MF by calf AE-STEC and EPEC O80:H2 and identify their MIC to tellurite; and (iii) perform a preliminary survey to assess these two properties to isolate AE-STEC and/or EPEC O80:H2 from feces collected from healthy cattle in one slaughterhouse.

2. Materials and Methods

2.1. Escherichia coli O80 Strains

A total of 44 *E. coli* O80 isolated in Belgium were studied for MF and TeR: 10 AE-STEC and 11 EPEC O80:H2 isolated from diarrheic < 3 month-old calves, 19 AE-STEC O80:H2 isolated from humans with (bloody) diarrhea and sometimes HUS, and four non-EPEC non-STEC *E. coli* O80:H6 and O80:H45 isolated from healthy adult cattle in slaughterhouses and in farms. All genome sequences are already available on the National Centre for Biotechnology Information (NCBI), BioProjects PRJNA606200 and PRJNA906740 [13,20,21].

The 40 calf and human AE-STEC and EPEC O80:H2 studied are classified into two main lineages (L) in a Single Nucleotide Polymorphism (SNP)-based phylogenetic tree [13]. The L1 lineage is subdivided into four sub-lineages (SL): SL1.1 with eight calf EPEC and five calf and human *stx1a* or *stx2a* AE-STEC, SL1.2 with 22 calf and human *stx2d* AE-STEC and three calf EPEC, SL1.3 with one human *stx2a* AE-STEC, and SL1.4 with 2 calf *stx2d* AE-STEC. The L2 lineage comprises two *stx1a* AE-STEC isolated from the same calf in 1987. The four *E. coli* O80:non-H2 are genetically not related to *E. coli* O80:H2 [21].

2.2. Genetic Studies

2.2.1. MF-Encoding *mel* Operon and the *70mel* DNA Sequence

The DNA sequences of the three genes of the *mel* operon (*melA*, *melB*, and *melR*) and of the *70mel* fragment were obtained from the genome sequences of the *E. coli* K-12 MG1655 laboratory strain (BioProject accession number SAMN13412807) and of the AE-STEC O80:H2 RDEx444 strain (BioProject accession number SAMN08915508), respectively [15]. The detection of the *mel* operon and of the *70mel* DNA fragment in the 44 *E. coli* O80 strains was performed using the Basic Local Alignment Search tool for DNA comparison (BLASTN). The cut-off values were ≥90%, as much for the query coverage rate than for the percentage identity (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 25 January 2023).

2.2.2. Tellurite Resistance-Encoding *ter* Operon

The original DNA sequences of the six genes of the *ter* operon (*terA*, *terB*, *terC*, *terD*, *terE*, and *terZ*) were obtained from the genome sequence of the AE-STEC O157:H7 Sakai strain (Bioproject accession number SAMN01911278) [19] and of the six genes of each of the four types of the *ter* operon from the BioProject PRJDB10561 [22]: NZ_CP02355.1 (*ter*-type1); MH208235.1 (*ter*-type2); NZ_CP027591 (*ter*-type3); CP0232000.1 (*ter*-type4). The detection of the *ter* operon and of the four *ter*-types in the 44 *E. coli* O80 strains was also performed using BLASTN with the same query coverage rate and percentage identity (≥90%) as for the *mel* operon and *70mel* DNA sequence (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 31 October 2023).

2.3. Phenotypic Assays

The 21 calf *E. coli* O80:H2 and the four bovine *E. coli* O80:non-H2, but not the 19 human AE-STEC O80:H2 were phenotypically studied.

2.3.1. Melibiose Fermentation

The 25 calf and bovine *E. coli* O80 strains were streaked on MacConkey agar (Fisher Scientific, Bruxelles, Belgium) plates containing 10% D(+)-melibiose monohydrate (Thermo Scientific, Geel, Belgium), as described elsewhere [15]. MF was read after overnight growth at 37 °C.

2.3.2. Potassium Tellurite Minimal Inhibitory Concentrations

The MIC of the 25 calf and bovine *E. coli* O80 strains to tellurite were determined by the two-fold dilution method in 96-well micro-titre plates (VWR International, Leuven, Belgium) in Mueller-Hinton broth (VWR International, Leuven, Belgium) in presence of potassium tellurite (K_2TeO_3) (SIGMA-ALDRICH Chemistry, Overijse, Belgium) (256 µg/mL to 0.5 µg/mL) and of bromocresol purple as pH indicator (SIGMA-ALDRICH Chemistry, Oversijse, Belgium). After overnight incubation at 37 °C with shaking, the MIC of each strain was determined by observation of color change in the wells containing potassium tellurite (Figure 1). One *E. coli* O157:H7 strain of the Bacteriology laboratory collection harboring the six *ter* genes (*terA*, *terB*, *terC*, *terD*, *terE*, and *terZ*) and the *E. coli* K-12 DH10B laboratory strain lacking those genes, as determined by specific PCR, were the positive and negative controls, respectively [16,17,23].

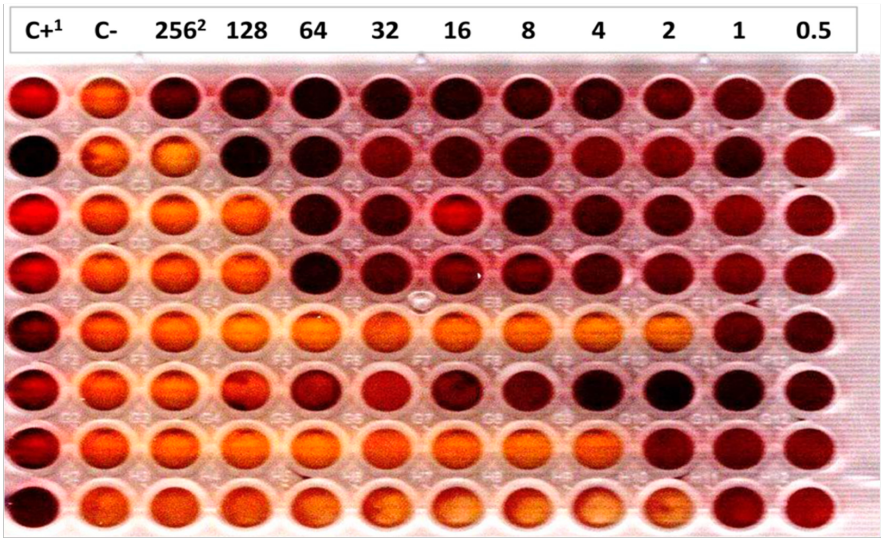

Figure 1. Minimal Inhibitory Concentration (MIC) determination to potassium tellurite in presence of bromocresol purple as a pH indicator. The change of color from yellow-orange to dark purple marks bacterial growth and by deduction the MIC (one strain per row). [1] C+: positive growth control (column 1); C-: negative growth control (column 2). [2] Potassium tellurite concentrations (µg/mL) in columns 3 to 12.

2.4. Detection Limit of E. coli O80:H2 in Fecal Material in Presence of Potassium Tellurite

Two EPEC and AE-STEC O80:H2 with different MIC to potassium tellurite were grown overnight in lauryl-sulfate enrichment (LSE) broth for enterobacteria (VWR Life Science, Leuven, Belgium). Ten-fold dilutions were performed to obtain bacterial concentrations ranging from 10^8 to 10^2 CFU per mL, as previously described [24]. One mL of each ten-fold dilution was added to eight mL of LSE broth containing two µg/mL of potassium tellurite along with one g of an O80 PCR-negative fecal sample. One g of the same fecal sample was added to nine mL of un-inoculated LSE broth containing two µg/mL of potassium tellurite, as a negative control. After overnight growth at 37 °C with shaking, total DNA was extracted from two mL of each LSE broth and tested with the O80 PCR, as previously described [21].

2.5. Attempts to Isolate of AE-STEC and EPEC O80:H2 from Bovine Fecal Samples

One g of 96 fecal samples collected in July 2023 from the rectum of healthy adult cattle at one slaughterhouse in the province of Liège, Belgium were distributed in nine mL of two LSE broths, one containing two µg/mL of potassium tellurite, as in Section 2.4. O80 PCR-positive LSE broths and colonies were identified as previously described, except that only mel-MAC agar plates were used to streak the O80 PCR-positive LSE broths. O80 PCR-positive colonies were PCR tested for the *fliC*$_{H2}$ gene encoding the H2 antigen and O80:H2-positive colonies, if any were genome sequenced for additional typing [21].

3. Results

3.1. Genetic Analysis

All 40 calf and human AE-STEC and EPEC O80:H2 strains harbored the *70mel* DNA sequence, but not the *mel* operon, while the *mel* operon, but not the *70mel* DNA sequence was detected in the four bovine *E. coli* O80:non-H2 strains (Table 1).

The six genes of the *ter* operon were detected and identified to *ter*-type 1 in the three calf *stx1a* AE-STEC, in the five human *stx1a* or *stx2a* AE-STEC and in eight of the 11 calf EPEC O80:H2 belonging to L1/SL1.1, L1/SL1.3 and L2 (Table 1). A *ter* operon was also detected and identified to *ter*-type 3 in three of the 14 human *stx2d* AE-STEC belonging to L1/SL1.2. Conversely, no *ter* operon could be detected in any of the remaining seven calf *stx2d* AE-STEC, 11 human *stx2d* AE-STEC and three calf EPEC, belonging to L1/SL1.2 and L1/SL1.4 (Table 1). The *ter* operon was not detected in the four bovine *E. coli* O80:non-H2 strains.

Table 1. Melibiose fermentation and tellurite resistance of the 44 calf and *human E. coli* O80 according to their classification in (sub-)lineages in a Single Nucleotide Polymorphism (SNP)-based phylogenetic tree, and detection of the *iha* gene.

Serotype [1]	Source [1] (No. Strains)	Virulotype [1]	Melibiose Fermentation			Tellurite Resistance		(Sub-)Lineage (L/SL) [1]	No. Strains	*iha* Gene (WGS) [1,2]
			mel Operon (WGS) [2]	70mel DNA Sequence (WGS) [2]	Melibiose McConkey [3]	*ter* Type Operon (WGS) [2,4]	Te^{++} MIC (ug/mL) [5]			
O80:H2	Calves (21)	*eae*ξ	-	+	-	+ (t1)	>256	L1/SL1.1	1	+
							256		4	
							128		2	
							8		1	
						-	2	L1/SL1.2	3	-
		*eae*ξ *stx1a*	-	+	-	+ (t1)	>256	L1/SL1.1	1	+
								L2 [6]	2	
		*eae*ξ *stx2d*	-	+	-	-	2	L1/SL1.2	5	-
								L1/SL1.4	2	+
	Humans (19)	*eae*ξ *stx1a*	-	+	ND	+ (t1)	ND	L1/SL1.1	3	+
		*eae*ξ *stx2a*	-	+	ND	+ (t1)	ND	L1/SL1.1	1	+
								L1/SL1.3	1	
		*eae*ξ *stx2d*	-	+	ND	+ (t3)	ND	L1/SL1.2	3	-
						-			11	-
O80:non-H2	Bovines	-	+	-	+	-	2–4	-	4	-

[1] from reference [13]. [2] operon/DNA sequence/gene detected (+) or not (-) after Whole Genome Sequencing (WGS). [3] melibiose fermentation (+) or not (-) after overnight growth at 37 °C on mel-MAC agar plates; ND: not done. [4] + (t1): the *ter*-type 1 operon was detected with >99.9% homology; + (t3): the *ter*-type 3 operon was detected with >99.9% homology. [5] Te^{++} MIC: Minimal Inhibitory Concentration of potassium tellurite; ND: not done.[6] *stx1a* AE-STEC EH2282 strain was erroneously referred to as *stx2a* in Figure 1 of reference [13].

3.2. Phenotypic Assays

None of the 21 calf AE-STEC and EPEC O80:H2 studied fermented melibiose on mel-MAC agar plates after overnight incubation at 37 °C (Table 1), in contrast to the four *E. coli* O80:non-H2 strains.

Regarding the MIC to potassium tellurite, the *E. coli* O157:H7 strain (positive control) had a MIC ≥ 256 µg/mL, while the *E. coli* K-12 DH10B laboratory strain (negative control) had a MIC of one µg/mL. Of the 11 calf *ter*-type 1-positive *stx1a* AE-STEC and EPEC, 10 had a very high MIC (≥128 µg/mL), while one EPEC had an intermediate MIC (eight µg/mL) (Table 1). Conversely, the 10 calf *ter*-negative *stx2d* AE-STEC and EPEC had low MIC (one-two µg/mL), like the four *ter*-negative *E. coli* O80:non-H2 (two-four µg/mL) and the negative control.

3.3. Detection Limit of E. coli O80:H2 in Bovine Fecal Material

One high MIC *stx1a* AE-STEC (MIC > 256 µg/mL) and one low MIC EPEC O80:H2 (MIC = two µg/mL) were chosen for this study. After overnight growth at 37 °C, the six enrichment broths inoculated with the highest concentrations of the *stx1a* AE-STEC tested positive with the O80 PCR. On the other hand, no positive amplification results were obtained with the seven enrichment broths inoculated with the EPEC and the negative control enrichment broth.

3.4. Attempts to Isolate AE-STEC and EPEC O80:H2 from Bovine Fecal Samples

After overnight growth at 37 °C in the presence or not of two µg/mL of potassium tellurite, 11 of the 96 (11.5%) enrichment broths (six with and five without tellurite) tested positive with the O80 PCR and were streaked on mel-MAC agar plates. After overnight growth at 37 °C, 13 of the 42 non-MF colonies picked-up (10 from tellurite-containing broths and three from tellurite-non-containing broths) tested positive with the O80 PCR, but none with the H2 PCR.

4. Discussion

Healthy cattle are the main reservoir of the major and several minor AE-STEC serotypes worldwide [1,25]. Nevertheless, the majority of the attempts using non-selective procedures to isolate AE-STEC O80:H2 from healthy cattle at slaughterhouses and from healthy cows and calves in farms have failed [8,9,14,21,26–28].

Even if *E. coli* O80 were identified like previously [21], no AE-STEC or EPEC O80:H2 could be recovered during this preliminary survey using a selective procedure based on non-MF and TeR. The reasons for these unsuccessful results can be several: (i) bovine AE-STEC and/or EPEC O80:H2 ferment melibiose and/or are sensitive to potassium tellurite; (ii) AE-STEC and/or EPEC O80:H2 are present in (very) low numbers in feces from healthy cattle; (iii) healthy cattle are not the primary reservoir of AE-STEC and/or EPEC O80:H2; (iv) the sizes of the samples during the different surveys are too small.

MF is not one of the reasons since the results of the genetic and phenotypic studies are straightforward and identical to those previously obtained on French human AE-STEC [15]: all tested Belgian calf and human EPEC and AE-STEC O80:H2 harbor the 70*mel* DNA fragment, but not the *mel* operon and no calf AE-STEC or EPEC ferment melibiose on mel-MAC agar plates.

In contrast, tellurite sensitivity is one reason, at least in part. Indeed, if all but one calf and human AE-STEC and EPEC belonging to L1/SL1.1, L1/SL1.3, and L2 (16 strains) harbor a *ter*-type1 operon, none of the calf and human AE-STEC and EPEC belonging to L1/SL1.2 and L1/SL1.4 (24 strains) do and only three human *stx2d* AE-STEC harbor a *ter*-type3 operon (Table 1). In agreement with the genetic results, all but one of the

11 *ter*-type 1-positive calf AE-STEC and EPEC tested have MIC ≥ 128 µg/mL, while the 10 *ter*-negative calf AE-STEC and EPEC tested have low MIC (one-two µg/mL) (Table 1). Lower MIC of *ter*-positive *E. coli*, like of the calf EPEC (MIC = eight µg/mL) have already been observed with some AE-STEC strains within the same serotype [16].

A second reason to explain the failure of isolating AE-STEC and EPEC O80:H2 from healthy cattle is that their numbers in feces do not reach the detection limit even after overnight growth, because their ecological niche would not be, in contrast to AE-STEC O157:H7, the recto-anal junction, but small or large intestinal segments, similarly to some other major serotypes, like O26:H- [25,29,30].

A third possible reason is that healthy cattle are not the primary reservoir of AE-STEC and/or EPEC O80:H2, in contrast to the major AE-STEC serotypes [1,25]. Not only other domestic and wild ruminants like sheep, goat, or deer, but also non-ruminants (pigs, wild boars), or even humans may represent their actual primary reservoir, like for some other AE-STEC serotypes [9,31].

Finally, the limited sizes of the fecal samples taken at slaughterhouses and farms, including in this study, could also explain the scarcity of positive isolation of AE-STEC and EPEC O80:H2 [8,9,14,21,26–28], especially since infections in humans and in calves tend to be sporadic [8,9,12,13].

Whatever the actual reason, these results imply that potassium tellurite cannot be used as a selective agent for the isolation of the great majority of AE-STEC O80:H2. This assumption is supported by the observation that two µg/mL potassium tellurite-containing enrichment broths inoculated with all but the lowest CFU concentration of the high MIC (>256 µg/mL) *stx1a* AE-STEC test positive with the O80 PCR after overnight growth, while all enrichment broths inoculated with the low MIC (two µg/mL) EPEC do not.

Besides those results, an interesting parallel observation is the simultaneous presence/absence of the *ter*-type1 operon (this study) and of the *iha* gene [13] in all but two calf and human AE-STEC and EPEC (Table 1). The two exceptions are the calf *stx2d* AE-STEC of L1/SL1.4 harboring the *iha* gene but no *ter* operon. Conversely, the *iha* gene was not detected in the three human *stx2d* AE-STEC of L1/SL1.2 harboring a *ter*-type 3 operon. The Iha (after IrgA Homologue Adhesin) adhesin protein is homologous of the Iron Regulatory Gene A (IrgA) adhesin of *Vibrio cholera* and may play a role in the intestinal colonization by AE-STEC O157:H7, although this has not been definitely demonstrated yet [32,33]. This simultaneous presence/absence of the *ter*-type 1 operon and of the *iha* gene is probably linked to their association with the prophage-like SpLE-1-like element (SpLE-1), one approximately 90 kbp prophage-like element initially identified in the AE-STEC O157:H7 Sakai strain [19,22,34–37]. Future genomic and phenotypic studies would help to confirm this hypothesis and, hopefully, to understand actual reasons, such an association between genes coding for heavy metal resistance and putative virulence factor.

5. Conclusions

As far as AE-STEC and EPEC O80:H2 are concerned, the selective isolation procedure based on non-MF and TeR may help to isolate most *stx1a* and *stx2a* AE-STEC and EPEC, but not *stx2d* AE-STEC that are not TeR.

Nevertheless, future surveys will be worth performing using either selective with different antibiotics, or non-selective procedures with fecal samples and/or samples taken from different intestinal segments (when possible) not only of healthy cattle in slaughterhouses and farms but also of other domestic and wild animal species in European countries and even of healthy humans [31,38,39].

Finally, future genome and phylogenetic analysis of more AE-STEC and EPEC O80:H2 from calves, humans and other sources should help to decipher and understand the molecular evolution of the different (sub-)lineages of this still emerging serotype.

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References

1. Tozzoli, R.; Scheutz, F. Diarrheagenic *Escherichia coli* infections in humans. In *Pathogenic Escherichia coli: Molecular and Cellular Microbiology*; Morabito, S., Ed.; Caister Academic Press: Norfolk, UK, 2014; pp. 1–18. ISBN 978-1-908230-37-9.
2. Mainil, J.G.; Fairbrother, M.J. Pathogenic *Escherichia coli* in domestic mammals and birds. In *Pathogenic Escherichia coli: Molecular and Cellular Microbiology*; Morabito, S., Ed.; Caister Academic Press: Norfolk, UK, 2014; pp. 19–43. ISBN 978-1-908230-37-9.
3. Piérard, D.; De Greve, H.; Haesebrouck, F.; Mainil, J. O157:H7 and O104:H4 Vero/Shiga toxin-producing *Escherichia coli* outbreaks: Respective role of cattle and humans. *Vet. Res.* **2012**, *43*, 13. [CrossRef] [PubMed]
4. Karmali, M.A.; Mascarenhas, M.; Shen, S.; Ziebell, K.; Johnson, S.; Reid-Smith, R.; Isaac-Renton, J.; Clark, C.; Rahn, K.; Kaper, J.B. Association of genomic O island 122 of *Escherichia coli* EDL 933 with Verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J. Clin. Microbiol.* **2003**, *41*, 4930–4940. [CrossRef]
5. Fakih, I.; Thiry, D.; Duprez, J.-N.; Saulmont, M.; Iguchi, A.; Piérard, D.; Jouant, L.; Daube, G.; Ogura, Y.; Hayashi, T.; et al. Identification of Shiga toxin-producing (STEC) and enteropathogenic (EPEC) *Escherichia coli* in diarrhoeic calves and comparative genomics of O5 bovine and human STEC. *Vet. Microbiol.* **2017**, *202*, 16–22. [CrossRef]
6. Navarro-Garcia, F. *Escherichia coli* O104:H4 pathogenesis: An enteroaggregative *E. coli* /Shiga toxin-producing *E. coli* explosive cocktail of high virulence. *Microbiol. Spectr.* **2014**, 2, EHEC-0008-2013. [CrossRef]
7. Bruyand, M.; Mariani-Kurkdjian, P.; Le Hello, S.; King, L.-A.; Van Cauteren, D.; Lefevre, S.; Gouali, M.; Jourdan-da Silva, N.; Mailles, A.; Donguy, M.-P.; et al. Paediatric haemolytic uraemic syndrome related to Shiga toxin-producing *Escherichia coli*, an overview of 10 years of surveillance in France, 2007 to 2016. *Euro. Surveill.* **2019**, *24*, 1800068. [CrossRef] [PubMed]
8. Soysal, N.; Mariani-Kurkdjian, P.; Smail, Y.; Liguori, S.; Gouali, M.; Loukiadis, E.; Fach, P.; Bruyand, M.; Blanco, J.; Bidet, P.; et al. Enterohemorrhagic *Escherichia coli* hybrid pathotype O80:H2 as a new therapeutic challenge. *Emerg. Infect. Dis.* **2016**, *22*, 1604–1612. [CrossRef] [PubMed]
9. Cointe, A.; Birgy, A.; Mariani-Kurkdjian, P.; Liguori, S.; Courroux, C.; Blanco, J.; Delannoy, S.; Fach, P.; Loukiadis, E.; Bidet, P.; et al. Emerging multidrug-resistant hybrid pathotype Shiga toxin-producing *Escherichia coli* O80 and related strains of Clonal Complex 165, Europe. *Emerg. Infect. Dis.* **2018**, *24*, 2262–2269. [CrossRef]

10. ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail), Avis relatif à la définition des souches pathogènes d'*Escherichia coli* productrices de Shiga-toxines, Saisine n° 2020-SA-0095, n°2016-SA-0121, 2010-SA-0031. 2023. Available online: https://www.anses.fr/fr/system/files/BIORISK2020SA0095.pdf (accessed on 3 June 2024).
11. EFSA-ECDC (European Food Safety Authority—European Centre for Disease Prevention and Control). The European Union one health 2022 zoonoses report. *EFSA J.* **2023**, *21*, e8442. [CrossRef]
12. Thiry, D.; Saulmont, M.; Takaki, S.; De Rauw, K.; Duprez, J.-N.; Iguchi, A.; Piérard, D.; Mainil, J.G. Enteropathogenic *Escherichia coli* O80:H2 in young calves with diarrhea, Belgium. *Emerg. Infect. Dis.* **2017**, *23*, 2093–2095. [CrossRef]
13. Habets, A.; Crombé, F.; Nakamura, K.; Guérin, V.; De Rauw, K.; Piérard, D.; Saulmont, M.; Hayashi, T.; Mainil, J.G.; Thiry, D. Genetic characterization of Shigatoxigenic and enteropathogenic *Escherichia coli* O80:H2 from diarrhoeic and septicaemic calves and relatedness to human Shigatoxigenic *E. coli* O80:H2. *J. Appl. Microbiol.* **2021**, *130*, 258–264. [CrossRef]
14. Soleau, N.; Ganet, S.; Werlen, S.; Collignon, L.; Cointe, A.; Bonacorsi, S.; Sergentet, D. First Isolation of the heteropathotype Shiga toxin-producing and extra-intestinal pathogenic (STEC-ExPEC) *E. coli* O80:H2 in French healthy cattle: Genomic characterization and phylogenetic position. *Int. J. Mol. Sci.* **2024**, *25*, 5428. [CrossRef] [PubMed]
15. Bizot, E.; Cointe, A.; Smadja, N.; Sergentet, D.; Lefèvre, S.; Weill, F.-X.; Levy, C.; Cohen, R.; Mariani-Kurkdjian, P.; Bonacorsi, S. Improved molecular diagnosis and culture of the emerging heteropathotype enterohemorrhagic *Escherichia coli* O80:H2 using its non-melibiose-fermenting and antibiotic-resistance properties. *J. Clin. Microbiol.* **2022**, *60*, e0153021. [CrossRef]
16. Kerangart, S.; Douëllou, T.; Delannoy, S.; Fach, P.; Beutin, L.; Sergentet-Thévenot, D.; Cournoyer, B.; Loukiadis, E. Variable tellurite resistance profiles of clinically-relevant Shiga toxin-producing *Escherichia coli* (STEC) influence their recovery from foodstuffs. *Food Microbiol.* **2016**, *59*, 32–42. [CrossRef] [PubMed]
17. Lewis, G.L.; Jorgensen, Q.R.; Loy, J.D.; Moxley, R.A. Tellurite resistance in Shiga toxin-producing *Escherichia coli*. *Curr. Microbiol.* **2018**, *75*, 752–759. [CrossRef] [PubMed]
18. Fan, R.; Bai, X.; Fu, S.; Xu, Y.; Sun, H.; Wang, H.; Xiong, Y. Tellurite resistance profiles and performance of different chromogenic agars for detection of non-O157 Shiga toxin-producing *Escherichia coli*. *Int. J. Food. Microbiol.* **2018**, *266*, 295–300. [CrossRef]
19. Hayashi, T.; Makino, K.; Ohnishi, M.; Kurokawa, K.; Ishii, K.; Yokoyama, K.; Han, C.G.; Ohtsubo, E.; Nakayama, K.; Murata, T.; et al. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res.* **2001**, *8*, 11–22. [CrossRef]
20. De Rauw, K.; Thiry, D.; Caljon, B.; Saulmont, M.; Mainil, J.; Piérard, D. Characteristics of Shiga toxin-producing and enteropathogenic *Escherichia coli* of the emerging serotype O80:H2 isolated from humans and diarrheic calves in Belgium. *Clin. Microbiol. Infect.* **2019**, *25*, 111.e5–111.e8. [CrossRef]
21. Ikeda, R.; Nakamura, K.; Saulmont, M.; Habets, A.; Duprez, J.-N.; Korsak, N.; Hayashi, T.; Thiry, D.; Mainil, J.G. *Escherichia coli* O80 in healthy cattle: Absence of Shigatoxigenic and enteropathogenic *E. coli* O80:H2 and (phylo)genomics of non-clonal complex 165 *E. coli* O80. *Microorganisms* **2023**, *11*, 230. [CrossRef]
22. Nguyen, T.T.H.; Kikuchi, T.; Tokunaga, T.; SIyoda, S.; Iguchi, A. Diversity of the tellurite resistance gene operon in *Escherichia coli*. *Front. Microbiol.* **2021**, *12*, 681175. [CrossRef]
23. Taylor, D.E.; Rooker, M.; Keelan, M.; Ng, L.-K.; Martin, I.; Perna, N.T.; Burland, N.T.V.; Blattner, F.R. Genomic variability of O islands encoding tellurite resistance in enterohemorrhagic *Escherichia coli* O157:H7 isolates. *J. Bacteriol.* **2002**, *184*, 4690–4698. [CrossRef]
24. Ikeda, R.; Laforêt, F.; Antoine, C.; Adachi, M.; Nakamura, K.; Habets, A.; Kler, C.; De Rauw, K.; Hayashi, T.; Mainil, J.M.; et al. Virulence of Shigatoxigenic and enteropathogenic *Escherichia coli* O80:H2 in *Galleria mellonella* larvae: Comparison of the roles of the pS88 plasmids and STX2d phage. *Vet. Sci.* **2023**, *10*, 420. [CrossRef] [PubMed]
25. Menge, C. The role of *Escherichia coli* Shiga toxins in STEC colonization of cattle. *Toxins* **2020**, *12*, 607. [CrossRef]
26. Blanco, M.; Blanco, J.E.; Mora, A.; Dahbi, G.; Alonso, M.P.; González, E.A.; Bernárdez, M.I.; Blanco, J. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (*eae-xi*). *J. Clin. Microbiol* **2004**, *42*, 645–651. [CrossRef]
27. Thiry, D.; De Rauw, K.; Takaki, S.; Duprez, J.-N.; Iguchi, A.; Piérard, D.; Korsak, N.; Mainil, J.G. Low prevalence of the "gang of seven" and absence of the O80:H2 serotypes among Shigatoxigenic and enteropathogenic *Escherichia coli* (STEC and EPEC) in intestinal contents of healthy cattle at two slaughterhouses in Belgium in 2014. *J. Appl. Microbiol.* **2018**, *124*, 867–873. [CrossRef]
28. Habets, A.; Engelen, F.; Duprez, J.-N.; Devleesschauwer, B.; Heyndrickx, M.; De Zutter, L.; Thiry, D.; Cox, E.; Mainil, J. Identification of Shigatoxigenic and enteropathogenic *Escherichia coli* serotypes in healthy young dairy calves in Belgium by recto-anal mucosal swabbing. *Vet. Sci.* **2020**, *7*, 167. [CrossRef] [PubMed]
29. van Diemen, P.M.; Dziva, F.; Stevens, M.P.; Wallis, T.S. Identification of enterohemorrhagic *Escherichia coli* O26:H- genes required for intestinal colonization in calves. *Infect. Immun.* **2005**, *73*, 1735–1743. [CrossRef] [PubMed]
30. Girard, F.; Dziva, F.; van Diemen, P.; Phillips, A.D.; Stevens, M.P.; Frankel, G. Adherence of enterohemorrhagic *Escherichia coli* O157, O26, and O111 strains to bovine intestinal explants *ex vivo*. *Appl. Environ. Microbiol.* **2007**, *73*, 3084–3090. [CrossRef]

31. Ray, R.; Singh, P. Prevalence and implications of Shiga toxin-producing *E. coli* in farm and wild ruminants. *Pathogens* **2022**, *11*, 1332. [CrossRef]
32. Tarr, P.I.; Bilge, S.S.; Vary, J.C.; Jelacic, S.; Habeeb, R.L.; Ward, T.R.; Baylor, M.R.; Besser, T.E. Iha: A novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect. Immun.* **2000**, *68*, 1400–1407. [CrossRef]
33. McWilliams, B.D.; Torres, A.G. Enterohemorrhagic *Escherichia coli* adhesins. *Microbiol. Spectrum* **2014**, *2*, EHEC-0003-2013. [CrossRef]
34. Ohnishi, M.; Terajima, J.; Kurokawa, K.; Nakayama, K.; Murata, T.; Tamura, K.; Ogura, Y.; Watanabe, H.; Hayashi, T. Genomic diversity of enterohemorrhagic *Escherichia coli* O157 revealed by whole genome PCR scanning. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 17043–17048. [CrossRef] [PubMed]
35. Ogura, Y.; Ooka, T.; Asadulghani, M.; Terajima, J.; Nougayrède, J.-P.; Kurokawa, K.; Tashiro, K.; Tobe, T.; Nakayama, K.; Kuhara, S.; et al. Extensive genomic diversity and selective conservation of virulence-determinants in enterohemorrhagic *Escherichia coli* strains of O157 and non-O157 serotypes. *Genome Biol.* **2007**, *8*, R138. [CrossRef] [PubMed]
36. Bielaszewska, M.; Middendorf, B.; Tarr, P.I.; Zhang, W.; Prager, R.; Aldick, T.; Dobrindt, U.; Karch, H.; Mellmann, A. Chromosomal instability in enterohaemorrhagic *Escherichia coli* O157:H7: Impact on adherence, tellurite resistance and colony phenotype. *Mol. Microbiol.* **2011**, *79*, 1024–1044. [CrossRef] [PubMed]
37. Lorenz, S.C.; Gonzalez-Escalona, N.; Kotewicz, M.L.; Fischer, M.; Kase, J.A. Genome sequencing and comparative genomics of enterohemorrhagic *Escherichia coli* O145:H25 and O145:H28 reveal distinct evolutionary paths and marked variations in traits associated with virulence & colonization. *BMC Microbiol.* **2017**, *17*, 183. [CrossRef]
38. Martin, A.; Beutin, L. Characteristics of Shiga toxin-producing *Escherichia coli* from meat and milk products of different origins and association with food producing animals as main contamination sources. *Int. J. Food. Microbiol.* **2011**, *146*, 99–104. [CrossRef]
39. Díaz-Sánchez, S.; Sánchez, S.; Sánchez, M.; Herrera-León, S.; Hanning, I.; Vidal, D. Detection and characterization of Shiga toxin-producing *Escherichia coli* in game meat and ready-to-eat meat products. *Int. J. Food Microbiol.* **2012**, *160*, 179–182. [CrossRef]

PART 2: Confirmation by whole genome sequencing analysis of the distribution and intermixing of human and calf AE-STEC and EPEC O80:H2 in a Single Nucleotide Polymorphism (SNP)-based phylogenetic classification, of the identity and distribution of the virulence-associated genes, including the differences in pS88 plasmid gene profiles, and of the localization of different virulence-associated genes in two calf *stx2f* AE-STEC O80:H2.

FOREWORD

In Europe, AE-STEC O80:H2 has been reported as the second or third leading cause of HUS, alongside O157 and O26 (EFSA, 2021, 2022, 2023). Due to their specific pS88 plasmid encoding putative virulence genes of ExPEC, AE-STEC O80:H2 also contribute to systemic infections such as septicaemia and bacteraemia (Peigne et al., 2009; Soysal et al., 2016; Wijnsma et al., 2017). In Belgium, AE-STEC and EPEC O80:H2 strains from humans and calves are genetically close at the SNP level, with a specific gene profile particularly involving the presence/absence of two pS88 plasmid virulence-associated genes (*etsC* and *iucC*) and of two other genes (*cma* and *iha*) (Habets et al., 2021).

The aim of this study was to confirm the phylogenetic classification of the population of AE-STEC and EPEC O80:H2 by analysing more strains isolated from humans and calves in Belgium and to analyse the complete genomes of two calf *stx2f* AE-STEC with different virulotypes, to characterize the localization of several virulence-associated genes, including *stx2f*, and other genes, including *cma* and *iha*, after long read sequencing.

The re-constructed maximum likelihood tree showed that 96% of the total 129 AE-STEC and EPEC O80:H2 strains were distributed into two major sub-lineages, as previously reported by Habets and collaborators (Habets et al., 2021): L1/SL1.1 (“EPEC lineage”) and L1/SL1.2 (“AE-STEC lineage”). All but one strains consistently harbored T3SS-encoding genes (non)-LEE and (*eaeξ*, *espA*, *espB*, *espF*, *tir*/*nleA*, *nleB*, *nleC*) and pS88-associated genes (*hlyF*, *ompTp*, *iss*, *iroN*, and *sitA*). However, the *etsC* and *iucC*

genes were predominantly found in L1/SL1.2 strains, while they were almost undetectable in L1/SL1.1 strains. Conversely, the *cma* and *iha* genes were frequently observed in L1/SL1.1 strains and not in L1/ SL1.2 strains. The *ter* resistance operon was also much more frequently identified in L1/SL1.1 strains and not in L1/SL1.2 strains. The simultaneous presence/absence of the *iha* gene and *ter* operon is probably linked with their association on the Sakai Prophage-like element 1 (SpLE1) (Bielaszewska et al., 2011; Hayashi et al., 2000).

The complete genome sequences of the two calf *stx2f* AE-STEC strains belonging to L1/SL1.1 and SL1.2 respectively showed both similarities and differences; one of the duplicated Stx2f phage was integrated into another prophage genome, which itself was integrated into the chromosome. The LEE were identical, and both strains harboured one pS88 plasmid. Nevertheless, the *etsC* and *iucC*, but not the *cma* genes were detected and located on the pS88 plasmid in the *stx2f* AE-STEC O80:H2 belonging to L1/SL1.2, while only the *cma* genes was detected and also located on the pS88-like plasmid in the *stx2f* AE-STEC O80:H2 belonging to L1/SL1.1. The *iha* gene and *ter* operon were also detected in the *stx2f* AE-STEC O80:H2 belonging to L1/SL1.1, but not in the *stx2f* AE-STEC O80:H2 belonging to L1/SL1.2.

These findings confirm population structure and the genetic characteristics of Belgian human and calf AE-STEC and EPEC O80:H2. It suggests that the differences in the gene profiles between AE-STEC and EPEC O80:H2 were influenced by their chromosomal and plasmid localization. Moreover, the analysis of STX2f phage emphasizes the need for further studies to elucidate the evolutionary dynamics and clinical implications of AE-STEC and EPEC O80:H2 strains.

STUDY 3. Population structure and comparative genomics of AE-STEC and EPEC O80:H2 isolated in Belgium from calves and humans.

- POPULATION STRUCTURE: RESULTS PRESENTED IN PART IN A POSTER DURING THE "COLLOQUE INTERNATIONAL FRANCOPHONE EN MALADIES INFECTIEUSES ANIMALES", RABAT 2025.

- COMPARATIVE GENOMICS: UNPUBLISHED STUDY

Phylogenetic Population Structure of Enteropathogenic and Shigatoxigenic *Escherichia coli* O80:H2 Isolated in Belgium from Diarrheic Calves and Diseased Humans and Comparative Genomics of Two *stx2f* Shigatoxigenic *E. coli* O80:H2

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Keywords: *Escherichia coli*, EPEC and AE-STEC, serotype O80:H2, *stx2f,* humans, calves

Abstract

Attaching-effacing and Shiga toxin-producing *Escherichia coli* (AE-STEC) serotype O80:H2 has emerged as a significant public health concern in Europe, ranking as the second or third leading cause of hemolytic uremic syndrome (HUS) alongside O157 and O26 (EFSA, 2023). These strains possess the pS88 plasmid, which encodes putative virulence genes also associated with extraintestinal pathogenic *E. coli* (ExPEC), contributing to severe systemic infections such as sepsis and bacteremia. In Belgium, AE-STEC and Enteropathogenic *E. coli* (EPEC) O80:H2 from humans and calves exhibit high genetic similarity at the SNP level, with specific virulence gene profiles involving *etsC*, *iucC*, *cma*, and *iha* genes.

This study aimed to perform the phylogenetic classification of AE-STEC and EPEC O80:H2 from humans and calves in Belgium by analyzing additional isolates and investigating the complete genomes of two calf *stx2f* AE-STEC using long-read sequencing. Phylogenetic analysis confirmed the division of 96% of AE-STEC and EPEC O80:H2 strains into two primary sub-lineages: L1/SL1.1 and SL1.2. Genes encoding type III secretion system (*eae*ξ, *espA*, *espB*, and *tir*) and pS88-associated virulence genes (*hlyF*, *iroN*, *iss*, *ompTp*, and *sitA*) were widely conserved across both sub-lineages. However, the differences in virulence gene distribution were noted: L1/SL1.2 strains predominantly carried *etsC* and *iucC* genes, while L1/SL1.1 strains frequently harbored *cma* and *iha* genes.

Comparative genomic analysis revealed that the two *stx2f* AE-STEC O80:H2 carried STX2f phages, pS88-like plasmid respectively, and Sakai prophage-like element (SpLE) 1 in SES0057 strain belonging SL1.1. Duplicated phages within other prophage elements were found in both strains, suggest a unique prophage structure which may play a role in promoting the diversity and evolution of virulence factors in AE-STEC and EPEC strains. Subsequently, the pS88-like plasmid with distinct virulence gene profiles was confirmed. In L1/SL1.2, the *etsC* and *iucC* genes were plasmid-localized, while in L1/SL1.1, only the *cma* gene was pS88 plasmid-associated. Finally, the simultaneous presence of the *iha* gene and *ter* operon were linked to the SpLE1 in L1/SL1.1, which suggested that sub-lineage-specific gene loss or acquisition may have occurred. These

findings confirm the distinct phylogenetic population structure and genetic characteristics of Belgian AE-STEC and EPEC O80:H2, which emphasize the impact of chromosomal and plasmid localization on virulence gene profiles. Further study of more comprehensive genome study, including the pathogenicity and host adaptation would be needed, and important for public health risk assessment not only in European countries but also worldwide.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is one of the most important pathogenic bacteria causing life-threatening human diseases, like Hemorrhagic Colitis (HC) and Hemolytic-Uremic Syndrome (HUS) worldwide. Although the abbreviated name EHEC has been prevalent for many years, it can today be referred to as 'AE-STEC', because EHEC produce both the Shiga toxins (Stx) of Shigatoxigenic *E. coli* (STEC) and the Attaching-Effacing lesion (A/E lesion) of enteropathogenic *E. coli* (EPEC), as major pathogenic factors (Piérard et al., 2012). Among these AE-STEC, seven serotypes are recognized as highly pathogenic (O26:H11, O103:H2, O111:H-, O121:H19, O145:H28, O165:H25, and O157:H7), known as the 'gang of seven', and are given priority in routine diagnostic procedures in many countries due to their impact on human health (Karmali, 1989; Tozzoli and Scheutz et al., 2014).

In the early 2010s, the serotype AE-STEC O80:H2 began to emerge in France and is today, recognized as a frequent HUS-associated serotype in Western European countries (EFSA, 2023; Soysal et al., 2016). AE-STEC O80:H2 have indeed also been isolated and reported in Switzerland, Italy, the Netherlands and Belgium (De Rauw et al., 2019; Gigliucci et al., 2021; Nüesch-Inderbinen et al., 2018; Rodwell et al., 2021). In Europe, AE-STEC O80:H2 has become one of the top five causative organism of HUS, indicating its increasing frequency (EFSA, 2023). In addition to HC and HUS, AE-STEC O80:H2 are also responsible for invasive infection and internal organ colonization (Mariani-Kurkdjian et al., 2014; Soysal et al., 2016). In addition, AE-STEC and EPEC O80:H2 have also emerged in young diarrheic calves in Belgium since 2016 and 2009 respectively, although invasion has only very rarely been reported (Thiry et al., 2017; Habets et al., 2021).

Genomic analyses have been conducted on human and calf AE-STEC and EPEC O80:H2 in those different countries (Bruyand et al., 2019; Cointe et al., 2018; De Rauw et al., 2019; Gigliucci et al., 2021; Habets et al., 2021; Nüesch-Inderbinen et al., 2018). All AE-STEC and EPEC O80:H2 studied belong to the MultiLocus Sequence Type 301 (ST301), a member of the Clonal Complex 165 (CC165) and contains the rare *eaeξ* gene encoding the intimin adhesin involved in the production of the A/E lesion and located on

the LEE (after Locus of Enterocyte Effacement) pathogenicicity island. AE-STEC O80:H2 also harbor different *stx* genes (*stx1a*, *stx2a*, *stx2d,* and *stx2c*) encoding Shiga toxins that are located on bacteriophages (Cointe et al., 2018; Mariani-Kurkdjian et al., 2014). Moreover, their great majority harbor one virulence-associated plasmid, the pS88 plasmid, previously identified in Neonatal Meningitis *E. coli* (NMEC) and Avian pathogenic *E. coli* (APEC) (Peigne et al., 2009). This plasmid indeed carries genes associated with invasiveness properties (Blanco et al., 2004; Peigne et al., 2009; Soysal et al., 2016) coding for: iron uptake systems including aerobactin and salmochelin siderophores (*iutA, iucA,B,C*, *D*; *sitA*, *B*,*C*, *D*; *iroB,C,D,E,N*; etc), increased serum survival (*iss*), an outer membrane protein (*ompTp*), the avian hemolysin (*hlyF*), colicins V and 1a (*cva A,B*,*C* and *cia* ..), and presumptive type I secretion system (*etsA*,*B*,*C*). A pS88-like plasmid was also identified in the human AE-STEC O80:H2 RDEx444 strain under the name of pR444_A plasmid that not only encodes the invasiveness properties of the pS88 plasmid, but also carries antibiotic and antiseptic resistance genes grouped on one integrated resistance cassette.

Human and calf AE-STEC and EPEC are also similar regarding the presence of LEE-located and non-LEE-located genes coding for a Type III Secretion System and Type III-secreted effectors also involved in the production of the A/E lesion, and of several chromosome and other plasmid-located genes coding for putative virulence factors, like the enterohemolysin (Ehly) and different adhesins, and for bacteriocins (Habets et al., 2021).

In addition to having very similar virulotypes and gene profiles, the 52 Belgian human and calf AE-STEC and EPEC studied by Habets and collaborators (Habets et al., 2021) are very closely related and even mingle in Single Nucleotide Polymorphism (SNP)-based phylogenetic trees forming two main lineages (L1 and L2). Most isolates belong to L1, which comprises four sub-lineages (SL). L1/SL1.1 and L1/SL1.2 account for the majority of strains with difference in the distribution of pathotypes: L1/SL1.1 was defined as 'EPEC', while L1/SL1.2 was as '*stx2d* AE-STEC' lineage (Habets et al., 2021). Such a classification was recently confirmed for French and Belgian human and calf AE-STEC by Soleau and collaborators who, however, did not include calf EPEC in their phylogenetic tree (Soleau et al., 2024).

Nevertheless, the AE-STEC and EPEC O80:H2 have different gene profiles dpending on their place in L1/SL1.1 or L1/SL1.2, with regard to not only two pS88-located genes (*iuc/iut* and *ets* genes), but also two genes located either on the Sakai prophage-Like Element 1 (SpLE1) in AE-STEC O157:H7 Sakai strain (*iha* gene coding for an Iron regulated gene <*irg*> Homologue Adhesin, subtype *iha* $_{EDL933}$; Colello et al., 2019) or on another plasmid (*cma* gene coding for the colicin M ; Köck et al., 1987). The majority of *stx2d* AE-STEC and of EPEC belonging to L1/SL1.2 harbor the former (*iuc/iut* and *ets*) but not the latter (*iha* and *cma*) genes, whereas the majority of EPEC and of *stx1a* or *stx2a* AE-STEC belonging to L1/SL1.1 have the opposite gene profile (Habets et al., 2021).

Bacterial species interact with each other to acquire virulence factors via horizontal gene transfers, like the pS88 plasmids and the Stx phages, respectively. Plasmid transfer by cell-to-cell contact is called conjugation, while phage trasnfer is called transduction (Landy and Ross, 1977; Schmidt et al., 1999; Virolle et al., 2020). Recently, thanks to long read sequencing technology, the genome structures of plasmids and bacteriophages can today be visualized. However, since few study reports using long read sequencing technology have been published yet, the complete genome structure of AE-STEC and EPEC O80:H2, including the pathogenicity islands like the LEE, the phages like the Stx and the prophage-like elements like SpLE-1, and the different virulence-associated plasmids like the pS88 and pO157 plasmids are poorly understood, compared to different major AE-STEC and EPEC serotypes (Cointe et al., 2018; Habets et al., 2022).

Therefore, the purpose of this study was two-fold: i) extending the number of Belgian human and calf AE-STEC and EPEC O80:H2 sequenced to confirm, or not the results published by Habets and collaborators (Habets et al., 2021) regarding not only the distribution and profiles of the virulence-associated genes (virulotypes) but also the population structure and genetic relationship in a Single Nucleotide Polymorphism (SNP)-based phylogenetic tree; and ii) identifying and comparing the actual distribution of several identified genes on pathogenicity islands, plasmids and prophages(-like elements) in two newly identified calf *stx2f* AE-STEC O80:H2 belonging to L1/SL1.1 and L1/SL1.2, respectively, after long read sequencing.

2. Materials and methods

2.1 E. coli *Strains*

A total of 129 Belgian *E. coli* O80:H2 isolates were analyzed, comprising 88 calf and 41 human isolates. Of them, 50 isolated between 2008 and 2019, and two isolated in 1987 (33 AE-STEC and EPEC from diarrheic calves and 19 AE-STEC from humans) were previously sequenced and published (De Rauw et al., 2019; Habets et al., 2021) with the BioProject PRJNA606200. The remaining 77 (55 from diarrheic calves and 22 from humans) strains were new isolates from 2018 to 2024, as well as previously uncharacterized isolates between 2009 and 2014, but still un-sequenced and unpublished, with the exceptions of two AE-STEC isolated from diarrheic calves in 2019 (see study 4) with BioSample accession numbers, SAMN35130764; SAMN35130765, respectively. The calf AE-STEC and EPEC were isolated from fecal and/or internal organ samples from diarrheic and/or septicemic calves (< three months old) after overnight growth on Entero-Haemolysin (EHly) blood agar plates at the *Association Régionale de Santé et d'Identification Animale* (ARSIA, Ciney, Belgium) following the methodology already described (Habets et al., 2021). Three colonies were subsequently tested with PCR for the *wzy*O80 and *fliC*H2, for the *eaeξ* gene and for the presence of *stx1* or *stx2* genes. O80 and H2 antigen PCR-positive colonies were subsequently genome sequenced by using two Illumina instruments (MiSeq and NovaSeq) to confirm the PCR results, identify the other genes present and perform the single nucleotide polymorphism (SNP) analysis.

Five *E. coli* non-O80:H2, one O80:H19 and four O80:H26, were used to root the phylogenetic tree.

All Accession number of Belgian O80:H2 and O80:nonH2 strains used for this study are listed in **Table 2 (see STUDY 3 Annexe)**

2.2 Virulotyping and Phylogenetic analysis

The virulence(-associated) genes of the remaining 77 new isolates were detected

as previously (Habets et al., 2021), by VirulenceFinder 2.0 (%ID threshold 85%, minimum length 60%) available from the Center for Genomic Epidemiology (https://cge.food.dtu.dk/). The phylogenetic analysis was conducted to investigate the evolutionary relationships among the 129 Belgian calf and human AE-STEC and EPEC O80:H2 strains. A total of 4,165 SNP sites were identified from the PP/IE-free and recombination-free chromosome conserved core genome sequence (3,677,600 bp) by Gubbins (v3.3.0) and the Nucmer program part of the MUMmer package (v3) (Croucher et al., 2015; Kurtz et al., 2004) using two human *stx2d* AE-STEC O80:H2 strains from USA (strain 2013C-4991) and France (strain RDEx444) for comparison (Cointe et al., 2018; Patel et al., 2018). The GenBank accession numbers for these two strains are CP027355 to CP027358 and QBDM01000001.1to QBDM01000004.1, respectively.

Maximum Likelihood (ML) tree was constructed using RAxML (v8.2.12) based on the identified Single Nucleotide Polymorphism (SNP) sites (Stamatakis et al., 2006). The tree was rooted by the five non-O80:H2 strains, one O80:H19 and four O80:H26. Finally, MLtree was displayed by FigTree (v1.4.4, http://tree.bio.ed.ac.uk/software/figtree/) and iTOL (https://itol.embl.de/ ; Letunic and Bork, 2021)

2.3 Genome assembly and annotation of two calf stx2f *AE-STEC*

The workflow was performed to achieve high-quality genome assembly for the two *stx2f* AE-STEC O80:H2 strains. DNA purification was performed by Genomic-tip 100/G (Qiagen**)** and subsequently sequenced on a MinION platform (Oxford Nanopore Technologies**)** with R9.4.1 flow cells over a 24-48 h runtime (Nishida et al., 2021; Wick et al., 2017). Base calling was performed by Guppy (v6.5.7: Oxford Nanopore Technologies, https://nanoporetech.com/). After FASTQ file generated, raw long reads were trimmed using Porechop **(**https://github.com/rrwick/Porechop**)** with default parameters and filtered with NanoFilt (v2.8.0), with a threshold of >3 kb and a quality score of >10 (De Coster et al., 2018). The previous short reads obtained from Illumina sequencers for these two strains were trimmed by Atria (v3.2.2-1) (Chuan et al., 2021**)**.

Filtered long reads and trimmed short reads were assembled using the microPIPE

pipeline (Murigneux et al., 2021) resulting in circularized genomes for strain SES0108, including small plasmids. Since the chromosome of strain SES0057 was split into two contigs, Circlator (v1.5.5) was used for gap closure with long reads followed by sequence correction with NextPolish (v1.4.0) utilizing Illumina short reads (Hu et al., 2020; Hunt et al., 2015). After complete genome sequences were obtained, gene annotation was performed by DFAST (https://dfast.ddbj.nig.ac.jp/dfc/; Tanizawa et al., 2016). Genome comparison was performed using GenomeMatcher (v3.10, with cut off value > 70%), the software which graphically displays the results (Ohtsubo et al., 2008).

3. Results

3.1 SNP-Based Phylogenetic Analysis

Of the 129 isolates analyzed (excluding the SNP-reference USA strain 2013C-4991 and comparison French strain RDEx444), 124 strains (96%) were classified in the major sub-lineages L1/SL1.1 (53 from calves and 7 from humans) and L1/SL1.2 (31 from calves and 33 from humans), previously defined (Habets et al., 2021). No additional isolate was assigned to the minor (sub-)lineages (L1/SL1.3, L1/SL1.4) and to L2. Of the 60 strains in L1/SL1.1, 46 (77%) were identified as EPEC. Similarly, of the 64 strains in L1/SL1.2, 51 strains (80%) were classified as AE-STEC. Of these, 41 strains (80%) carried the *stx2d* genes (**Fig.1**). The distribution trend of pathotype between the two sub-lineages was maintained through the study period from 2008 to 2024 (**Fig. 2**).

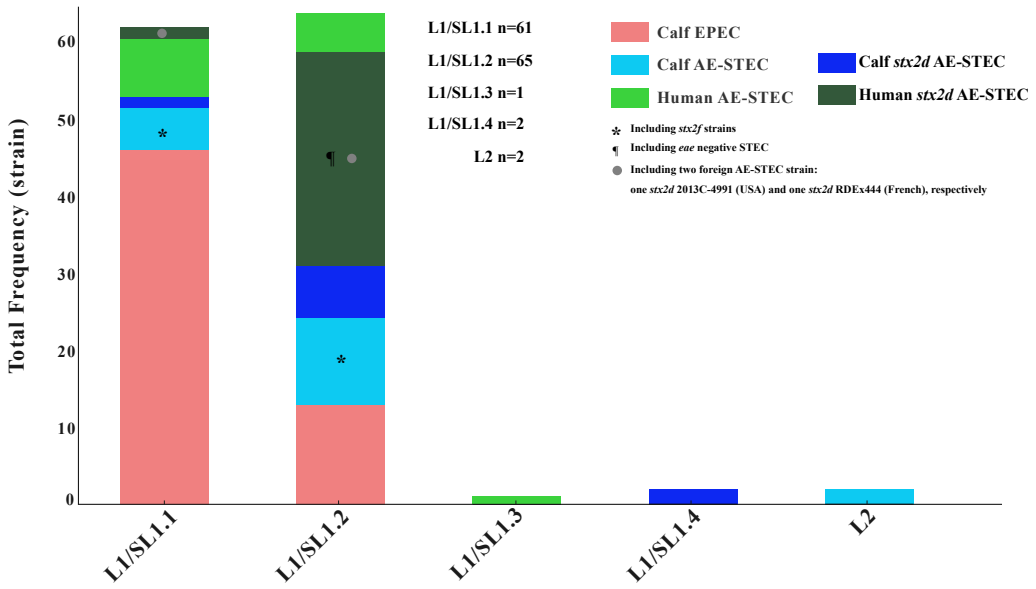


Figure 1. Distribution trends by (sub)-lineage of AE-STEC and EPEC O80:H2 isolated from calf and human in Belgium. Bars indicate the total frequency of strains per (sub-)lineage (L1/SL1.1, L1/SL1.2, L1/SL1.3, L1/SL1.4, L2), with colors representing the source and *stx* profile: calf EPEC, calf AE-STEC, calf *stx2d* AE-STEC, human AE-STEC, and human *stx2d* AE-STEC. Symbols denote specific cases: * indicates *stx2f* strains; ¶ indicates one *eae*-negative STEC; ● indicates two foreign AE-STEC strains (one *stx2d* 2013C-4991 from the USA and one *stx2d* RDEx444 from France).

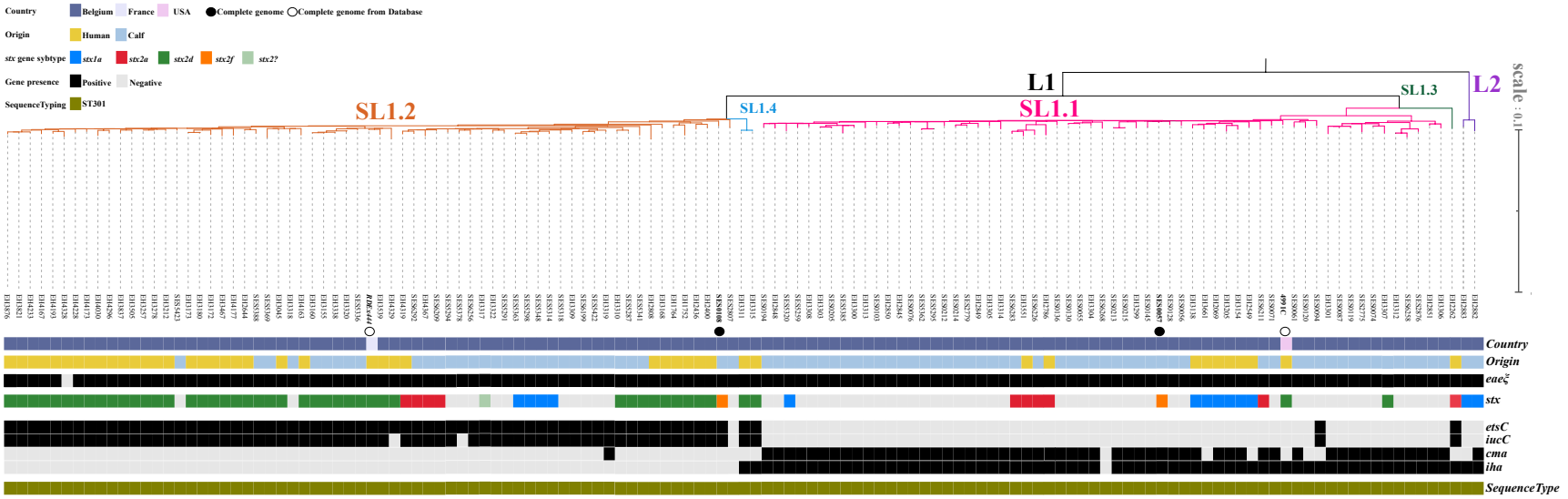


Figure 2. Maximum-likelihood (ML) tree consists of all Belgian AE-STEC and EPEC strains isolated from calf and humans, isolated in 1987 (L2), and from 2008 to 2024 (L1). Tree branches are colored by (sub-)lineage. Panels below the tree show information on country of isolation, host origin (human or calf), *eaeξ* and *stx1*/*stx2* gene subtypes, presence or absence of *etsC*, *iucC*, *cma*, and *iha*, and sequence type (ST301). Filled and open circles indicate complete genomes generated in this study (black) and from public databases (clear), respectively.

3.2 Genomic Identification and Prevalence of Virulence(-associated) Genes

All 129 calf and human AE-STEC and EPEC O80:H2 of this study belonged to ST301. The presence or absence of the different virulence genes are described in **Table 2 (see STUDY 3 Annexes).**

3.2.1. Distribution of stx *Gene Subtypes*

The chronological distribution of the *stx2* gene subtypes was as follows. The *stx2d* gene was by far the most frequent and was consistently detected in 13 calf AE-STEC strains from 2016 to 2020 and in human AE-STEC since 2008, demonstrating its predominant presence over the study period. Conversely, the *stx2a* gene was detected more sporadically: five calf strains in 2021 (3 strains) and 2023 (2 strains) and also five human strains in 2013, 2016, and 2021 (1 strain each year), and in 2024 (2 strains). The other *stx2* gene detected was the *stx2f* gene, only in two calf strains isolated in 2010 and 2011. Regarding the *stx1* gene subtypes, only the *stx1a* gene was identified in calf strains isolated in 1987 (2 strains), 2018 (1 strain), 2019 (5 strains), and also in human strains, one strain for each of the following years: 2011, 2015, 2018, 2019, and 2022 (**Figs. 3a, 3b**).

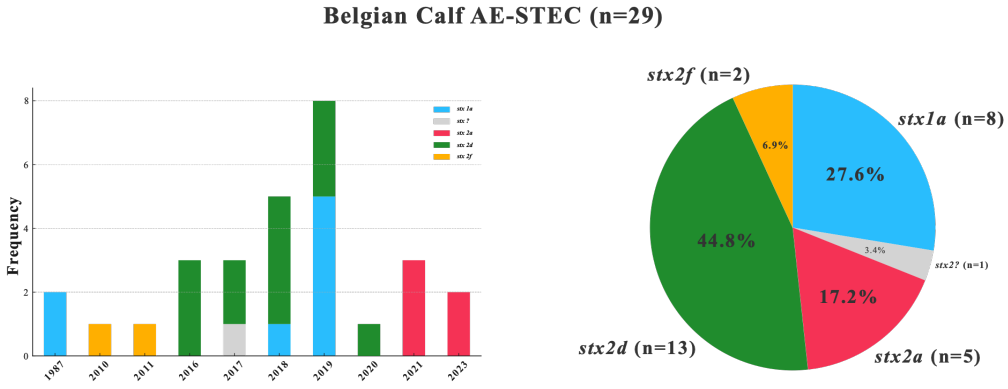


Figure 3a. Frequency of *stx* subtypes among Belgian calf AE-STEC strains (n = 29) isolated in 1987 and 2010–2023. The bar chart (left) shows yearly distribution by *stx* subtype, color-coded as: blue (*stx1a*), red (*stx2a*), green (*stx2d*), yellow (*stx2f*), and gray (*stx2*?). The pie chart (right) displays the proportion of each subtype, with numbers and percentages indicated.

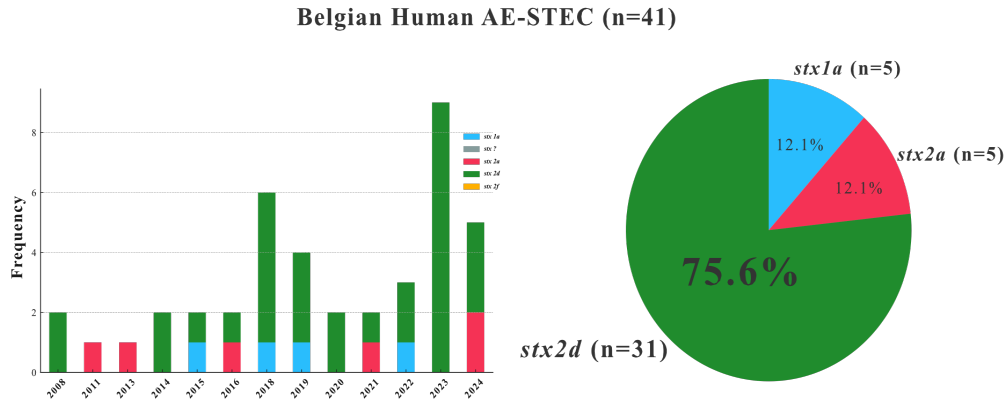


Figure 3b. Frequency of *stx* subtypes among Belgian human AE-STEC strains (n = 41) isolated in 2008–2024. The bar chart (left) shows yearly distribution by *stx* subtype, using the same color scheme as in **Figure 3a**. The pie chart (right) displays the proportion of each subtype, with numbers and percentages indicated.

3.2.2. Distribution of (Non)-LEE-Located T3SS- and T3-Secreted Effector-Encoding Genes

Apart from the one human LEE-negative STEC isolated in 2024 (human *stx2d* EH4328), all calf and human AE-STEC and EPEC O80:H2 strains harbored the *eae*ξ gene and different LEE-located T3SS-encoding genes, including *espA*, *espB*, *espF* and *tir*. Non-LEE-located genes *nleA*, *nleB*, *nleC* and *espP* genes were also detected in all or most of the isolates (the human *stx2a* EH3661 lacked *nleC* gene). The *espI*, a T3SS effector-encoding gene outside the LEE region, was identified in one calf EPEC (EH3305) and 34% of human AE-STEC strains, but was not detected in any calf AE-STEC strains. The *cif* and *espJ* genes were not detected in any of the 129 strains (**Fig. 4a**).

3.2.3. pS88 Plasmid-Associated Genes

Of the nine pS88 plasmid-associated genes, *hlyF*, *iroN*, *iss*, *ompTp*, and *sitA* were present in over 90% of both calf and human AE-STEC and EPEC O80:H2 strains. Of the colicin-encoding genes associated with the pS88 plasmid, the *cia* gene was detected in the great majority of calf (83 % of EPEC and 97% of AE-STEC) and human AE-STEC (98%) strains. Although the *cvaA* gene was also detected in the great majority of calf strains (93% of EPEC and 83% of AE-STEC), it was present in only in 46% of human AE-STEC strains, indicating a slightly different distribution tendency between calf and human strains (**Fig. 4b**).

The analysis of the distribution of the *etsC* and *iucC* genes between AE-STEC and EPEC confirmed the gene profiling reported in the previous study (Habets et al., 2021). The *etsC* and *iucC* genes were detected in 76% of calf AE-STEC and 83% of human AE-STEC strains with the majority belonging to L1/SL1.2, whereas their detection rate in EPEC strains belonging to L1/SL1.1 was lower at 20%. In addition, 78% of AE-STEC strains with the *etsC* and *iucC* genes carried the *stx2d* gene.

3.2.4. Other Putative Virulence- and Bacteriocin-Encoding Genes

In contrast to the *etsC* and *iucC* genes, the *cma* and *iha* genes were detected in 76% of calf EPEC strains while their prevalence was much lower in calf (28%) and human (12%) AE-STEC strains. Interestingly, the detection rate of the *iha* gene was different between calf and human AE-STEC strains: approximately 40% in calf AE-STEC and 20% in human isolates (**Fig. 4c**). The majority of AE-STEC strains in which the *iha* gene was detected belonged to L1/SL1.1., like the EPEC strains. Moreover, the *stx* subtype of *iha*-detected AE-STEC strains were *stx1a*, *stx2a*, or *stx2f*, and the only *stx2d* strain (EH3307) which belonged to L1/SL1.1 (**Figs. 1 and 2**; **Table 2; see Annexes**).

Finally, of the other putative virulence genes searched for, the *ehxA* gene was detected in all but one (EH2883) calf EPEC and AE-STEC strains and in all but two (EH2436 and EH4328) human AE-STEC strains and the *efa1* gene was identified in all strains. Additional putative virulence genes, including *gad* (Glutamate decarboxylase), *mchB/C/F* (Microcin), *astA* (Heat stable enterotoxin 1: EAST 1), *cba* (Colicin B), and *ireA* (Iron-regulated outer membrane protein) were identified in several strains (**Table 2; see Annexes**).

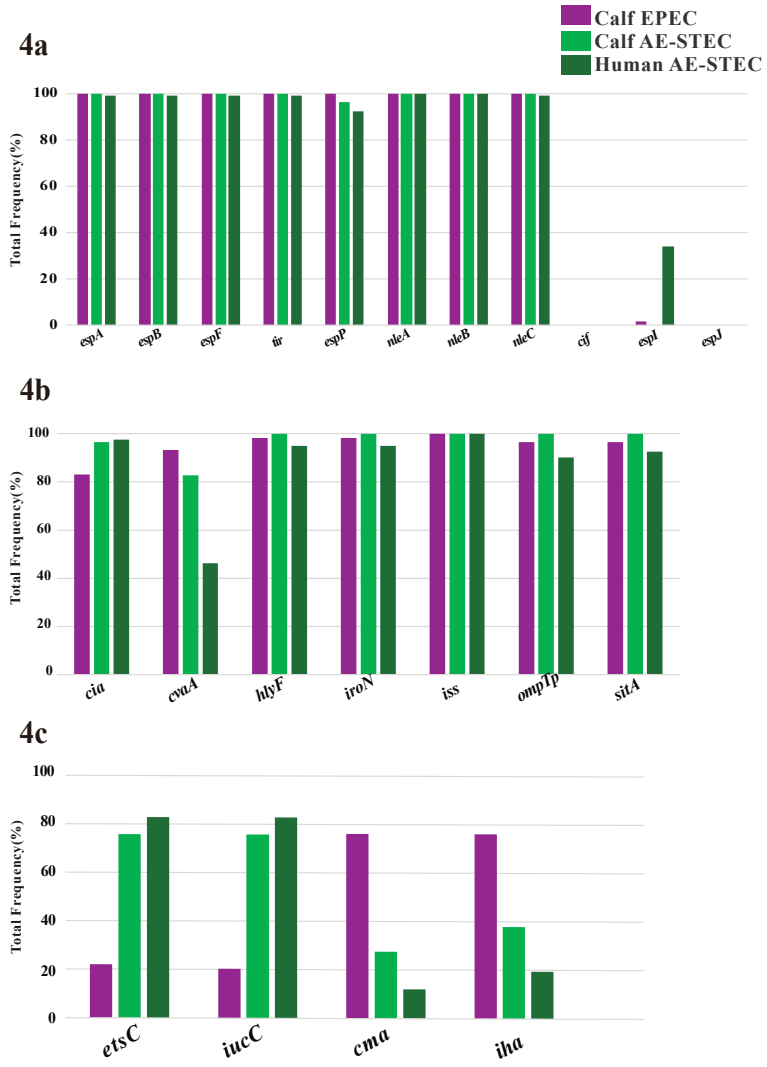
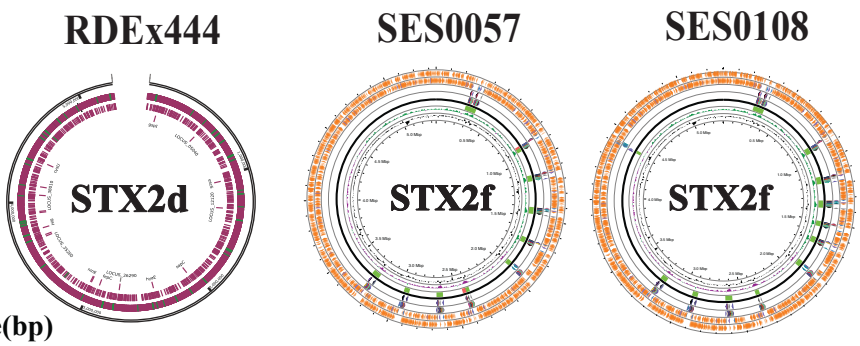


Figure 4. Frequency of virulence-associated genes of calf and human AE-STEC and EPEC O80:H2 in Belgium; (a) (Non)-LEE-located T3SS- and T3-secreted effectors-encoding genes; (b) pS88 Plasmid-Associated Genes; (c) *etsC*, *iucC*, *cma*, and *iha* genes. Each color indicates calf EPEC (purple); calf AE-STEC (light green); human AE-STEC (dark green).

3.3 Complete Genome Analysis of the Two Calf stx2f *AE-STEC*

3.3.1 General Chromosome Analysis

After complementing long read sequencing of the two calf *stx2f* AE-STEC O80:H2 belonging to L1/SL1.1 (strain SES0057) and L1/SL1.2 (strain SES0108) (**Figs. 1 and 2**), hybrid genome assembly revealed lengths of 5,348,840 base pairs for strain SES0057 and 5,305,731 base pairs for strain SES0108. The detailed and general features of the complete circular genomes comparison with RDEx444 (length of 5,256,050 base pairs) are described in **Fig. 5**. Nucleotide sequence identity (described in sections 3.3.2 and 3.3.3) values were obtained using web BLASTN. Accession numbers and detailed descriptions are provided in **Table 2.**



Genome size(bp)	RDEx444	SES0057	SES0108
Chromosome	**5,256,050**	**5,348,840**	**5,305,731**
Plasmid_1	176,500	153,597	151,146
Plasmid_2	117,090	112,522	112,532
Plasmid_3	95,050	91,101	93,667
Plasmid_4			90,885
Plasmid_5			48,852
Plasmid_6			6,637

Figure 5. General features of the complete circular genomes of the two calf *stx2f* AE-STEC O80:H2 and comparison with human *stx2d* AE-STEC RDEx444 (Cointe et al., 2018). Genome sizes of chromosomes and plasmids are shown in base pairs.

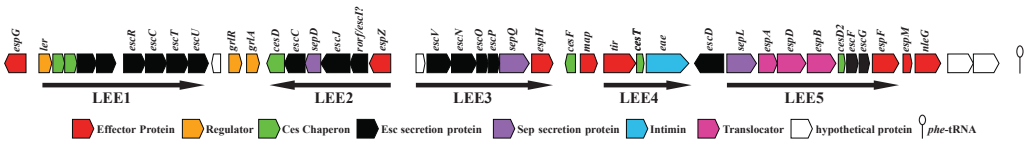


Figure 6. Whole-genome structure of the Locus of Enterocyte Effacement (LEE) region identified in two calf *stx2f* O80:H2 strains, integrated into *phe*-tRNA. Genes are color-coded by function as shown in the legend: effector proteins, regulators, Ces chaperones, Esc secretion proteins, Sep secretion proteins, intimin, translocators, and hypothetical proteins. The five LEE operons (LEE1–LEE5) are indicated by black arrow.

3.3.2. Virulotypes of Two Calf Stx2f *AE-STEC O80:H2 Strains*

The virulotypes of the two *stx2f* strains belonging to L1/SL1.1 (SES0057) and L2/SL1.2 (SES0108) were compared based on the presence and localization (chromosome, Plasmid_1, and Plasmid_3; **see Figure 5**, section *3.3.1 General Chromosome Analysis*) of virulence-associated genes of O80:H2 **(Table 1)**.

Table 1. Comparison of virulotype and its asscoiasted-genes and their genomic localization in the two *stx2f* AE-STEC strains SES0057 (L1/SL1.1) and SES0108 (L2/SL1.2). Presence (+) or absence (−) of selected chromosomal- and plasmid-encoding genes is indicated. Truncated genes are specified where applicable.

Localyzation	Genes	SES0057(L1/SL1.1)	SES0108(L2/SL1.2)
Chromosome	*eae* and *stx*	*eaeξ*/*stx2f*	
	LEE located	*espA*,*B*,*F*,and *tir*	
	Non-LEE located	*nleA*,*B*,*C*	
	iha	+	-
	ter operon (*terZABCDE*)	+	-
Plasmid_1	*iss*, *hlyF*, *iroN*, and *sitA*	+	
	cvaA,*B*,*C* (colicinV)	+	
	cia and *imm* (colicin1a)	-	+
	cma and *cmi* (colicinM)	+	-
	etsC/*iucC*	+	-
Plasmid_3	*ehxA*/*espP*/*efa1*	+	
	cia and *imm* (colicin1a)	+	
	cma and *cmi* (colicinM)	+ (Truncated)	

3.3.3. LEE and SpLE1

Both strains exhibited the integration of a Locus of Enterocyte Effacement (LEE) of approximately 35 kbp in the *phe*-tRNA locus. The LEE region of two calf *stx2f* AE-STEC consists of five operons (LEE1 to LEE5) and carry classical LEE-located genes, such as *ler*, *ces*, *esc*, *tir*, *eaeξ* and T3SS-associated genes such as *espA*, *espB*, *espD*, and *espF* (**Fig. 6, Table 2; see STUDY 3 Annexes**).

In the case of strain SES0057, a Sakai prophage-like element (SpLE1) of approximately 86,747 bp was identified. This element was inserted in the same *ser*-tRNA locus as in AE-STEC Sakai O157:H7 and other major non-O157 AE-STEC serotypes (e.g., O111 and O26). Moreover, the SpLE1 of strain SES0057 included the tellurite resistance operon (*terZABCDEF*) and the IrgA homologous adhesin factor (*iha*). When comparing the genomes of SpLE1 of Sakai O157:H7 and of SES0057 O80:H2 strains, homologous regions were limited to specific genes rather than being broadly conserved across the entire element. Specifically, high sequence identity (95 to 100%) was observed in certain genes such as *xerC* (coding for the integrase), *iha*, and in *ter* operon, whereas large portions of the two SpLE1 lacked detectable identity (**Fig. 7**). Conversely, no

SpLE1-like element was detected on the chromosome of strain SES0108. The structures of the Stx2f phages and of the pS88 plasmids of these two strains will now be detailed.

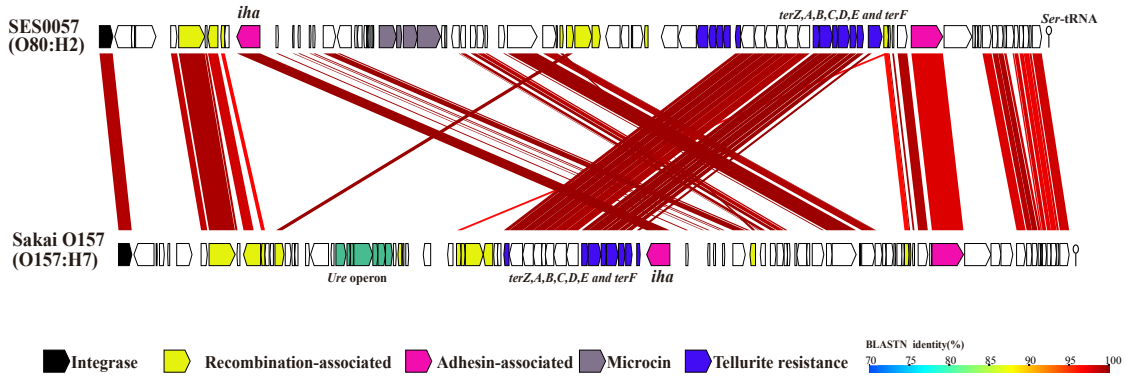


Figure 7. Comparative genomic structure and identity of the SpLE1-like elements identified in AE-STEC SES0057 (O80:H2) and Sakai O157 (O157:H7). Shared homologous regions are indicated by red bars, with color intensity reflecting BLASTN identity (70–100%).

3.3.4. Stx2f-*Converting Phages*

3.3.4.1. Comparison Between SES0057 and SES0108

In both SES0057 and SES0108, two Stx2f phages; Stx2f-1 and Stx2f-2, each approximately 42 kbp in size, were identified. These two phages showed almost 100% identity within each strain and were therefore regarded as duplicated Stx2f phages (**Fig. 8a**). The two phages Stx2f-2 are integrated into tmRNA encoding *ssrA* gene, while the two Stx2f-1 are located upstream in the bacterial genomes and are integrated within another prophage, itself located at the height of the *thrW*-tRNA locus (**Fig. 8b**).

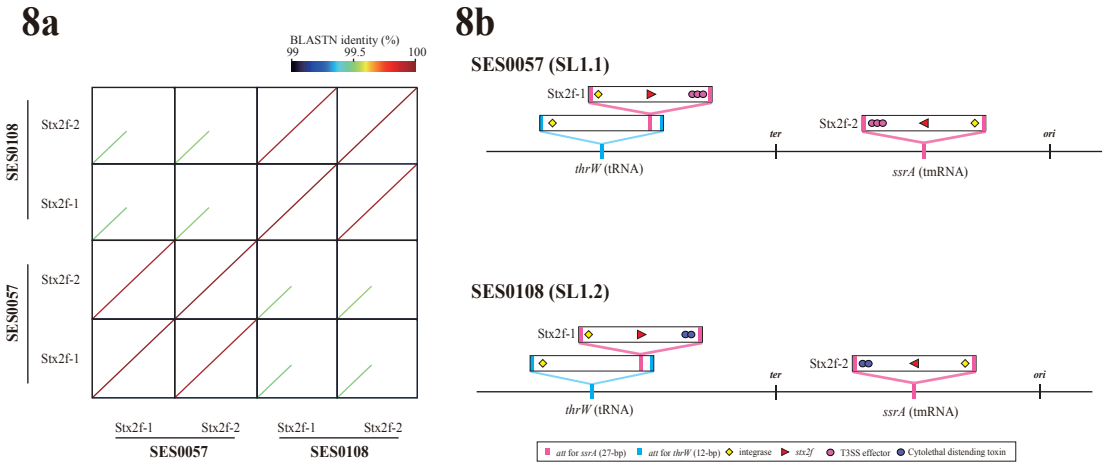


Figure 8. (a) Pairwise sequence comparison Stx2f phages in strain SES0057 and SES0108: dot plots show pairwise alignments of two Stx2f phages (Stx2f-1 and Stx2f-2) from each strain (SES0057 and SES0108) by BLASTN (> 99%). Diagonal lines indicate homologous regions; (b) Schematic diagram of the genome structure on chromosome level of the two Stx2f phages in SES0057 (L1/SL1.1) and SES0108 (L1/SL1.2). The integration sites, phage-associated genes (integrase-encoding genes, *stx2f* genes, T3SS effector-encoding genes, cytolethal distending toxin-encoding genes), and *att* sequences are indicated.

The Stx2f phage genomes of strain SES0057 and SES0108 were divided into three regions (**Fig. 9a**):

(i) a first highly homologous region (> 99.5% identity),

(ii) a second poorly homologous region (< 70% identity)

(iii) a third partially homologous region (from 70 to 99% identity).

The first highly homologous region (i) extended from the integrase-encoding gene to the lysis-associated gene and included the *stx2f* gene.

The second poorly homology region (ii) was located after the lysis-associated genes and mainly contained the phage morphogenesis-encoding gene clusters (phage terminase subunit, prohead protease, DNA packaging, major capsid, major tail, and minor tail genes...).

The third partially homologous region (iii) corresponding to the terminal part of the phage genome also contained morphogenesis genes (such as tail assembly, host specificity protein, and tail fiber genes....). In addition, this region also contains some virulence-associated genes: SES0057 possesses three *nleG* genes encoding T3SS effectors, while SES0108 possesses the *cdtA* and *cdtB* genes encoding a cytolethal distending toxin (**Figs. 8b, 9b**). According to BLASTN search, these two *cdt* genes were identical to those of CDT type I. However, the *cdtC* gene, which normally occurs in the *cdtA–cdtB–cdtC* cluster, was not detected in strain SES108. Interestingly, the second and third genome regions of strain SES0057 were highly homologous (>99.7%) to a prophage integrated near the *ttcA* gene within the same strain SES0057, including the presence of three *nleG* genes as well (**Fig. 9c**).

Moreover, Web BLASTN search for similar sequences of those virulence-associated genes revealed that the *cdtA* and *cdtB* genes of strain SES0108 were identical to those present in human *stx2f* STEC strains O145:H34, O63:H63, O157:H16 and other Gram-negative bacteria like *Escherichia albertii*, and that the *nleG* genes of strain SES0057 were identical to those of major (AE-)STEC serotypes O157:H7 and O26:H11 (**Table 3; see STUDY 3 Annexes**).

3.3.4.2. Comparison With The Stx2f Phages of Other STEC Serotypes

Subsequently, the genome comparison with Stx2f phages of other STEC serotypes was performed: the Stx2f phage genome of SES0108 shared over 99.5% identity across the entire genome with both Stx2f phages of two other STEC serotypes, O145:H34 and O63:H6 isolated from human stools. Logically, as observed in the previous section (3.3.2.1), the second and third genome regions of strain SES0057 do not share high homology with the corresponding genome regions of these other two *stx2f* STEC serotypes (**Fig. 9b**).

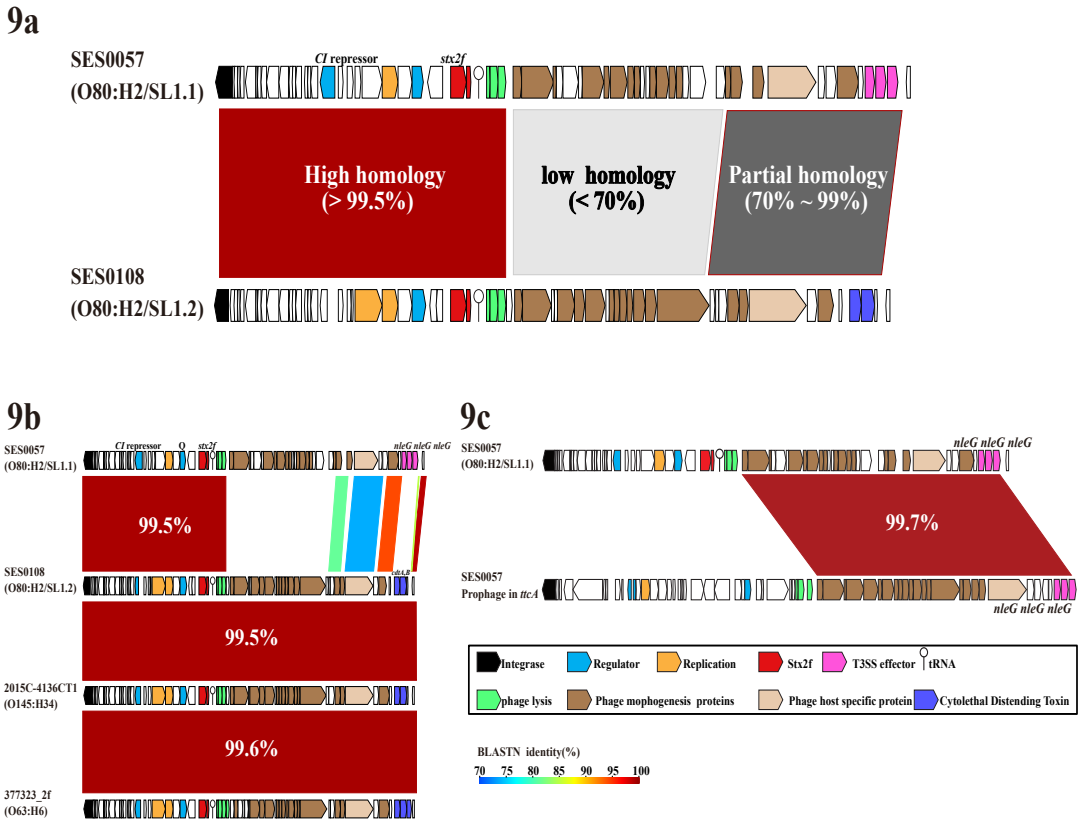


Figure 9: Genome structure and comparison of *stx2f* phages. (a) Comparison between two calf *stx2f* O80:H2 strains, SES0057 (O80:H2/SL1.1) and SES0108 (O80:H2/SL1.2), showing three regions of high nucleotide identity (>99.5%), low nucleotide identity (<70%), and partial nucleotide identity (from 70 to 99%); (b) Comparison of Stx2f phages of AE-STEC O80:H2 with Stx2f phages from other STEC serotypes (O145:H34 and O63:H6) isolated from human stools; (c) Genomic structure of a prophage integrated into the *ttcA* gene in strain SES0057 with a 99.7% homologous to the second and third regions of the strain SES0057 Stx2f phage. BLASTN identity (70–100%) is shown by the color scale of the alignment regions between genomes.

3.3.5. PS88-Located and Other Virulence-Associated- and Bacteriocin-Encoding Genes

Both *stx2f* AE-STEC harbored a pS88 plasmid of 153,759 bp (strain SES0057) and 151,146 bp (strain SES0108), respectively. These plasmids included two replicons (RepFIIA and RepFIB) and one Tra region with more than 30 F-pilus factor genes for bacterial conjugation. Along with colicin V-encoding genes (*cvaA*/*B*/*C* operon), the following ExPEC-associated genes were also detected on both plasmids: *hlyF*, *iss*, *iroN/B/C/D/E*, *ompTp*, and *sitA*/*B*/*C*/*D*.

On the other hand, several differences were observed: the pS88 plasmid of strain SES0057 lacked several virulence-associated genes: (i) the *iutA* and *iuc A/B/C/D* genes coding for iron uptake; (ii) the *etsA/B/C* genes for putative type I secretion system; (iii) the *cia* gene coding for colicin 1a and the *imm* gene coding for the immunity protein. Indeed, although VirulenceFinder 2.0 detected the *cia* and *imm* genes in strain SES0057 at the strain level (**Table 2; see STUDY 3 Annexes**), these colicin 1a-encoding genes were absent from the pS88 plasmid of SES0057, which was contrary to expectation. In addition, the pS88 plasmid of strain SES0057 carried the *cma and cmi* genes coding for colicin M with 100% identity to the *cma* and *cmi* genes of APEC and ExPEC strains (**Table 3; see STUDY 3 Annexes**), which was also contrary to expectation. In contrast, the pS88 plasmid of strain SES0108 carried: (i) the *iutA* and *iuc A/B/C/D* genes; (ii) the *etsA/B/C* gene; and (iii) the *cia* and *imm* genes; but (iv) not the *cma* and *cmi* genes, as expected since VirulenceFinder 2.0 did not detect them in SES0108.

The comparison between the two pS88 plasmids identified in strains SES0057 and SES0108, the original pS88 plasmid of NMEC O45:K1:H7 strain S88 (CU928146) and the pS88-like pR444_A plasmid (QBDM01000004.1) of AE-STEC O80:H2 strain RDEx444, also revealed that, like thepS88-like pR444_A plasmid of RDEx444, both SES0057 and SES0108 pS88 plasmids carried additional genes conferring resistance to tetracycline (*tetA*), trimethoprim (*dfrA5*), sulfonamide (*sul2*), β-lactam ($bla_{TEM\text{-}IB}$), kanamycin (*aph[3']-Ia*), and streptomycin (*strA* and *strB*).

Moreover, two other plasmids which are present in strain RDEx444 were also identified in both SES0057 and SES0108 strains, with >99 % identity (**Fig. 11**): the

cryptic pR444_B plasmid (QBDM01000003.1) and the pO157-like pR444_C plasmid which harbour several genes coding for virulence factors and bacteriocins, e.g. enterohemolysin (*ehxA*), serin protease (*espP*), and two of colicin-encoding genes (colicin1a: *cia*; colicinM: *cma*) (QBDM01000002.1). The pR444_C-like plasmids of both SES0057 and SES0108 carried the *cia* (colicin 1a) and *imm* (colicin 1a immunity protein), the *espP* gene (serin protease), and the *ehxA* (enterohemolysin) genes, but the *katP* (catalase peroxidase) gene was absent. The detection of colicin 1a-encoding genes on strain SES0057 by VirulenceFinder (**Table 2; see STUDY 3 Annexes**) can be explained because they were not on the pS88 plasmid but on the pR444_C like plasmid. Furthermore, in addition to the pS88 plasmid of SES0057, the pR444_C-like plasmids in the two calf *stx2f* AE-STEC also carried the *cma* and *cmi* genes. However, in both strains, these *cma* gene sequences were markedly shorter (285 bp) than the full-length *cma* gene (816 bp) identified on the pS88 plasmid of SES0057(**Fig. 11**).

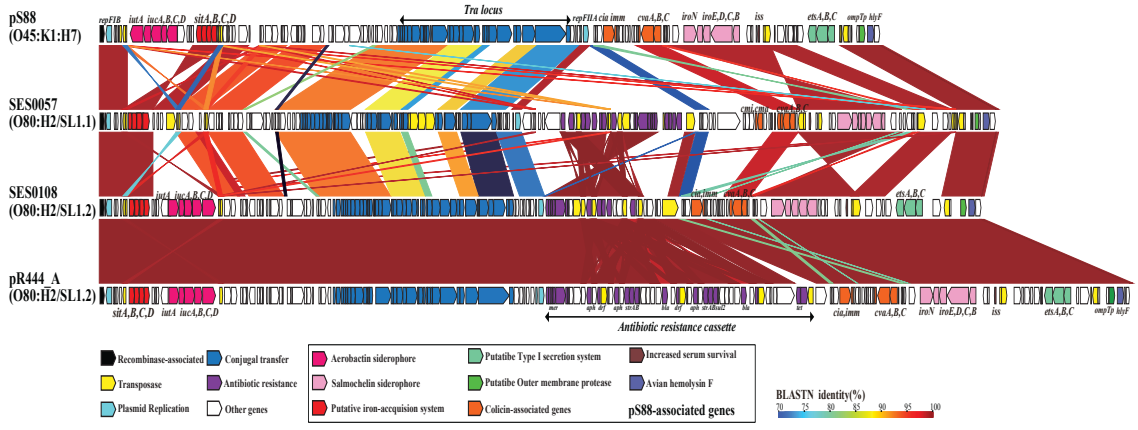


Figure 10. Comparative genomic structure of the pS88 plasmids from NMEC O45:K1:H7 strain K88, from the two calf *stx2f* AE-STEC O80:H2 strains (SES0057 and SES0108), and from the human *stx2d* AE-STEC O80:H2 strain RDEx444. Shared homologous regions are indicated by connecting blocks, with BLASTN identity (70–100%) shown by the color scale. Notable loci corresponding to pS88-associated genes such as *sitABCD*, *etsABC*, *iucABCD*, *iss*, *hlyF*, *ompTp*, *iroBCDEN*, and bacteriocin related (*cvaABC*, *cia*/*imm*, and *cma*/*cmi*) are annotated.

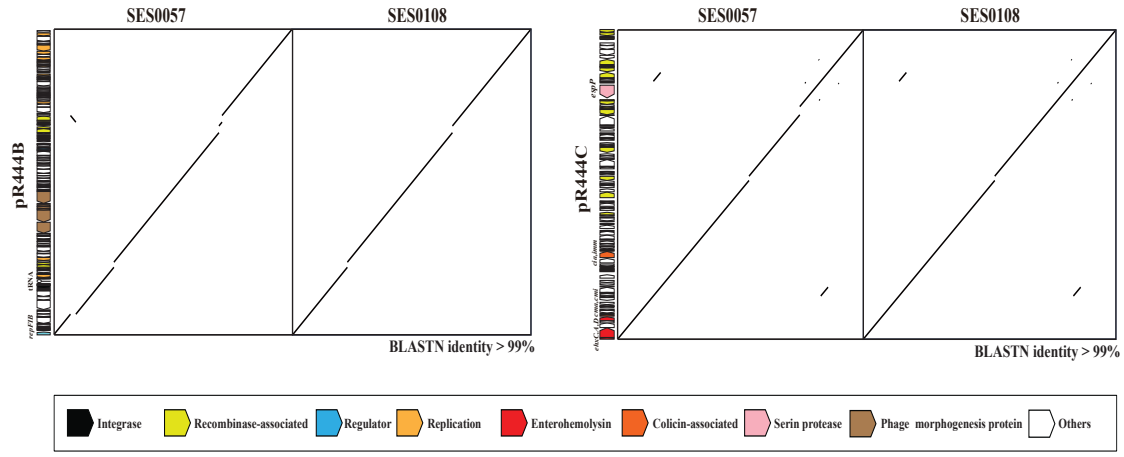


Figure 11. Comparative genomic structure of the two additional plasmids identified in *stx2d* AE-STEC strain RDEx444 and their counterparts in the two *stx2f* AE-STEC O80:H2 calf strains (SES0057 and SES0108): pR444_B-like cryptic plasmids (left) and pR444_C pO157-like plasmid (right). BLASTN identity (>99%) is shown for aligned regions. Genes are color-coded according to predicted functions, including integrase-recombinase-, replication-, virulence factor-, phage morphogenesis protein-encoding genes.

4. Discussion

The pathogenicity of AE-STEC and EPEC serotypes depends on genes located on mobile genetic elements (MGE), such as pathogenicity islands (e.g. the LEE), prophages (e.g. the Stx-converting phages) and plasmids (e.g. pO157 and pS88), that also contribute to their diversity and evolution. The emerging AE-STEC and EPEC serotype O80:H2 are no exception (Frost et al., 2005). They are associated not only with HC, HUS and bacteremia in humans (Soysal et al., 2016; Cointe et al., 2018), but also with diarrhea and septicemia in calves (Thiry et al., 2017; De Rauw et al., 2019; Habets et al., 2021). Moreover, the similar combinations of the virulence-associated genes, including those located on the pS88 plasmids, and the phylogenetic relatedness of human and calf strains were demonstrated in these two previous studies (De Rauw et al., 2019; Habets et al., 2021). However, due to its not-outbreak-associated sporadic occurrence and to the fact that it has been rarely isolated from food sources or healthy cattle, the number of research reports on the AE-STEC and EPEC O80:H2 remains much lower compared to other AE-STEC serotypes, such as O157:H7 and O26:H11 (EFSA, 2023; EFSA BIOHAZARD Panel Koutsoumanis et al., 2020). Whole genome sequencing (WGS) has today become a crucial tool for understanding the detailed genetic information of bacteria in general and of pathogenic bacteria in particular and for revealing the dynamics of mobile genetic elements and their contribution to pathogenicity (EFSA BIOHAZ Panel Koutsoumanis et al., 2019). Yet, in the case of AE-STEC and EPEC O80:H2, comprehensive genomic information is still lacking. Therefore, this study aimed to extend the number of analyzed genomes of Belgian human and calf AE-STEC and EPEC O80:H2 to uncover additional insights into their genetics and evolution.

4.1 Population structure

At first, the Maximum Likelihood (ML) SNP-based phylogenetic constructed tree confirms previously published results (Habets et al., 2021) on 52 Belgian calf and human AE-STEC and EPEC O80:H2. All 129 Belgian calf and human AE-STEC and EPEC O80:H2 isolated between 2008 and 2024 indeed belong to the L1 evolutionary lineage that comprises four lineages (L1/SL1.1 to SL1.4; **Fig. 2**). Moreover, all 77 additional

human and calf AE-STEC and EPEC O80:H2 strains belong to L1/SL1.1 and SL1.2. These results mean that (i) no strain was added to the L1/SL1.3 or SL1.4 minor sub-lineages; (ii) L2 lineage contains no other strain either than the two calf *stx1a* AE-STEC O80:H2 isolated in 1987; and (iii) no new (sub-)lineage is emerging right now in Belgium. The presence of (sub)-lineages to which the majority of strains belong is not limited to Belgian strains. A similar population structure was observed in one cgMLST-based tree, including human and calf AE-STEC O80:H2 strains from Belgium, France, Switzerland and the UK and a few healthy cattle-isolated strains, although no calf EPEC was included (Soleau et al., 2024). This implies that AE-STEC and EPEC O80:H2 strains are genetically highly related, regardless of their isolation country, and what is more, similar strains spread widely in different neighbor European countries. In 2025, Mainil and collaborators carried out extended phylogenetic analysis using of genomes of O80:H2 strains, from not only European countries, but also other continents (e.g. Asia and South America.). From this study, two new (sub)-lineages were observed that were not described in European O80:H2 population (Mainil et al., 2025). This new finding gives two insights: (i) AE-STEC and EPEC O80:H2 are not European specific serotype; (ii) more genomes of O80:H2 strains originating from non-European countries must be studied and analyzed to fully understand their origin and evolution.

In addition, other previous observations on Belgian AE-STEC and EPEC O80:H2 (Habets et al., 2021) were also confirmed. For instance, most of the strains belonging to L1/SL1.1 are calf EPEC (77%), while most of those belonging to L1/SL1.2 are human or calf *stx2d* AE-STEC (64%). Of the 13 calf *stx2d* AE-STEC strains, 12 belong to L1/SL1.2 whereas only one strain belongs to L1/SL1.1, confirming that *stx2d* AE-STEC O80:H2 strains tend to be distributed within L1/SL1.2 whatever their origin. Compared to the *stx2d* gene, the *stx1a* and *stx2a* gene sub-types were less frequent (**Fig. 2; Fig. 12**). These two *stx* subtypes belonged to not only both L1/SL1.1 and L1/SL1.2, but also to the minor (sub)-lineages, L1/SL1.3 and L2 (Habets et al., 2021). Finally, the only two *stx2f* AE-STEC that were identified belong to either L1/SL1.1 or L1/SL1.2 (**Fig. 2; Fig.12**). Besides their relationship in the phylogenetic tree, Belgian calf and human AE-STEC and EPEC O80:H2 are also highly related by their virulotypes and other gene profiles, here too as previously described (De Rauw et al., 2019; Habets et al., 2021).

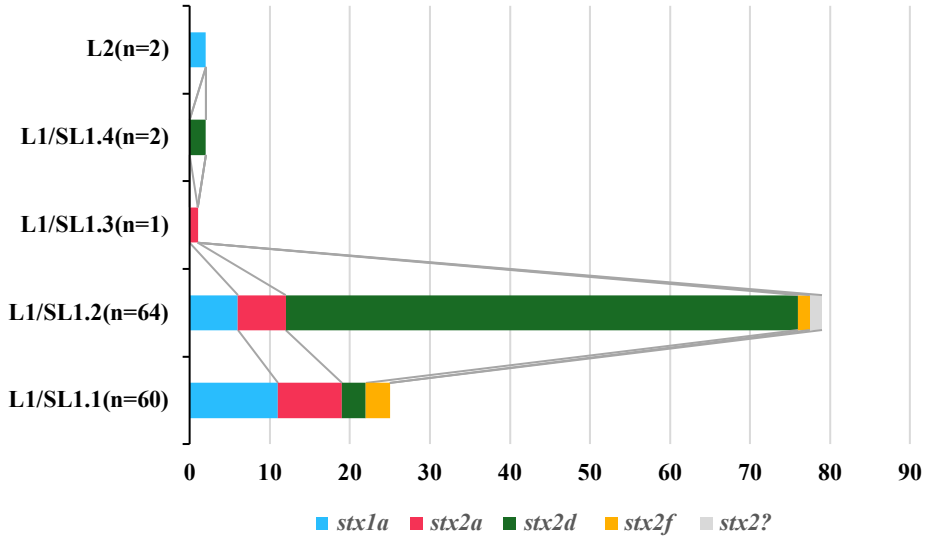


Figure 12. Distribution of *stx* gene subtypes among Belgian AE-STEC O80:H2 strains of each (sub)-lineages isolated in 1987 (the two calf *stx1a* AE-STEC in L2) and from 2008 to 2024 (all in L1).

Most of the 129 human and calf strains analyzed by VirulenceFinder2.0 indeed harbor (non)-LEE-located T3SS-encoding genes, *stx* genes (in STEC strains) and pS88-associated genes, consistent with previous studies on AE-STEC human strains from other countries showing similar virulence gene profiles (Mariani-Kurkdjian et al., 2014; Soysal et al., 2016; Rodwell et al., 2021). Of the 129 analyzed Belgian strains, only one (*stx2d* EH4328 isolate) identified in 2024 and belonging to L1/SL1.2 was *eae*-negative, without any of the LEE-located genes searched for (*eae*, *espA*, *espB*, *espF* and *tir*) being detected either. The intimin-encoding *eaeξ* gene subtype of the serotype O80:H2 is widely regarded as an important characteristic (Blanco et al., 2004; Cointe et al., 2021). Since STEC strains which carry *eae* gene are responsible for severe diseases like HC or HUS, the presence of the LEE island is an important virulence factor and marker of AE-STEC (Alhadlaq et al., 2024; Paton and Paton, 1998). However, the pathogenicity of *eae*-

negative STEC strains remains a subject of debate since their primary identification (Paton et al., 1999). For instance, the Agg-STEC strain O104:H4 without the *eae* gene, caused an outbreak affecting approximately 4,000 peoples in Germany (Ibarra et al., 2013). The clinical symptoms of the elderly patient (71 years old, female) from whom *stx2d* STEC EH4328 was isolated was non-bloody diarrhea and she developed no HUS (**Table 2; see STUDY 3 Annexes**). The possible emergence of *eae*-negative STEC O80:H2 after loss of the LEE, or before its acquisition, warrants continuous attention and investigations (Krause et al., 2018).

Of the pS88 plasmid-located virulence genes, five genes (*hlyF*, *iroN, iss*, *ompTp*, and *sitA*) were detected in more than 90% of calf and human O80:H2 strains regardless of the lineage, virulotype and time of isolation. On the other hand, the frequency of *cvaA* and *cia* bacteriocin-encoding genes was lower. The majority of AE-STEC and EPEC O80:H2 strains harbor the *cvaA* (88% to 100%) and *cia* (84% to 96%) genes, but the *cvaA* gene was especially not detected in both calf and human AE-STEC strains identified between 2021 to 2024 **(Table 2; see STUDY3 Annexes)**. Although these two different types of bacteriocin-encoding genes, coding for colicin1a and colicin V have been previously considered as important virulence markers for AE-STEC O80:H2, this might not the case for the strains of this study (Cointe et al., 2018; Soysal et al., 2016). As far as the two pS88-located *iuc/iut* and *ets* gene clusters are concerned, the majority of EPEC and AE-STEC O80:H2 belonging to L1/SL1.1 and to SL1.2 have different profiles, confirming the previously reported observation (Habets et al., 2021): the majority AE-STEC and EPEC of L1/SL1.2 harbor both gene clusters (*iuc/iut* and *ets*), while the majority of EPEC and AE-STEC of L1/SL1.1 do not. Conversely, the former do not harbor the other plasmid-located *cma* gene and the SpLE1-located *iha* gene, whereas the latter do. These results nevertheless confirmed that the presence of pS88-associated genes is an essential marker of O80:H2 strains, especially AE-STEC (De Rauw et al., 2019; Soysal et al., 2016; Rodwell et al., 2021).

Other putative virulence-associated genes were detected in all O80:H2 strains, like the *efa1* gene coding for EHEC factor for adherence, or in all but one calf *stx1a* and two human *stx2d* AE-STEC (EH2883 belonging to L2 and EH2436 and EH4328 belonging to L1/SL1.2, respectively) like the *ehxA* gene coding for the enterohemolysin. The *ehxA*

gene is often located on the pO157 plasmid among AE-STEC strains along with *espP* gene (Brunder et al., 1997; Cointe et al., 2018; Tatsuno et al., 2001). Interestingly, the simultaneous absence of *espP* and *ehxA* genes was observed in these three AE-STEC. Therefore, the lack of these genes suggesting that they possibly do not harbor any pO157-like plasmid.

4.2 Comparative genomics

The identification of two calf *stx2f* AE-STEC belonging to L1/SL1.1 (strain SES0057) and L1/SL1.2 (strain SES0108) isolated in 2010 and 2011 was notable, since this Stx sub-type was originally identified in AE-STEC from bird species, including pigeons and poultry and is still rarely identified in bovines and humans, although it has recently been associated with HUS in humans (Cointe et al., 2020b; Grande et al., 2016; Den Ouden et al., 2023 ; van Hoek et al., 2019). The whole genome sequences obtained by long reads of these two *stx2f* AE-STEC O80:H2 strains confirm the presence of identical LEE regions, different Stx2f phages, multiple plasmids, including pS88 and pO157 plasmids, and an SpLE1-like integrative element in one of them.

At first, the LEE regions are identical regarding the gene contents, including the *eae* gene and are inserted into *phe*-tRNA locus, like the LEE regions of other non-O157 STEC strains (Ogura et al., 2009).

In both SES0057 and SES0108 strains, the Stx2f phages were identified in duplicates with almost 100% identity (Stx2f-1 and Stx2f-2). Unlike the Stx2d phage of a calf O80:H2 strain which was integrated into the *yecE* locus (Habets et al., 2022), the Stx2f-2 phages were inserted into *ssrA* tmRNA loci, one of the common insertion sites for Stx phages (Carter et al., 2023; Bonanno et al., 2015). Interestingly, the Stx2f-1 phages were integrated not directly on the chromosome, but within another prophage. This type of insertion is called 'prophage in prophage' and was first reported in other STEC strains and for another Stx2 phage, as the Stx2a phage in STEC O145:H28 (Nakamura et al., 2021). This rarely phenomenon may confer more opportunities of integration if the insertion hot spots (*yecE*, *yehV*, *wrbA*...) are already occupied by other prophages. Following successful integration, Stx phages may contribute to the accumulation and

duplication of virulence-associated genes in (AE-)STEC.

Sequence identity of the Stx2f phages was not constant between strains SES0057 and SES0108; (i) the first region including *stx2f* and lysis-associated genes showed more than 99.5% of nucleotide identity, whereas (ii) the second region and (iii) the third region including mainly phage morphogenesis-encoding genes showed <70% and from 70% to 99% identity, respectively (**Figs. 9a, 9b**). Phage morphogenesis-encoding genes, such as those involved in head and tail formation, are essential for producing new phage particles (Mondal et al., 2016). Among them, tail fiber proteins are often associated with host recognition (Chibani-Chennoufi et al., 2004). In the third region, the genes encoding phage host specificity proteins have relatively low sequence identity (70–75%), whereas the tail fiber-encoding genes were more conserved (90–95%) (**Fig. 9b**). According to Chibani-Chennoufi and collaborators, even subtle differences in morphogenesis proteins, especially in the host specificity-related regions can result in changes of host range. Therefore, the lowly conserved host specificity-related genes suggest differences in phage structure between the two calf Stx2f phages, possibly reflecting adaptation to different ecological niches or preferential host(s).

Furthermore, genome comparison of the Stx2f phages of these two AE-STEC O80:H2 with Stx2f phages of other (AE-)STEC serotypes revealed additional information: the phage of strain SES0108 (L1/SL1.2) showed high nucleotide identity (>99.5%) with the phages of human (AE-)STEC O145:H34 and O63:H3 across the entire genome regions. Those three Stx2f phages possessed the *cdtA/B* genes that are identical to the *cdtA/B* genes present in the operon coding for the Cytolethal Distending Toxin (CDT) type 1 variant which has previously been reported in human Stx2f *E. coli* isolates (Crombé et al., 2024; van Hoek et al., 2019). However, the CDT is a tripartite holotoxin coded by the *cdtA/B/C* operon and associated with invasion, increased persistence, and severity of diseases caused by Gram-negative bacterial pathogens (Carter et al., 2023; Hinenoya, 2021; Mainil, 2013; Tóth et al., 2009). In this STUDY 3, the *cdtC* gene, which is essential to display full activity, was not detected in strain SES0108 (Guerra et al., 2011). The absence of the *cdtC* gene suggests that CDT-1 might have contributed at some stage to the virulence of some AE-STEC O80:H2 in diarrheic calves, as well as humans, but was lost e.g. during phage transmission process.

On his side, the Stx2f phage of SES0057 (L1/SL1.1) carry three copies of the *nleG* genes in its (iii) third region. Although this third region does not show high similarity to that of Stx2f phages of SES0108 and of other STEC serotypes, another *nleG*-carrying prophage detected in SES0057 showed >99.7% identity. Notably, this observation implicated that the Stx2f phage of SES0057 may have undergone recombination with another prophage, resulting in a mosaic structure. Moreover, this *nleG*-carrying prophage shares >96% identity with prophage sequences identified in O26:H11 and O103:H2 AE-STEC strains (data not shown in STUDY 3). Accordingly, the recombination event may have involved prophages circulating between diverse *E. coli* serotypes.

The presence of pS88 plasmids carrying gene coding for invasive properties is one of the important characteristics that define O80:H2 as hybrid pathotype strain, ExPEC-(AE)-STEC (Cointe et al., 2018; Peigne et al., 2009; Soysal et al., 2016). However, the gene profiles of the pS88 plasmids are different regarding the *iut/iuc* and *ets* genes depending on their association with L1/SL1.1 or L1/SL1.2. Moreover, the presence or absence of these two genes is inversely related to the presence or absence of two other genes, *cma* and *iha*, classically not associated with pS88 plasmids (Habets et al., 2021; STUDY 3). The comparison of the two *stx2f* AE-STEC not only confirms that the pS88 plasmid of strain SES0108 (L1/SL1.2) carries the *etsC* and *iucC* genes whereas the pS88 plasmid of strain SES0057 (L1/SL1.1) does not, but also reveals the presence of the *cma* gene on the latter which was not expected.

The *cma* gene encodes the colicin M and the adjacent *cmi* gene encodes its immunity protein. These genes are commonly found on different plasmids in Extra-intestinal *Escherichia coli* (ExPEC), particularly in Avian Pathogenic *Escherichia coli* (APEC). Colicin M is one of the antibacterial properties which inhibit O antigen and peptidoglycan synthesis (Chérier et al., 2021; Harkness and Braun, 1989; Johnson et al., 2006). Therefore, the presence of *cma* and *cmi* genes on the pS88 plasmid of SES0057 suggests that they act by killing other *E. coli* strains to help colonizing and living in the intestinal ecological niche of hosts, e.g. humans and calves.

On the other hand, the *cma* and *cmi* genes on the pS88 plasmid were not detected in strain SES0108 (L1/SL1.2), or on the pR444_A pS88-like plasmid of strain RDEx444

(L1/SL1.2) (Cointe et al 2018), or in the NMEC O45:K1:H7 strain K88 (Peigne et al., 2009). This finding leads to the question of how these genes were acquired by the pS88 plasmid of SES0057. The *cma* and *cmi* genes are often co-occuring with colicin B-encoding genes (*cba* and *cbi*) in conjugative plasmids (Chérier et al., 2021; Christenson and Gordon, 2009), but no *cba* and *cbi* genes were identified on the pS88 plasmid of SES0057. Although pS88 plasmids have been defined as ColV plasmids (Cointe et al., 2018), they also share similarity with the structure of the pAPEC-O1-ColBM plasmid (Peigne et al., 2009). Despite not being any ColV plasmid, the pAPEC-O1-ColBM plasmids carries ColV-associated virulence genes, such as *sitA/B/C/D*, *iss*, *iroB/C/D/E/N*, and *cvaA/B/C*, likely resulting from gene transfer events between ColV and ColBM plasmids (Johnson et al., 2006). In such plasmids, colicin B-encoding genes are often truncated and inactivated (Christenson and Gordon, 2009).

Accordingly, the following scenario can be proposed: (i) SES0057 strain acquired a ColBM-like plasmid, which had already lost colicin B-encoding genes, but not the colicin M-encoding genes (*cma* and *cmi*); (ii) this ColBM-like plasmid acquired ColV-associated virulence properties from the ColV pS88 plasmid via gene transfer; (iii) the *cma* and *cmi* genes (possibly of APEC origin) were transferred onto the ColV pS88 plasmid of SES0057 after acquisition. Interestingly enough, the genome region including the *cma* and *cmi* genes is flanked by IS elements and displays a lower GC content compared to surrounding sequences (data not shown). A homologous recombination event between ColV and ColBM plasmids cannot be excluded. However, at the present stage, this possibility is not supported by any evidence beyond the shared bacteriocin- and virulence-associated genes identified in this study.

The *etsC* and *iucC* genes, encoding putative type I secretion system and an iron acquisition system (aerobactin siderophore), respectively, are virulence properties often found in ExPEC, including APEC strains (Ling et al., 2013; Peigne et al., 2009; Van Goor et al., 2017). These genes are present on the pS88 plasmid in SES0108 (L1/SL1.2), while the pS88 plasmid of SES0057 (L1/SL1.1) lacks both *etsC* and *iucC*. According to Cointe and collaborators (Cointe et al., 2018), two types of pS88-like plasmids have been reported among human O80:H2 strains: the “complete” form, typically associated with *stx2d* AE-STEC strains which is more similar in structure to the original pS88 plasmid

(Peigne et al., 2009), and the "incomplete" form found in other strains. These observations suggest two contrasting evolutionary paths within the L1 sublineages: the pS88 plasmid of SES0108 (L1/SL1.2) may have retained a complete plasmid type which highly conserved ExPEC-like structure, while the pS88 plasmid of SES0057 (L1/SL1.1) likely reflects an incomplete plasmid type, possibly derived from a plasmid that had already integrated ExPEC virulence properties before its acquisition by SES0057 and subsequently lost the *etsC* and *iucC* genes. These differences identified in the two calf *stx2f* AE-STEC pS88 plasmids might also reflect adaptation to distinct ecological niches or hosts.

Regarding bacteriocin-encoding genes, the *cvaA/B/C* operon coding for colicin V is present on the pS88 plasmid in both SES0057 and SES0108. By contrast, the *cia* and *imm* genes coding for colicin Ia were detected on the pS88 plasmid and also on the pR444_C pO157-like plasmid in strain SES0108, whereas in SES0057, these two genes were detected only on the pR444_C pO157-like plasmid. Previous studies have shown that colicin Ia- and colicin V-encoding genes are often located together on a single conjugative plasmid (Jeziorowski and Gordon, 2007). In SES0108, the co-occurrence of the *cva* and *cia* operons on the pS88 plasmid confirms this close association between colicin V- and colicin Ia-encoding loci on a single plasmid backbone, that was not observed in the pS88 plasmid of SES0057. Together, these differences between SES0108 and SES0057 suggest that bacteriocin-encoding regions within their pS88 plasmids may have undergone distinct gene acquisition and/or rearrangement events.

The simultaneous detection of the *ter* operon and *iha* gene in SES0057 strain belonging to L1/SL1.1 (**Figs. 1, 8**) correlates with the presence of an SpLE1-like element, while their absence in SES0108 strain belonging to L1/SL1.2 strains would correspond to the lack of this element. This observation suggests that the genetic differentiation between L1/SL1.1 and L2/SL1.2 sub-lineages of AE-STEC and EPEC O80:H2 is parallel to the acquisition or loss of the SpLE1 element (Bielaszewska et al., 2011). As for SpLE1 element of other (AE-)STEC serotypes, Nakamura and collaborators reported that SpLE1 element was lost by strains of the Clonal Complex CC119, including STEC O165:H25, but was still present in closely related serotypes, such as O121:H19 (Nakamura et al., 2023). This raises the question of how the SpLE1 element emerged in O80:H2 AE-STEC

and/or EPEC. One hypothesis is that the ancestral lineage of *E. coli* O80:H2 originally acquired the SpLE1 element and that certain sub-lineage, such as L1/SL1.2 lost this element during evolution, maybe as a result of adaptation to specific environments or hosts. Alternatively, the ancestral lineage of *E. coli* O80:H2 did not harbor any SpLE1 element, with specific sub-lineages, like L1/SL1.1 later acquiring it through horizontal gene transfer, also as a part of their adaptation to ecological niches. Given that O80:H2 belongs to CC165, a detailed comparative analysis of SpLE1 in the related serotypes is necessary to elucidate whether the retention of this region was a common ancestral trait or a (sub)lineage-specific adaptation.

5. Conclusions

Re-constructed SNP-based phylogenetic tree show that 96% of the 129 AE-STEC and EPEC O80:H2 belong to L1/SL1.1 and L1/SL1.2, suggesting the population structure is highly conserved even with the expanded dataset. Since limited information can be obtained by draft genome, full-length genomes remain essential to clarify the evolutionary background of mobile genetic elements acquired through horizontal gene transfer (HGT). The results of long read complete genome sequencing of two *stx2f* AE-STEC O80:H2 belonging to two different sub-lineages (L1/SL1.1 and L1/SL1.2) provide new clues about the genetic characteristics and evolution of these two major sub-lineages of human and calf AE-STEC and EPEC O80:H2 isolated in Belgium. However, since only two AE-STEC O80:H2 were sequenced and compared, more comprehensive studies should be performed including larger numbers of not only *stx2f* but also *stx1a*, *stx2a*, and *stx2d* AE-STEC, without forgetting EPEC strains from different isolation sources and countries, to understand the overall population structure, origin and evolution of this serotype O80:H2.

5. References

Ahmed, S.A., Awosika, J., Baldwin, C., Bishop-Lilly, K.A., Biswas, B., Broomall, S., Chain, P.S.G., Chertkov, O., Chokoshvili, O., Coyne, S., Davenport, K., Detter, J.C., Dorman, W., Erkkila, T.H., Folster, J.P., Frey, K.G., George, M., Gleasner, C., Henry, M., Hill, K.K., Hubbard, K., Insalaco, J., Johnson, S., Kitzmiller, A., Krepps, M., Lo, C.-C., Luu, T., McNew, L.A., Minogue, T., Munk, C.A., Osborne, B., Patel, M., Reitenga, K.G., Rosenzweig, C.N., Shea, A., Shen, X., Strockbine, N., Tarr, C., Teshima, H., van Gieson, E., Verratti, K., Wolcott, M., Xie, G., Sozhamannan, S., Gibbons, H.S., Threat Characterization Consortium, 2012. Genomic comparison of *Escherichia coli* O104:H4 isolates from 2009 and 2011 reveals plasmid, and prophage heterogeneity, including shiga toxin encoding phage stx2. *PLoS One* 7, e48228. https://doi.org/10.1371/journal.pone.0048228

Alhadlaq, M.A., Aljurayyad, O.I., Almansour, A., Al-Akeel, S.I., Alzahrani, K.O., Alsalman, S.A., Yahya, R., Al-Hindi, R.R., Hakami, M.A., Alshahrani, S.D., Alhumeed, N.A., Al Moneea, A.M., Al-Seghayer, M.S., AlHarbi, A.L., Al-Reshoodi, F.M., Alajel, S., 2024. Overview of pathogenic *Escherichia coli*, with a focus on Shiga toxin-producing serotypes, global outbreaks (1982-2024) and food safety criteria. *Gut Pathog* 16, 57. https://doi.org/10.1186/s13099-024-00641-9

Blanco, M., Blanco, J.E., Mora, A., Dahbi, G., Alonso, M.P., González, E.A., Bernárdez, M.I., Blanco, J., 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (*eae*-xi). *J Clin Microbiol* 42, 645–651. https://doi.org/10.1128/JCM.42.2.645-651.2004

Bonanno, L., Loukiadis, E., Mariani-Kurkdjian, P., Oswald, E., Garnier, L., Michel, V., Auvray, F., 2015. Diversity of Shiga Toxin-Producing *Escherichia coli* (STEC) O26:H11 Strains Examined via *stx* Subtypes and Insertion Sites of Stx and EspK Bacteriophages. *Appl Environ Microbiol* 81, 3712–3721. https://doi.org/10.1128/AEM.00077-15

Brunder, W., Schmidt, H., Karch, H., 1997. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. *Mol Microbiol* 24, 767–778. https://doi.org/10.1046/j.1365-2958.1997.3871751.x

Bruyand, M., Mariani-Kurkdjian, P., Le Hello, S., King, L.-A., Van Cauteren, D., Lefevre, S., Gouali, M., Jourdan-da Silva, N., Mailles, A., Donguy, M.-P., Loukiadis, E., Sergentet-Thevenot, D., Loirat, C., Bonacorsi, S., Weill, F.-X., De Valk, H., Réseau hospitalier de surveillance du SHU pédiatrique, 2019. Paediatric haemolytic uraemic syndrome related to Shiga toxin-producing *Escherichia coli,* an overview of 10 years of surveillance in France, 2007 to 2016. *Euro Surveill* 24, 1800068. https://doi.org/10.2807/1560-7917.ES.2019.24.8.1800068

Carter, M.Q., Quiñones, B., He, X., Pham, A., Carychao, D., Cooley, M.B., Lo, C.-C., Chain, P.S.G., Lindsey, R.L., Bono, J.L., 2023. Genomic and Phenotypic Characterization of Shiga Toxin-Producing *Escherichia albertii* Strains Isolated from Wild Birds in a Major Agricultural Region in California. *Microorganisms* 11, 2803. https://doi.org/10.3390/microorganisms11112803

Chérier, D., Patin, D., Blanot, D., Touzé, T., Barreteau, H., 2021. The Biology of Colicin M and Its Orthologs. *Antibiotics (Basel)* 10, 1109. https://doi.org/10.3390/antibiotics10091109

Christenson, J.K., Gordon, D.M., 2009. Evolution of colicin BM plasmids: the loss of the colicin B activity gene. Microbiology 155, 1645–1655. https://doi.org/10.1099/mic.0.026666-0

Chibani-Chennoufi, S., Bruttin, A., Dillmann, M.-L., Brüssow, H., 2004. Phage-Host Interaction: an Ecological Perspective. J Bacteriol 186, 3677–3686. https://doi.org/10.1128/JB.186.12.3677-3686.2004

Chuan, J., Zhou, A., Hale, L.R., He, M., Li, X., 2021. Atria: an ultra-fast and accurate trimmer for adapter and quality trimming. *GigaByte* 2021, gigabyte31. https://doi.org/10.46471/gigabyte.31

Cointe, A., Birgy, A., Mariani-Kurkdjian, P., Liguori, S., Courroux, C., Blanco, J., Delannoy, S., Fach, P., Loukiadis, E., Bidet, P., Bonacorsi, S., 2018. Emerging

Multidrug-Resistant Hybrid Pathotype Shiga Toxin-Producing *Escherichia coli* O80 and Related Strains of Clonal Complex 165, Europe. *Emerg Infect Dis* 24, 2262–2269. https://doi.org/10.3201/eid2412.180272

Cointe, A., Birgy, A., Pascault, A., Louillet, F., Dufougeray, A., Mariani-Kurkdjian, P., Bonacorsi, S., 2020. Be aware of Shiga-toxin 2f-producing *Escherichia coli*: case report and false-negative results with certain rapid molecular panels. *Diagn Microbiol Infect Dis* 98, 115177. https://doi.org/10.1016/j.diagmicrobio.2020.115177

Croucher, N.J., Page, A.J., Connor, T.R., Delaney, A.J., Keane, J.A., Bentley, S.D., Parkhill, J., Harris, S.R., 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res* 43, e15. https://doi.org/10.1093/nar/gku1196

De Coster, W., D'Hert, S., Schultz, D.T., Cruts, M., Van Broeckhoven, C., 2018. NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics* 34, 2666–2669. https://doi.org/10.1093/bioinformatics/bty149

De Rauw, K., Thiry, D., Caljon, B., Saulmont, M., Mainil, J., Piérard, D., 2019. Characteristics of Shiga toxin producing- and enteropathogenic *Escherichia coli* of the emerging serotype O80:H2 isolated from humans and diarrhoeic calves in Belgium. *Clin Microbiol Infect* 25, 111.e5-111.e8. https://doi.org/10.1016/j.cmi.2018.07.023

Den Ouden, A., Greig, D.R., Rodwell, E.V., Tripodo, F., Olonade, I., Swift, C., Jenkins, C., 2023. *Escherichia coli* encoding Shiga toxin subtype Stx2f causing human infections in England, 2015-2022. *J Med Microbiol* 72. https://doi.org/10.1099/jmm.0.001707

Deng, W., Puente, J.L., Gruenheid, S., Li, Y., Vallance, B.A., Vázquez, A., Barba, J., Ibarra, J.A., O'Donnell, P., Metalnikov, P., Ashman, K., Lee, S., Goode, D., Pawson, T., Finlay, B.B., 2004. Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc Natl Acad Sci* U S A 101, 3597–3602. https://doi.org/10.1073/pnas.0400326101

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2023. The European Union One Health 2022 Zoonoses Report. EFS2 21. https://doi.org/10.2903/j.efsa.2023.8442

European Food Safety Authority, European Centre for Disease Prevention and Control, 2021. The European Union One Health 2019 Zoonoses Report. EFS2 19. https://doi.org/10.2903/j.efsa.2021.6406

Friesema, I.H.M., Keijzer-Veen, M.G., Koppejan, M., Schipper, H.S., van Griethuysen, A.J., Heck, M.E.O.C., van Pelt, W., 2015. Hemolytic uremic syndrome associated with *Escherichia coli* O8:H19 and Shiga toxin 2f gene. *Emerg Infect Dis* 21, 168–169. https://doi.org/10.3201/eid2101.140515

Frost, L.S., Leplae, R., Summers, A.O., Toussaint, A., 2005. Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol* 3, 722–732. https://doi.org/10.1038/nrmicro1235

Gigliucci, F., van Hoek, A.H.A.M., Chiani, P., Knijn, A., Minelli, F., Scavia, G., Franz, E., Morabito, S., Michelacci, V., 2021. Genomic Characterization of hlyF-positive Shiga Toxin-Producing *Escherichia coli*, Italy and the Netherlands, 2000-2019. *Emerg Infect Dis* 27, 853–861. https://doi.org/10.3201/eid2703.203110

Guerra, L., Cortes-Bratti, X., Guidi, R., Frisan, T., 2011. The Biology of the Cytolethal Distending Toxins. Toxins 3, 172–190. https://doi.org/10.3390/toxins3030172

Habets, A., Antoine, C., Wagemans, J., Vermeersch, M., Laforêt, F., Diderich, J., Lavigne, R., Mainil, J., Thiry, D., 2022. Impact of Shiga-toxin encoding gene transduction from O80:H2 Shiga toxigenic *Escherichia coli* (STEC) on non-STEC strains. *Sci Rep* 12, 21587. https://doi.org/10.1038/s41598-022-26198-8

Habets, A., Crombé, F., Nakamura, K., Guérin, V., De Rauw, K., Piérard, D., Saulmont, M., Hayashi, T., Mainil, J.G., Thiry, D., 2021. Genetic characterization of Shigatoxigenic and enteropathogenic *Escherichia coli* O80:H2 from diarrhoeic and septicaemic calves and relatedness to human Shigatoxigenic E. coli O80:H2. *J. Appl. Microbiol*. 130, 258–264. https://doi.org/10.1111/jam.14759

Habets, A., Engelen, F., Duprez, J.-N., Devleesschauwer, B., Heyndrickx, M., De Zutter, L., Thiry, D., Cox, E., Mainil, J., 2020. Identification of Shigatoxigenic and Enteropathogenic *Escherichia coli* Serotypes in Healthy Young Dairy Calves in Belgium by Recto-Anal Mucosal Swabbing. *Vet Sci* 7, 167. https://doi.org/10.3390/vetsci7040167

Harkness, R.E., Braun, V., 1989. Inhibition of lipopolysaccharide O-antigen synthesis by colicin M. *J Biol Chem* 264, 14716–14722.

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C.G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M., Shinagawa, H., 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res* 8, 11–22. https://doi.org/10.1093/dnares/8.1.11

Hinenoya, A., 2021. [Molecular epidemiology of *Escherichia albertii*, emerging zoonotic enteropathogen]. *Nihon Saikingaku Zasshi* 76, 175–185. https://doi.org/10.3412/jsb.76.175

Hu, J., Fan, J., Sun, Z., Liu, S., 2020. NextPolish: a fast and efficient genome polishing tool for long-read assembly. *Bioinformatics* 36, 2253–2255. https://doi.org/10.1093/bioinformatics/btz891

Hunt, M., Silva, N.D., Otto, T.D., Parkhill, J., Keane, J.A., Harris, S.R., 2015. Circlator: automated circularization of genome assemblies using long sequencing reads. *Genome Biol* 16, 294. https://doi.org/10.1186/s13059-015-0849-0

Ibarra, C., Amaral, M.M., Palermo, M.S., 2013. Advances in pathogenesis and therapy of hemolytic uremic syndrome caused by Shiga toxin-2. *IUBMB Life* 65, 827–835. https://doi.org/10.1002/iub.1206

Ikeda, R., Nakamura, K., Crombé, F., Diderich, J., Saulmont, M., Thiry, D., Hayashi, T., Mainil, G.J., n.d. Génomique comparative et phylogénie de 127 souches Shigatoxinogènes et entéropathogènes d'*Escherichia coli* O80:H2 isolées de veaux et

d'humains en Belgique entre 2008 et 2024 ; Colloque International Francophone en Maladies Infectieuses Animales 2025(CIFMIA)

Ikeda, R., Nakamura, K., Korsak, N., Duprez, J.-N., Hayashi, T., Thiry, D., Mainil, J.G., 2025. Non-Melibiose Fermentation and Tellurite Resistance by Shigatoxigenic and Enteropathogenic *Escherichia coli* O80:H2 from Diseased Calves: Comparison with Human Shigatoxigenic *E. coli* O80:H2. *Vet Sci* 12, 274. https://doi.org/10.3390/vetsci12030274

Ikeda, R., Nakamura, K., Saulmont, M., Habets, A., Duprez, J.-N., Korsak, N., Hayashi, T., Thiry, D., Mainil, J.G., 2023. *Escherichia coli* O80 in Healthy Cattle: Absence of Shigatoxigenic and Enteropathogenic *E. coli* O80:H2 and (Phylo) Genomics of Non-Clonal Complex 165 *E. coli* O80. Microorganisms 11, 230. https://doi.org/10.3390/microorganisms11020230

Jeziorowski, A., Gordon, D.M., 2007. Evolution of microcin V and colicin Ia plasmids in *Escherichia coli*. J Bacteriol 189, 7045–7052. https://doi.org/10.1128/JB.00243-07

Johnson, T.J., Johnson, S.J., Nolan, L.K., 2006. Complete DNA sequence of a ColBM plasmid from avian pathogenic *Escherichia coli* suggests that it evolved from closely related ColV virulence plasmids. *J Bacteriol* 188, 5975–5983. https://doi.org/10.1128/JB.00204-06

Karmali, M.A., 1989. Infection by verocytotoxin-producing *Escherichia coli. Clin Microbiol Rev* 2, 15–38. https://doi.org/10.1128/CMR.2.1.15

Köck, J., Olschläger, T., Kamp, R.M., Braun, V., 1987. Primary structure of colic in M, an inhibitor of murein biosynthesis. J Bacteriol 169, 3358–3361. https://doi.org/10.1128/jb.169.7.3358-3361.1987

Krause, M., Barth, H., Schmidt, H., 2018. Toxins of Locus of Enterocyte Effacement-Negative Shiga Toxin-Producing *Escherichia coli*. *Toxins (Basel)* 10, 241. https://doi.org/10.3390/toxins10060241

Kurtz, S., Phillippy, A., Delcher, A.L., Smoot, M., Shumway, M., Antonescu, C., Salzberg, S.L., 2004. Versatile and open software for comparing large genomes. *Genome Biol* 5, R12. https://doi.org/10.1186/gb-2004-5-2-r12

Landy, A., Ross, W., 1977. Viral integration and excision: structure of the lambda *att* sites. *Science* 197, 1147–1160. https://doi.org/10.1126/science.331474

Letunic, I., Bork, P., 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res* 49, W293–W296. https://doi.org/10.1093/nar/gkab301

Ling, J., Pan, H., Gao, Q., Xiong, L., Zhou, Y., Zhang, D., Gao, S., Liu, X., 2013. Aerobactin synthesis genes *iucA* and *iucC* contribute to the pathogenicity of avian pathogenic *Escherichia coli* O2 strain E058. *PLoS One* 8, e57794. https://doi.org/10.1371/journal.pone.0057794

Mainil, J., 2013. *Escherichia coli* virulence factors. *Vet Immunol Immunopathol* 152, 2–12. https://doi.org/10.1016/j.vetimm.2012.09.032

Mainil, J.G., Nakamura, K., Ikeda, R., Crombé, F., Diderich, J., Saulmont, M., Piérard, D., Thiry, D., Hayashi, T., 2025. Emerging hybrid shigatoxigenic and enteropathogenic *Escherichia coli* serotype O80:H2 in humans and calves. *Clin Microbiol Rev* e0001125. https://doi.org/10.1128/cmr.00011-25

Mariani-Kurkdjian, P., Lemaître, C., Bidet, P., Perez, D., Boggini, L., Kwon, T., Bonacorsi, S., 2014. Haemolytic-uraemic syndrome with bacteraemia caused by a new hybrid *Escherichia coli* pathotype. *New Microbes and New Infections* 2, 127–131. https://doi.org/10.1002/nmi2.49

Mondal, S.I., Islam, M.R., Sawaguchi, A., Asadulghani, M., Ooka, T., Gotoh, Y., Kasahara, Y., Ogura, Y., Hayashi, T., 2016. Genes essential for the morphogenesis of the Shiga toxin 2-transducing phage from *Escherichia coli* O157:H7. Sci Rep 6, 39036. https://doi.org/10.1038/srep39036

Murigneux, V., Roberts, L.W., Forde, B.M., Phan, M.-D., Nhu, N.T.K., Irwin, A.D., Harris, P.N.A., Paterson, D.L., Schembri, M.A., Whiley, D.M., Beatson, S.A., 2021.

MicroPIPE: validating an end-to-end workflow for high-quality complete bacterial genome construction. *BMC Genomics* 22, 474. https://doi.org/10.1186/s12864-021-07767-z

Nakamura, K., Murase, K., Sato, M.P., Toyoda, A., Itoh, T., Mainil, J.G., Piérard, D., Yoshino, S., Kimata, K., Isobe, J., Seto, K., Etoh, Y., Narimatsu, H., Saito, S., Yatsuyanagi, J., Lee, K., Iyoda, S., Ohnishi, M., Ooka, T., Gotoh, Y., Ogura, Y., Hayashi, T., 2020. Differential dynamics and impacts of prophages and plasmids on the pangenome and virulence factor repertoires of Shiga toxin-producing *Escherichia coli* O145:H28. *Microb Genom* 6, e000323. https://doi.org/10.1099/mgen.0.000323

Nakamura, K., Ogura, Y., Gotoh, Y., Hayashi, T., 2021. Prophages integrating into prophages: A mechanism to accumulate type III secretion effector genes and duplicate Shiga toxin-encoding prophages in *Escherichia coli*. *PLoS Pathog* 17, e1009073. https://doi.org/10.1371/journal.ppat.1009073

Nakamura, K., Seto, K., Lee, K., Ooka, T., Gotoh, Y., Taniguchi, I., Ogura, Y., Mainil, J.G., Piérard, D., Harada, T., Etoh, Y., Ueda, S., Hamasaki, M., Isobe, J., Kimata, K., Narimatsu, H., Yatsuyanagi, J., Ohnishi, M., Iyoda, S., Hayashi, T., 2023. Global population structure, genomic diversity and carbohydrate fermentation characteristics of clonal complex 119 (CC119), an understudied Shiga toxin-producing *E. coli* (STEC) lineage including O165:H25 and O172:H25. *Microb Genom* 9. https://doi.org/10.1099/mgen.0.000959

Nguyen, T.T.H., Kikuchi, T., Tokunaga, T., Iyoda, S., Iguchi, A., 2021. Diversity of the Tellurite Resistance Gene Operon in *Escherichia coli*. *Front Microbiol* 12, 681175. https://doi.org/10.3389/fmicb.2021.681175

Nishida, R., Nakamura, K., Taniguchi, I., Murase, K., Ooka, T., Ogura, Y., Gotoh, Y., Itoh, T., Toyoda, A., Mainil, J.G., Piérard, D., Seto, K., Harada, T., Isobe, J., Kimata, K., Etoh, Y., Hamasaki, M., Narimatsu, H., Yatsuyanagi, J., Kameyama, M., Matsumoto, Y., Nagai, Y., Kawase, J., Yokoyama, E., Ishikawa, K., Shiomoto, T., Lee, K., Kang, D., Akashi, K., Ohnishi, M., Iyoda, S., Hayashi, T., 2021. The global population structure and evolutionary history of the acquisition of major virulence factor-encoding genetic

elements in Shiga toxin-producing *Escherichia coli* O121:H19. *Microbial Genomics* 7. https://doi.org/10.1099/mgen.0.000716

Ogura, Y., Ooka, T., Iguchi, A., Toh, H., Asadulghani, M., Oshima, K., Kodama, T., Abe, H., Nakayama, K., Kurokawa, K., Tobe, T., Hattori, M., Hayashi, T., 2009. Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic *Escherichia coli*. *Proc Natl Acad Sci* U S A 106, 17939–17944. https://doi.org/10.1073/pnas.0903585106

Ohtsubo, Y., Ikeda-Ohtsubo, W., Nagata, Y., Tsuda, M., 2008. GenomeMatcher: a graphical user interface for DNA sequence comparison. *BMC Bioinformatics* 9, 376. https://doi.org/10.1186/1471-2105-9-376

Patel, P.N., Lindsey, R.L., Garcia-Toledo, L., Rowe, L.A., Batra, D., Whitley, S.W., Drapeau, D., Stoneburg, D., Martin, H., Juieng, P., Loparev, V.N., Strockbine, N., 2018. High-Quality Whole-Genome Sequences for 77 Shiga Toxin-Producing *Escherichia coli* Strains Generated with PacBio Sequencing. *Genome Announc* 6, e00391-18. https://doi.org/10.1128/genomeA.00391-18

Paton, A.W., Manning, P.A., Woodrow, M.C., Paton, J.C., 1998. Translocated Intimin Receptors (Tir) of Shiga-Toxigenic *Escherichia coli* Isolates Belonging to Serogroups O26, O111, and O157 React with Sera from Patients with Hemolytic-Uremic Syndrome and Exhibit Marked Sequence Heterogeneity. *Infect Immun* 66, 5580–5586. https://doi.org/10.1128/IAI.66.11.5580-5586.1998

Paton, A.W., Woodrow, M.C., Doyle, R.M., Lanser, J.A., Paton, J.C., 1999. Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. *J Clin Microbiol* 37, 3357–3361. https://doi.org/10.1128/JCM.37.10.3357-3361.1999

Peigne, C., Bidet, P., Mahjoub-Messai, F., Plainvert, C., Barbe, V., Médigue, C., Frapy, E., Nassif, X., Denamur, E., Bingen, E., Bonacorsi, S., 2009. The plasmid of *Escherichia coli* strain S88 (O45:K1:H7) that causes neonatal meningitis is closely related to avian pathogenic *E. coli* plasmids and is associated with high-level bacteremia

in a neonatal rat meningitis model. *Infect Immun* 77, 2272–2284. https://doi.org/10.1128/IAI.01333-08

Perna, N.T., Plunkett, G., Burland, V., Mau, B., Glasner, J.D., Rose, D.J., Mayhew, G.F., Evans, P.S., Gregor, J., Kirkpatrick, H.A., Pósfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E.J., Davis, N.W., Lim, A., Dimalanta, E.T., Potamousis, K.D., Apodaca, J., Anantharaman, T.S., Lin, J., Yen, G., Schwartz, D.C., Welch, R.A., Blattner, F.R., 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409, 529–533. https://doi.org/10.1038/35054089

Piérard, D., De Greve, H., Haesebrouck, F., Mainil, J., 2012. O157:H7 and O104:H4 Vero/Shiga toxin-producing *Escherichia coli* outbreaks: respective role of cattle and humans. *Vet Res* 43, 13. https://doi.org/10.1186/1297-9716-43-13

Purushothaman, S., Meola, M., Roloff, T., Rooney, A.M., Egli, A., 2024. Evaluation of DNA extraction kits for long-read shotgun metagenomics using Oxford Nanopore sequencing for rapid taxonomic and antimicrobial resistance detection. *Sci Rep* 14, 29531. https://doi.org/10.1038/s41598-024-80660-3

Rodwell, E.V., Vishram, B., Smith, R., Browning, L., Smith-Palmer, A., Allison, L., Holmes, A., Godbole, G., McCarthy, N., Dallman, T.J., Jenkins, C., 2021. Epidemiology and genomic analysis of Shiga toxin-producing *Escherichia coli* clonal complex 165 in the UK. *J Med Microbiol* 70. https://doi.org/10.1099/jmm.0.001471

Schmidt, H., Bielaszewska, M., Karch, H., 1999. Transduction of enteric *Escherichia coli* isolates with a derivative of Shiga toxin 2-encoding bacteriophage phi3538 isolated from Escherichia coli O157:H7. *Appl Environ Microbiol* 65, 3855–3861. https://doi.org/10.1128/AEM.65.9.3855-3861.1999

Soleau, N., Ganet, S., Werlen, S., Collignon, L., Cointe, A., Bonacorsi, S., Sergentet, D., 2024. First Isolation of the Heteropathotype Shiga Toxin-Producing and Extra-Intestinal Pathogenic (STEC-ExPEC) *E. coli* O80:H2 in French Healthy Cattle: Genomic Characterization and Phylogenetic Position. *Int J Mol Sci* 25, 5428. https://doi.org/10.3390/ijms25105428

Soysal, N., Mariani-Kurkdjian, P., Smail, Y., Liguori, S., Gouali, M., Loukiadis, E., Fach, P., Bruyand, M., Blanco, J., Bidet, P., Bonacorsi, S., 2016. Enterohemorrhagic *Escherichia coli* Hybrid Pathotype O80:H2 as a New Therapeutic Challenge. *Emerg Infect Dis* 22, 1604–1612. https://doi.org/10.3201/eid2209.160304

Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22, 2688–2690. https://doi.org/10.1093/bioinformatics/btl446

Stevens, M.P., Frankel, G.M., 2014. The Locus of Enterocyte Effacement and Associated Virulence Factors of Enterohemorrhagic *Escherichia coli*. *Microbiol Spectr* 2, EHEC-0007-2013. https://doi.org/10.1128/microbiolspec.EHEC-0007-2013

Tanizawa, Y., Fujisawa, T., Kaminuma, E., Nakamura, Y., Arita, M., 2016. DFAST and DAGA: web-based integrated genome annotation tools and resources. *Biosci Microbiota Food Health* 35, 173–184. https://doi.org/10.12938/bmfh.16-003

Tarr, P.I., Bilge, S.S., Vary, J.C., Jelacic, S., Habeeb, R.L., Ward, T.R., Baylor, M.R., Besser, T.E., 2000. Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect Immun* 68, 1400–1407. https://doi.org/10.1128/IAI.68.3.1400-1407.2000

Tatsuno, I., Horie, M., Abe, H., Miki, T., Makino, K., Shinagawa, H., Taguchi, H., Kamiya, S., Hayashi, T., Sasakawa, C., 2001. *toxB* gene on pO157 of enterohemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. *Infect Immun* 69, 6660–6669. https://doi.org/10.1128/IAI.69.11.6660-6669.2001

Thiry, D., De Rauw, K., Takaki, S., Duprez, J.-N., Iguchi, A., Piérard, D., Korsak, N., Mainil, J.G., 2018. Low prevalence of the “gang of seven” and absence of the O80:H2 serotypes among Shigatoxigenic and enteropathogenic *Escherichia coli* (STEC and EPEC) in intestinal contents of healthy cattle at two slaughterhouses in Belgium in 2014. J *Appl Microbiol* 124, 867–873. https://doi.org/10.1111/jam.13677

Thiry, D., Saulmont, M., Takaki, S., De Rauw, K., Duprez, J.-N., Iguchi, A., Piérard, D., Mainil, J.G., 2017. Enteropathogenic *Escherichia coli* O80:H2 in Young Calves with

Diarrhea, Belgium. *Emerg Infect* Dis 23, 2093–2095. https://doi.org/10.3201/eid2312.170450

Tóth, I., Nougayrède, J.-P., Dobrindt, U., Ledger, T.N., Boury, M., Morabito, S., Fujiwara, T., Sugai, M., Hacker, J., Oswald, E., 2009. Cytolethal Distending Toxin Type I and Type IV Genes Are Framed with Lambdoid Prophage Genes in Extraintestinal Pathogenic *Escherichia coli*. Infect Immun 77, 492–500. https://doi.org/10.1128/iai.00962-08

Tozzoli, R.; Scheutz, F. Diarrheagenic *Escherichia coli* infections in humans. In *Pathogenic Escherichia coli*: *Molecular and Cellular Microbiology*; Morabito, S., Ed.; Caister Academic Press: Norfolk, UK, 2014; pp. 1–18. ISBN 978-1-908230-37-9.

Van Goor, A., Stromberg, Z.R., Mellata, M., 2017. A recombinant multi-antigen vaccine with broad protection potential against avian pathogenic *Escherichia coli*. *PLoS One* 12, e0183929. https://doi.org/10.1371/journal.pone.0183929

van Hoek, A.H.A.M., van Veldhuizen, J.N.J., Friesema, I., Coipan, C., Rossen, J.W.A., Bergval, I.L., Franz, E., 2019. Comparative genomics reveals a lack of evidence for pigeons as a main source of *stx2f*-carrying *Escherichia coli* causing disease in humans and the common existence of hybrid Shiga toxin-producing and enteropathogenic E. coli pathotypes. *BMC Genomics* 20, 271. https://doi.org/10.1186/s12864-019-5635-z

Virolle, C., Goldlust, K., Djermoun, S., Bigot, S., Lesterlin, C., 2020. Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level. *Genes (Basel)* 11, 1239. https://doi.org/10.3390/genes11111239

Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E., 2017. Completing bacterial genome assemblies with multiplex MinION sequencing. *Microb Genom* 3, e000132. https://doi.org/10.1099/mgen.0.000132

Génomique comparative et phylogénie de 127 souches Shigatoxinogènes et entéropathogènes d'*Escherichia coli* O80:H2 isolées d'humains et de veaux en Belgique entre 2008 et 2024

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Introduction

Les *Escherichia coli* productrices de la lésion d'Attachement-Effacement (AE) et de toxines de Shiga (Stx) (souches AE-STEC), anciennement dénommées *E. coli* entéro-hémorragiques ou EHEC, sont responsables de colite hémorragique (CH) et de syndrome hémolytique-urémique (SHU) chez l'homme. De nombreux gènes, dont le gène *eae*, codant pour la lésion AE sont regroupés sur l'îlot de pathogénicité LEE (« Locus of Enterocyte Effacement »), tandis que d'autres sont localisés en dehors du LEE. Les gènes codant pour les toxines Stx sont situés sur des bactériophages lambdoïdes (Mainil et al., 2025).

Le sérotype AE-STEC O80:H2 a émergé depuis 2010 en Europe occidentale, principalement en France (Mainil et al., 2025). Les AE-STEC O80:H2 sont responsables non seulement de CH et de SHU, mais aussi de bactériémies et de septicémies, suite à la présence de plasmides pS88-like porteurs de gènes impliqué dans l'invasion et la survie dans l'hôte. Parallèlement, des *E. coli* entéro-pathogènes (souches EPEC), productrices de lésions AE, mais pas de Stx, et des souches AE-STEC O80:H2 ont émergé depuis 2009 en Belgique chez les jeunes veaux (<3 mois) souffrant de diarrhée, éventuellement de septicémies (Habets et al., 2021).

L'objectif de ce travail sur 127 *E. coli* O80:H2 isolées de veaux et d'humains entre 2008 et 2024 en Belgique était double: (i) identifier et comparer leurs « Multi-Locus Sequence Type » (MLST) et leurs virulotypes; et (ii) étudier leurs relations phylogénétiques dans un « Single Nucleotide Polymorphism (SNP)-based Maximum Likehood (ML) tree ».

Matériels et Méthodes

Les 59 EPEC et 27 AE-STEC de veaux isolées à l'ARSIA et les 40 AE-STEC et une STEC humaines provenant du CNR-STEC de Belgique ont été soumises au séquençage du génome total par Illumina. A ces 127 souches isolées entre 2008 et 2024, deux AE-STEC belges isolées en 1987 d'un même veau et deux AE-STEC non-belges de référence (2013C-4991 et RDEx444) ont été ajoutées pour comparaison (Habets et al., 2021; Ikeda et al., non publié).

Les gènes codant pour le sérotype O80:H2 et le MLST, les variants des gènes *eae* et *stx*, différents autres gènes situés ou non sur le LEE et les gènes localisés sur les plasmides pS88-like ont été détectés via SerotypeFinder-2.0, MLST-2.0, MyDbFinder-2.0 et VirulenceFinder-2.0 (https://cge.cbs.dtu.dk/) (seuils: ID85%; longueur: 60%) (Habets et al., 2021).

Les « Single Nucleotide Polymorphism » (SNP) présents sur les chromosomes des 127 souches belges O80:H2 ont été identifiés via Gubbins ver. 2.2.0 et Nucmer program ver. 3.1 (MUMmer package), en utilisant la séquence chromosomique de la souche américaine AE-STEC O80:H2 2013C-4991 après masquage des prophages (PP) et éléments intégratifs PP-like comme génome de référence pour des alignements un-par-un. Un « ML tree » a été construit par RAxML ver. 8.2.10 (Nakamura et al. 2020).

Résultats-1: VIRULOTYPES (Tableau 1)

- les 127 EPEC et (AE-)STEC veaux et humaines étudiés appartiennent bien au sérotype O80:H2 et au MLST301.
- les EPEC et AE-STEC veaux et humaines appartiennent à des virulotypes identiques.
- parmi les 68 (AE-)STEC, le gène *stx2d* est le plus fréquent (65%), suivi des gènes *stx1a* (16%), *stx2a* (15%) et *stx2f* (4%).
- 124 souches (98%) contiennent le gène *ehxA* codant pour une entérohémolysine et situé sur un plasmide pO157-like.
- les souches EPEC et AE-STEC *stx2d* diffèrent pour les présences de quatre gènes situés sur les plasmides pS88-like (*etsC* et *iucC*), un « prophage-like element » (*iha*) ou sur un plasmide (*cma*).

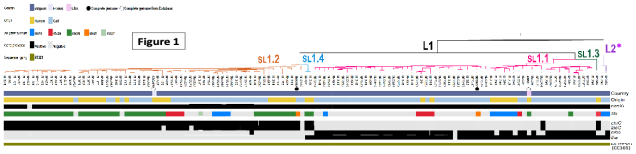
Tableau 1. Comparaison des principaux gènes détectés dans les 127 AE-STEC et EPEC O80:H2 veaux et humaines isolées entre 2008 et 2024 (* une souche humaine est *eae*- et LEE-négative = souche STEC)

Virulotypes (nbre souches)	Origine (nbre souches)	Gènes LEE		Gènes non-LEE		Gènes pS88			Autres gènes		
		*eae*ξ	*tir/espABF*	*nleABC*	*cif/espJI*	*hlyF*	*etsC*	*iucC*	*iha*	*cma*	*ehxA*
EPEC (59)	veau (59)	59	59	59	0	58	13	12	45	45	59
AE-STEC *stx1a* (11)	veau (6)	6	6	6	0	6	4	4	2	2	6
	humain (5)	5	5 (4)	5	0	3	0	0	3	5	5
AE-STEC *stx2a* (10)	veau (5)	5	5	5	0	5	2	2	3	3	5
	humain (5)	5	5	5	0	5	3	3	2	3	5
AE-STEC *stx2d* (44)	veau (13)	13	13	13	0	13	12	12	1	3	13
	humain (31)	30*	30*	31	0	31	31	30	0	0	29
AE-STEC *stx2f* (2)	veau (2)	2	2	2	0	2	1	1	1	1	2
AE-STEC *stx2*? (1)	veau (1)	1	1	1	0	1	1	1	1	0	0

Résultats-2: PHYLOGENIE (Figure 1)

- les 127 souches isolées entre 2008 et 2024 appartiennent à la lignée (L1) et les deux souches isolées en 1987 à la lignée (L2).
- 124 des souches de L1 (97,6%) appartiennent aux deux sous-lignées principales, SL1.1 (48,0%) et SL1.2 (49,6%).
- SL1.1 contient une majorité d'EPEC (78,7%) et SL1.2 une majorité d'(AE-)STEC (79,4%), essentiellement *stx2d* (63,5%).
- la majorité des 61 EPEC (78,7%) appartiennent à SL1.1 et des 66 AE-STEC (75,8%) à SL1.2.
- la majorité des 61 souches SL1.1 sont *etsC-iucC-iha+cma+* (90,2%) et des 63 souches SL1.2 sont *etsC+iucC+iha-cma-* (93,7%)

Figure 1: SNP-based « ML-tree » et virulotypes des 127 souches AE-STEC et EPEC O80:H2 veaux et humaines isolées entre 2008 et 2024, des deux AE-STEC belges isolées d'un même veau en 1987 (L2*) et des deux AE-STEC de référence°



Discussion

- Les souches AE-STEC humaines et veaux ne peuvent être différenciées, ni par leurs virulotypes (Tableau 1) et ni par leurs relations phylogénétiques (Figure 1).
- L'acquisition de LEE et du gène *eae*ξ s'est probablement produite précocement soit avant, soit après l'apparition du sérotype O80:H2.
- Les acquisitions du plasmide pS88-like, du plasmide pO157-like et du « prophage-like element » se seraient produites avant l'acquisition des phages Stx et gènes *stx*.
- Les acquisitions des phages Stx et gènes *stx* s'est produite et se produit encore à divers moments anciens ou récents (Figure 1).
- Les EPEC peuvent représenter des précurseurs (SL1.1) ou des dérivés (SL1.2) de souches AE-STEC par acquisiton / perte des phages Stx et gènes *stx*.
- En résumé, l'évolution des *E. coli* O80:H2 est non-linéaire et plus complexe qu'originellement anticipé.

REFERENCES: Habets et al. *J. Appl. Microbiol.*, 2021, **130**, 258-264 https://doi.org/10.1111/jam.14759 ; Mainil et al. *Clin. Microbiol. Rev.*, 2025, manuscript accepté pour publication ; *Nakamura et al. Microb. Genom.*, 2020, **6**, e000323. https://doi.org/10.1099/mgen.0.000323

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Annexes : Génomique comparative et phylogénie de 127 souches Shigatoxinogènes et entéropathogènes d'*Escherichia coli* O80:H2 isolées de veaux et d'humains en Belgique entre 2008 et 2024 : poster presentation in Colloque International Francophone en Maladies Infectieuses Animales (CIFMIA) Rabat, Maroc, 22th May, 2025

PART 3: Assessment and comparison of the roles of Stx-phages and pS88 plasmids in the pathogenicity of AE-STEC and EPEC O80:H2 in the *in vivo* model in larvae of *Galleria mellonella* moth.

FOREWORD

The emerging AE-STEC serotype O80:H2 is involved not only in HC and HUS, but also in bacteremia and internal organ infections in humans. Moreover, AE-STEC and EPEC are associated with diarrhea and sometimes septicemia in young calves (EFSA, 2020; Habets et al., 2021; Soysal et al., 2016). Their primary virulence properties include the production of: (i) AE lesions on enterocytes encoded by LEE-located genes including the *eae* gene coding for the intimin adhesion by AE-STEC and EPEC; (ii) Shiga toxins (Stx) encoded by phage-located *stx* genes by AE-STEC; and (iii) bacteremia/septicemia encoded by different genes located on the ColV-pS88 plasmids by AE-STEC and EPEC. Two types of pS88 plasmid gene profiles have been described based on the presence or absence of the *ets* and *iuc* genes and depending on the lineage to which the strains belong to (Habets et al., 2021; Ikeda et al., unpublished).

Despite the potential public health hazard, the respective roles of these properties of AE-STEC and EPEC O80:H2 have not been fully assessed and compared in animal models yet (Lemaître et al., 2012). Therefroe, the roles of two Stx, of one Stx2d-encoding phage and of the two types of pS88 plasmids, but not of the LEE in the pathogenicity island, were compared using a *Galleria mellonella* larvae model, whose innate immune system shares similarities with human innate immune system (Cutuli et al., 2019).

Among the 10 strains tested, the *stx2d* AE-STEC strain and the K12DH10B transfected with the Stx2d phage exhibited the highest lethality, with all larvae killed during the experimental period at the highest inoculated bacterial concentration (log6). While the *stx1a* AE-STEC and the EPEC strains were also lethal, they were less virulent with approximator 0-10% of larvae were survived compared to the *stx2d* AE-STEC strain. The pS88 plasmid contained two genes (*ets* and *iuc*) showed limited effects in Stx-producing strains, but slightly increased lethality in EPEC strains compared to the plasmid did not contain these two genes. More than 70% of larvae infected with the

K12DH10B strain carrying the pS88 plasmid conjugated by *stx1a* AE-STEC with *ets* and *iuc* genes survived, even at log6 concentrations.

These results suggested that Stx2d functions as a major virulence factor of AE-STEC O80:H2, at least in the *Galleria mellonella* model, while the role of the pS88 plasmids is observed in EPEC O80:H2 but more limited in AE-STEC strains. To better understand the pathogenic mechanisms and the role of each virulence factors of O80:H2, continuous efforts are needed, including more precise *in vivo* / *ex vivo* assessments beyond the insect model.

STUDY 4. Role of Stx toxins, STX2d phage and pS88 plasmids in the virulence of AE-STEC and EPEC O80:H2 in larvae of *Galleria mellonella*.

PUBLICATION 3

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Article

Virulence of Shigatoxigenic and Enteropathogenic *Escherichia coli* O80:H2 in *Galleria mellonella* Larvae: Comparison of the Roles of the pS88 Plasmids and STX2d Phage

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Simple Summary: Following the "Replacement/Reduction/Refinement" policy, insects are proposed to replace mammals and birds as experimental models to study the virulence of bacterial pathogens and to identify their virulence properties. The aim of this study was to assess in larvae of the *Galleria mellonella* moth the virulence of the Shigatoxigenic and enteropathogenic *Escherichia coli* O80:H2 and the respective roles of two virulence properties: the pS88 plasmid-encoded invasiveness properties and the phage-encoded Shiga toxin 2d. The objectives were to compare: (i) the virulence of bovine Shigatoxigenic and enteropathogenic *E. coli* O80:H2; (ii) the roles of the pS88 plasmid and Shiga toxin 2d-encoding phage; (iii) the virulence of *E. coli* O80:H2 and O80:non-H2. The results and the conclusions are: (i) *E. coli* O80:H2 and O80:non-H2 are lethal at log5 and log6 concentrations; (ii) the pS88 plasmids are partially responsible for the virulence of *E. coli* O80:H2; (iii) the phage-encoded Stx2d toxin is entirely responsible for the virulence of the Shigatoxigenic *Escherichia coli* O80:H2; (iv) the virulence properties of *E. coli* O80:non-H2 could not be identified. As a general conclusion, *G. mellonella* larvae represent a useful model to study the virulence of bacterial pathogens but are limited in identifying their virulence properties.

Abstract: The invasiveness properties of Shigatoxigenic and enteropathogenic *Escherichia coli* (STEC and EPEC) O80:H2 in humans and calves are encoded by genes located on a pS88-like ColV conjugative plasmid. The main objectives of this study in larvae of the *Galleria mellonella* moth were therefore to compare the virulence of eight bovine STEC and EPEC O80:H2, of two *E. coli* pS88 plasmid transconjugant and STX2d phage transductant K12 DH10B, of four *E. coli* O80:non-H2, and of the laboratory *E. coli* K12 DH10B strains. Thirty larvae per strain were inoculated in the last proleg with 10 µL of tenfold dilutions of each bacterial culture corresponding to 10 to 10^6 colony-forming units (CFUs). The larvae were kept at 37 °C and their mortality rate was followed daily for four days. The main results were that: (i) not only the STEC and EPEC O80:H2, but also different *E. coli* O80:non-H2 were lethal for the larvae at high concentrations (from 10^4 to 10^6 CFU) with some variation according to the strain; (ii) the Stx2d toxin and partially the pS88 plasmid were responsible for the lethality caused by the *E. coli* O80:H2; (iii) the virulence factors of *E. coli* O80:non-H2 were not identified. The general conclusions are that, although the *Galleria mellonella* larvae represent a useful

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first-line model to study the virulence of bacterial pathogens, they are more limited in identifying their actual virulence properties.

Keywords: *Galleria mellonella* larvae; virulence; Shigatoxigenic *Escherichia coli*; enteropathogenic *Escherichia coli*; O80:H2; O80:non-H2; pS88 plasmid; STX2d phage

1. Introduction

Escherichia coli (*E. coli*) is a Gram-negative bacterial species and a member of the human and animal intestinal microbiota. Although most *E. coli* strains are harmless to their hosts, some strains have acquired genes encoding virulence properties and can cause disease in both humans and animals: they are generically called pathogenic *E. coli* [1].

Pathogenic *E. coli* are classified into two main groups: diarrheagenic *E. coli* or DEC and extraintestinal *E. coli* or ExPEC. ExPEC comprise different invasive *E. coli* (septicemic or SePEC, neonatal meningitis-associated or NMEC) and uropathogenic *E. coli* (UPEC) whereas DEC are subdivided into six main pathotypes: enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC), Shigatoxigenic (STEC), enteroaggregative (EAEC), and Diffusely Adherent (DAEC) *E. coli* [1–4].

Moreover, "hybrid" pathogenic *E. coli* have been described that combine the properties of different pathotypes. One recent dramatic example is the *E. coli* O104:H4 that caused a short-lived outbreak of diarrhea and hemolytic–uremic syndrome in humans in Germany in 2011 and combined the typical properties of EAEC and STEC [5,6]. Nevertheless, the most frequent examples are still today the enterohemorrhagic *E. coli* (EHEC) that emerged in humans in the year 1980 and produced both the Attaching-Effacing (AE) lesion on enterocytes typical of EPEC and one or two Shiga toxins (Stx) typical of STEC [1,3]. Since the EHEC nomenclature is considered obsolete by EFSA [7], they will be named "Attaching-Effacing STEC" (AE-STEC) in this manuscript, as previously proposed [6]. The AE lesion is encoded by genes grouped together on one chromosomal pathogenicity island (Locus of Enterocyte Effacement or LEE) while the Stx are encoded by *stx* phage-located genes [1,3,4]. Two Stx families have been described: Stx1 with three subtypes, (Stx1a, Stx1c, Stx1d) and Stx2 with up to 11 subtypes so far (Stx2a to Stx2k) [8–11].

The most frequent and pathogenic AE-STEC serotypes in humans are O26:H11, O103:H2, O111:H-, O121:H9, O145:H- and O157:H7, and most frequently the contamination sources are meat, dairy, or vegetable foods contaminated with feces of ruminants, especially cattle, that are healthy carriers in their intestines. Nevertheless, other so-called "minor" serotypes can emerge from time to time and become epidemiologically important in some countries [3,12].

Serotype O80:H2 represents one of these "minor" serotypes that has been emerging since 2010 in France and other Western European countries in humans suffering (bloody) diarrhea, hemolytic–uremic syndrome, and bacteremia [13–18]. These AE-STEC O80:H2 combine not only the pathogenic properties of EPEC and STEC, but also of SePEC since they can invade the blood stream and colonize internal organs. The invasiveness properties are encoded by genes located on a pS88-like ColV conjugative plasmid [15,16,18,19].

In addition to their presence in humans, not only AE-STEC, but also EPEC O80:H2 have been emerging since 2009 in young calves in Belgium suffering from diarrhea and only very occasionally from septicemia [20,21]. Genetic analysis confirmed that calf AE-STEC and EPEC O80:H2 are highly related to human AE-STEC O80:H2 by their virulotypes, including the presence of the *eae*ξ intimin-encoding gene on the LEE and of a pS88-like ColV plasmid [21]. Ca. 80% of the bovine AE-STEC harbor the *stx2d* gene like the great majority of human AE-STEC O80:H2, while the remaining bovine and human AE-STEC O80:H2 harbor the *stx1a* or the *stx2a* gene [17,21,22]. Nevertheless, a difference in the virulotypes of AE-STEC and EPEC O80:H2 exists: the majority of *stx2d* AE-STEC harbor the *etsC* and *iucC* genes associated with the pS88 plasmid, but not the *cma* and *iha* chromosomal genes, while the EPEC and the *stx1a* and *stx2a* AE-STEC have the opposite gene profile [21].

Phylogenetically, the different AE-STEC and EPEC O80:H2 form different closely related sub-lineages in the single nucleotide polymorphism (SNP)-based phylogenetic tree and the bovine and human AE-STEC are intermixed in the same sub-lineages. The *stx2d*-positive AE-STEC group together, while the other AE-STEC are more closely related to EPEC [21].

No in vivo study has so far been performed to compare the respective role(s) of the AE lesion, Stx toxin, and pS88-encoded properties in the pathogenicity of AE-STEC and EPEC O80:H2 in humans and calves. Following the 3R policy (Replacement, Reduction and Refinement) [23], insects have been proposed as infectious models to replace mammals and birds for in vivo testing of not only virulence, but also therapy and prophylaxis of bacterial and viral pathogens [24,25]. Of them, larvae of the *Galleria mellonella* moth are frequently used to study bacterial species, including different pathotypes of *E. coli* [25–28]. Besides their low cost and the possibility of testing multiple groups of larvae in a short time, *G. mellonella* larvae possess an innate immune system similar to mammals and can be maintained at 37 °C, like the bacterial pathogens of mammals [25,29]. So, it was recently observed that the STX2d phage and the type 3 secretion system (T3SS) contribute to the virulence of AE-STEC O80:H2 and EPEC O127:H6, respectively, in *G. mellonella* larvae [25,30,31], while the contribution of the T3SS to the virulence of AE-STEC O157:H7 is less clear [32,33]. However, no study has been performed yet to assess the role of the pS88 plasmids of AE-STEC and EPEC O80:H2.

Therefore, the aim of this study in *G. mellonella* larvae was to compare the virulence of: (i) Belgian calf AE-STEC and EPEC O80:H2 harboring different *stx* genes and pS88 plasmids; (ii) laboratory *E. coli* K12 pS88 plasmid transconjugant and STX2d phage transductant; (iii) Belgian bovine AE-STEC and EPEC O80:H2 and *E. coli* O80:non-H2.

2. Materials and Methods

2.1. Bacterial Strains

The virulence of 11 wild-type Belgian bovine *E. coli* O80 strains was studied in *Galleria mellonella* larvae (Table 1): eight AE-STEC and EPEC O80:H2 strains with different virulotypes isolated from diarrheic calves at ARSIA ("Association Régionale de Santé et d'Identification animale") and three *E. coli* O80:H45 and O80:H6 strains isolated from healthy adult cattle [21,34]. The genomes of six AE-STEC and EPEC O80:H2 and of the three *E. coli* O80:H45 and O80:H6 strains were previously sequenced and analyzed. The remaining two AE-STEC O80:H2 were identified by PCR, as previously described [21], and were genome sequenced for confirmation (see Section 2.2). One of the AE-STEC O80:H2 (EH3320/SES3090) had been previously tested in larvae of *G. mellonella* [30] and was the O80:H2 positive control.

Table 1. Serotypes and virulotypes of the wild-type *E. coli* strains used in this study.

Isolate References [1]	Serotype	*stx* Genes	*eae* Gene	pS88 Plasmid Virulotype (*iucC*/*etsC* Genes)	Reference of Genome Sequencing
SES5320	O80:H2	1a	ξ	nd/nd [2]	This study
SES5363		1a	ξ	d/d	
EH2282 [3,4]		1a	ξ	d/d	[21]
EH3160 [4]		2d	ξ	d/d	
EH3307/SES2959		2d	ξ	nd/nd	
EH3320/SES3090 [5]		2d	ξ	d/d	
EH3308/SES2973		nd	ξ	nd/nd	
EH3322/SES3122		nd	ξ	d/d	
SES6039	O80:H6	nd	nd	--- [6]	[34]
SES5725	O80:H45	nd	nd	---	
SES6156		nd	nd	---	
Serotype collection	O80:H26 [5]	nd	nd	---	This study
	O78:H4	nd	nd	d/d	

[1] EH reference in [21]; SES reference is a laboratory new reference. [2] d: gene detected; nd: gene not detected. [3] AE-STEC O80:H2 EH2282 strain was erroneously referred to as *stx2a* instead of *stx1a* in Figure 1 of reference [21]. [4] AE-STEC O80:H2 EH2282 strain was the donor strain for the pS88 plasmid conjugation; AE-STEC O80:H2 EH3160 strain was the donor strain for STX2d phage transduction. [5] AE-STEC O80:H2 EH3320/SES3090 and serotype collection *E. coli* O80 strains were previously tested in *G. mellonella* [30]. [6] --- = no pS88 plasmid.

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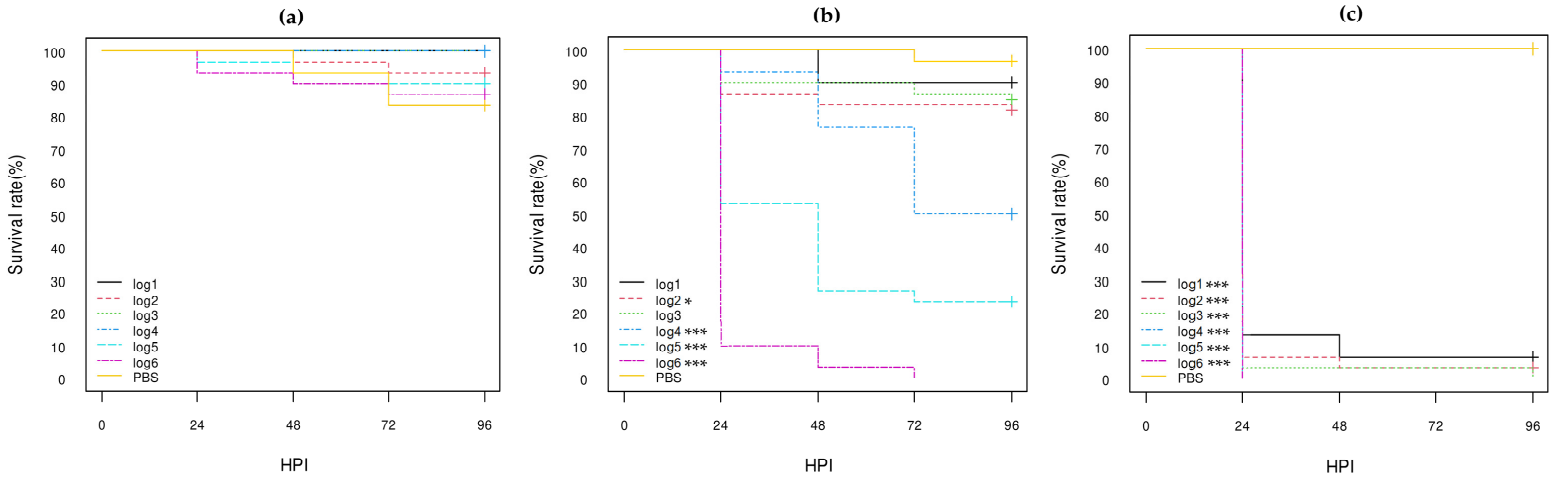

Figure 1. Kaplan–Meier survival curves of control *E. coli* strains: (**a**) K12-DH5α; (**b**) serotype O80:H26 (EH3161 strain); (**c**) serotype O78:H4 (pS88++). HPI = Hour Post-Inoculation. * *p*-value statistically significant at the threshold 0.05. *** *p*-value statistically significant at the threshold 0.001. pS88++: pS88 plasmid carrying the *ets*C/*iuc*C genes.

The laboratory *E. coli* K12 DH5α strain and one *E. coli* O80:H26 strain of the laboratory serotype collection were the non-O80:H2 negative and positive controls, also based on previously published results in larvae of *G. mellonella* [30]. One *E. coli* O78:H4 strain also of the laboratory serotype collection was added as a control strain after genome sequencing and preliminary testing in *G. mellonella* larvae (see Section 3.1).

Laboratory *E. coli* K12 DH10B and MG1655 strains were used in the pS88 plasmid conjugation and the former was also used in the STX phage transduction. The laboratory *E. coli* K12 strains harbor the genes for the production of the O16 surface antigen although they are phenotypically rough [35].

2.2. Genome Sequencing

The genomes of the two not-yet-sequenced AE-STEC O80:H2 and of the *E. coli* O80:H26 and O78:H4 were sequenced and analyzed to confirm the presence or absence of *stx*, *eae*, pS88-located and *cma*/*iha* genes using Virulence Finder 2.0 (https://cge.food.dtu.dk/services/VirulenceFinder/; accessed on 23 March 2023) and their O:H serotypes using SeroType Finder 2.0 (https://cge.food.dtu.dk/services/SerotypeFinder/; accessed on 23 March 2023), as previously described [21,34]. Sequencing data were submitted as NCBI BioProject PRJNA973567. The Genbank accession numbers are SAMN35130762 (O78C), SAMN35130763 (EH3161), SAMN35130764 (SES5320) and SAMN35130765 (SES5363).

2.3. Construction of DH10B Transconjugant and Transductant

The pS88 plasmid conjugation strategy was based on the presence of the ColV-encoding *cva* operon on the pS88 plasmid of bovine AE-STEC and EPEC O80:H2 [21–36]. The recipient strain was the *E. coli* K12 DH10B strain harboring the recombinant plasmid pAuto-ColV-Switch1.0 carrying a synthetic ColV-encoding locus (received from Syngulon Company, Seraing, Belgium; https://syngulon.com). The *cva*C ColV peptide-encoding gene is, however, disrupted by the insertion of an ampicillin resistance cassette (Amp^R) while the other genes of the locus remain functional. Therefore, the *E. coli* K12 DH10B strain is unable to produce the ColV peptide but remains immune to ColV since the *cvi* gene is intact.

To choose the donor strain, all 33 Belgian bovine AE-STEC and EPEC O80:H2 previously identified [21] were phenotypically tested for their antibiotic resistance profile by the disk diffusion assay [37] against 12 antibiotics on Mueller–Hinton (AxonLab, Machelen, Belgium) agar plates: ampicillin/amoxicillin (20 µg), amoxicillin/clavulanic acid (20 µg + 10 µg), cefoxitin (30 µg), ceftiofur (30 µg) and cefquinome (30 µg) (β lactams), kanamycin (30 µg) and gentamicin (30 µg) (aminosides), nalidixic acid (30 µg) and enrofloxacin (5 µg) (<fluoro>quinolones), florfenicol (30 µg) (phenicols), doxycycline (30 µg) (tetracyclins), and trimethoprim/sulfamethoxazole (1.25 µg + 23.75 µg) (AxonLab, Machelen, Belgium). According to the EUCAST CASFM and CASFMVET 2020 reference of values (https://www.sfm-microbiologie.org/2020/10/02/casfm-eucast-v1-2-octobre-2020/; https://www.sfm-microbiologie.org/2020/09/09/casfm-veterinaire-2020/; accessed on 15 February 2021), nine strains were sensitive to ampicillin. Of them, the AE-STEC EH2282 strain (Table 1) isolated in 1987 [20] was resistant to only three of the tested antibiotics (kanamycin, doxycycline, trimethoprim/sulfamethoxazole) compared to the other ampicillin-sensitive strains and was therefore chosen as the donor strain.

The pS88 plasmid conjugation was performed on Luria–Bertani (LB; VWR Chemicals, Leuven, Belgium) agar plates [38] by mixing 100 µL of an 8 h growth of the donor strain in 5 mL of LB broth and 500 µL of an 8 h growth of the recipient strain in LB broth with 100 µg/mL ampicillin. After overnight incubation at 37 °C, full loops of the macrocolony were streaked on 10 LB agar plates with 100 µg/mL ampicillin and incubated overnight at 37 °C. Isolated colonies were transferred to two 96-well microtiter plates containing 200 µL LB broth with 100 µg/mL ampicillin and grown overnight at 37 °C. The following day the colonies were transferred onto LB agar plates with 100 µg/mL ampicillin covered with a layer of the ColV-sensitive *E. coli* K12 MG1655 strain harboring the pKK223-3

plasmid [39] carrying an ampicillin resistance-encoding gene (received from Syngulon Company, Seraing, Belgium; https://syngulon.com). The colonies inhibiting the growth of the *E. coli* K12 MG1655 strain were sub-cultured in LB broth with 100 µg/mL ampicillin of which two mL were transferred into two CRYO tubes (Greiner Bio-One, Frickhausen, Germany) with two mL of sterile 80% glycerol that were stored at −20 °C and −80 °C, respectively, until further use.

These colonies were subsequently grown on LB agar plates with 100 µg/mL ampicillin and tested with PCR for the serotype O80-, H2-, and O16-encoding genes, the *cvaC* wild-type and Amp^R cassette-inserted genes and the pS88 plasmid-located *hlyF* gene (Table 2), using the FASTGENE2x Optima Hotstart kit (Nippon Genetics, Filter service, Eupen, Belgium) after DNA extraction from one colony by boiling [34].

Table 2. Target genes, primer sequences and amplified fragment lengths of the PCR applied on the DH10B pS88 plasmid transconjugant candidates.

PCR	Target Genes	Primers	Amplified Fragment Length	Reference
Serotype O80	*wzy*$_{O80}$	Og80-F: 5′-TGGTGTTGATTCCACTAGCGT-3′ Og80-R: 5′-CGAGAGTACCTGGTTCCCAAA-3′	285 bp	[40]
Serotype H2	*fliC*$_{H2}$	Hg2-F: 5′-TGATCCGACACTTCCTGATG-3′ Hg2-R: 5′-CCGTCATCACCAATCAACGC-3′	228 bp	[41]
Serotype O16	*wzx*$_{O16}$	Og16-F: 5′-GGTTTCAATCTCACAGCAACTCAG-3′ Og16-R: 5′-GTTAGAGGGATAATAGCCAAGCGG-3′	302 bp	[40]
Colicin V_wt Colicin V_M [1]	*cva*C_wt *cva*C_M	CvaC-F: 5′-TATGAGAACTCTGACTCTAAAT-3′ CvaC-R: 5′-ATTTATAAACAAACATCACTAA-3′	314 bp 1555 bp [1]	[42]
Avian hemolysin	*hlyF*	HlyF-F: 5′-GGCGATTTAGGCATTCCGATACTC-3′ HlyF-R: 5′-ACGGGGTCGCTAGTTAAGGAG-3′	599 bp	[19]

[1] The *cvaC* gene is disrupted by the insertion of an ampicillin resistance cassette (see Section 2.3).

STX2d phage transductants were constructed using the *E. coli* K12 DH10B strain free of the recombinant plasmid pAuto-ColV-Switch1.0 (received from the Syngulon Company, Seraing, Belgium; https://syngulon.com) as the recipient strain, as previously described [30]. STX2d phages were induced by UV radiation and isolated from the three bovine *stx2d* AE-STEC O80:H2 EH3155, EH3160 and EH3320/SES3090 strains. The transductant candidates were confirmed with a *stx2d* gene quantitative (q) PCR.

2.4. In Vivo Assay: The Galleria Mellonella Model

At first, the growth curve of all 17 *E. coli* strains tested in larvae of *G. mellonella* (eight *E. coli* O80:H2, two *E. coli* O80:H45, one *E. coli* O80:H6, the two *E. coli* O80:H26 and O78:H4, the two *E. coli* K12 DH5a and DH10B strains, and the two *E. coli* K12 DH10B pS88 plasmid-conjugated and STX2d phage-transduced strains) was followed by comparing the optical density of an LB broth culture at a wavelength of 600 nm (OD_{600}) and the number of colony-forming units (CFU) after plating 10 µL on LB agar and overnight growth at 37 °C. An OD_{600} between 0.2 and 0.35 corresponded to a concentration of 10^8 CFU/mL depending on the strain. Then, 10 µL of an overnight culture of each strain in LB broth at 37 °C was transferred to fresh LB broth that was incubated at 37 °C until reaching the appropriate OD_{600} corresponding to a concentration of 10^8 CFU/mL.

Thirty larvae per strain divided into three groups of 10 larvae (Animal Confort, Loncin, Belgium) were inoculated in the last proleg, as previously described [30], with 10 µL of each of the tenfold dilutions of the bacterial culture corresponding to 10 to 10^6 CFU using an automatic injector (ColeParmer, Vernon Hills, IL, USA). In addition, 30 larvae were injected with 10 µL of PBS as a control group. The larvae were kept at 37 °C and their mortality rate was followed daily for four days. In parallel, back-titration was performed

to confirm the actual inoculation dose by streaking 10 µL of each dilution on LB agar plates and incubating them overnight at 37 °C.

2.5. Statistical Analysis

All statistical analyses were performed using R software with "Rcmdr v2.6-0" and "survival" packages (https://www.john-fox.ca/RCommander/index.html; accessed on 1 April 2023) after pooling the different groups of larvae per strain, virulotype, or serotype. Kaplan–Meier curves were created to assess the survival of the larvae according to the inoculation doses of each bacterial strain. Log-rank tests were carried out to highlight significant differences in survival rates between the groups of larvae inoculated with the different concentrations (log1 to log6) of each *E. coli* strain and the PBS group. Hazard ratios (HRs) with a 95% confidence interval (HR-95%) were calculated to give the relative measure of the risk factor for death for the larvae when comparing the same concentration of two different *E. coli* strains or groups of strains, the reference and the test strains: when the HR-95% values included the value 1, the risk factor for death was the same with both strains; when both HR-95% values were >1 or <1, the risk factor for death was higher or lower, respectively, with the test strain(s) compared to the reference strain(s). The significant thresholds of 0.05, 0.01, and 0.001 were applied for all statistical analysis.

3. Results

3.1. Virulotypes and Serotypes of the Four Genome Sequenced E. coli Strains

The genome sequencing and analysis confirmed the virulotypes and serotypes of the two AE-STEC O80:H2 SES5320 and SES5363 strains using Virulence Finder 2.0 and SeroType Finder 2.0 (Tables 1 and 3). The *eaeξ* and *stx1a* genes and several genes located on the pS88 plasmid, including the specific *hlyF* gene, were detected. The pS88 plasmid-located *etsC*/*iucC* genes were detected in the AE-STEC O80:H2 SES5363 strain, but not in the AE-STEC O80:H2 SES5320 strain. Conversely, the chromosomal *cma*/*iha* genes were detected in the AE-STEC O80:H2 SES5320 strain, but not in the AE-STEC O80:H2 SES5363 strain. Therefore, three *stx1a* AE-STEC, three *stx2d* AE-STEC, and two EPEC O80:H2 strains with different pS88 plasmid and chromosomal gene profiles (Tables 1 and 3) were tested in *G. mellonella* larvae.

The serotypes of the *E. coli* O80:H26 and O78:H4 of the laboratory serotype collection were also confirmed (Tables 1 and 3). The *stx* genes and the LEE-located genes were not detected, identifying them as neither AE-STEC nor EPEC. Conversely, the pS88 plasmid-located genes, including the *etsC*/*iucC* genes, were detected in the *E. coli* O78:H4 strain, but not in the *E. coli* O80:H26 strain (Tables 1 and 3), whereas the *cma*/*iha* chromosomal genes were not detected. Since the *E. coli* O78:H4 was pathogenic for larvae of *G. mellonella* in preliminary testing and harbored one pS88-like plasmid, this strain was added as a non-O80 pS88 plasmid-positive control.

Table 3. Virulence genes detected after genome analysis with Virulence Finder 2.0 of the *E. coli* O80:H2, O80:H6, O80:H26, O80:H45 and O78:H4 strains.

***E. coli* Serotypes and Strains**		**O80:H2** [1,2]								**O80:H6** [3]	**O80:H26** [4]	**O80:H45** [3]		**O78:H4** [4]
Genes Detected after Genome Analysis		**SES5320**	**SES5363**	**EH2282** [5]	**EH3307/ SES2959**	**EH3320/ SES3090**	**EH3160**	**EH3308/ SES2973**	**EH3322/ SES3122**	**SES6039**	**EH3161**	**SES5725**	**SES6156**	**O78C**
Phage-located genes	*stx1a*	+ [6]	+	+										
	stx2d				+	+	+							
LEE-located genes	*eaeξ*	+	+	+	+	+	+	+	+					
	espA/B/F/P	+	+	+	+	+	+	+	+					
	tir	+	+	+	+	+	+	+	+					
pS88 plasmid-located genes	*cia*	+	+	+	+	+	+	+	+					+
	cvaA	+	+	+	+	+	+	+	+					+
	hlyF	+	+	+	+	+	+	+	+					+
	iroN	+	+	+	+	+	+	+	+					+
	iss	+	+	+	+	+	+	+	+	+		+	+	+
	ompT	+	+	+	+	+	+	+	+			+	+	+
	sitA	+	+	+	+	+	+	+	+			+	+	+
	etsC		+	+		+	+		+					+
	iucC		+	+		+	+		+					+
Chromosome-located genes	*cma*	+		+	+			+						
	iha	+		+	+			+						

[1] all but *stx1a* AE-STEC SES5320 and SES5363 strains were previously sequenced [21]. The Genbank accession numbers are SAMN35130764 (SES5320) and SAMN35130765 (SES5363). [2] EH numbers are listed in [21]; SES numbers are the new laboratory collection references. [3] all three *E. coli* O80:H6 and O80:H45 strains were previously sequenced [34]. [4] The Genbank accession numbers are SAMN35130763 (EH3161) and SAMN35130762 (O78C). [5] *stx1a* AE-STEC EH2282 strain was erroneously referred to as *stx2a* instead of *stx1a* in Figure 1 of reference [21]. [6] +: gene detected; empty box: gene not detected.

3.2. Identification of Transconjugant and Transductant

After growth in LB broth with 100 µg/mL ampicillin in microtiter plates, 50 colonies were transferred onto LB agar plates with 100 µg/mL ampicillin covered with a layer of the ColV-sensitive ampicillin-resistant *E. coli* K12 MG1655 strain. Eleven of these 50 colonies inhibited the growth of the *E. coli* K12 MG1655 strain and gave two amplification fragments (314 bp and 1555bp; Table 2) with the PCR for the *cva*C wild-type and Amp^R cassette-inserted genes, respectively. Of the 11 transconjugant candidates, two also tested positive with the PCR for the O16-encoding genes and the pS88 plasmid-located *hlyF* gene and negative with the PCR for the serotype O80- and H2-encoding genes. These two transconjugant candidates (F5 and D4) were sub-cultured on two LB agar plates with 100 µg/mL ampicillin. Ten colonies from each agar plate were chosen and re-tested with the same PCR. Of these 20 colonies, 12 once more gave unambiguous PCR results and 1 colony from the F5 transconjugant candidate was chosen for testing in *G. mellonella* larvae.

The STX2d phages isolated from the AE-STEC O80:H2 EH3155 and EH3160 strains, but not from the EH3320/SES3090 strain, produced plaque lysis on the *E. coli* K12 DH10B strain. Either phage and the *E. coli* K12 DH10B strain were mixed in LB broth and incubated overnight at 37 °C. The transductant candidates were recovered by centrifugation, re-suspended in LB broth, and spread on STEC agar plates. After overnight incubation at 37 °C, one colony from each plate was randomly picked up and confirmed by qPCR targeting the *stx2d* gene. These colonies were sub-cultured three times on STEC agar to confirm the stability of the transduced phages. At each stage, the transductant candidates were confirmed by the qPCR targeting the *stx2d* gene. *E. coli* K12 DH10B transductant from AE-STEC O80:H2 EH3160 strain was chosen for studies in *G. mellonella* larvae.

3.3. Virulence of *E. coli* Strains in *G. mellonella* Larvae

3.3.1. *E. coli* Non-O80:H2 Control Strains

Of these three control strains (*E. coli* K12 DH5α, *E. coli* O80:H26, and *E. coli* O78:H4), the *E. coli* O78:H4 strain was the most highly virulent, killing almost all larvae within 24 h post-inoculation (HPI) even at log1 concentration (p-value < 0.001), while more than 80% of larvae inoculated with the *E. coli* K12 DH5α strain still survived at 96 HPI at log6 concentration (Figure 1; Table S1). The *E. coli* O80:H26 strain was also virulent, killing about half and 75% of the larvae at log4 and log5 concentrations, respectively, at 96 HPI and all larvae at log6 concentration at 72 HPI (p-value < 0.001; Figure 1; Table S1). The *E. coli* K12 DH5α and O80:H26 strains gave similar results to those previously observed [30].

3.3.2. Comparison of the *E. coli* O80:H2 Strains Belonging to Different Virulotypes

According to the log-rank analysis of Kaplan–Meier curves (Figures 2–4; Table S1), the log5 and log6 concentrations of all eight *E. coli* O80:H2 strains gave significantly different results from the PBS group results with less than 40% survival at 96 HPI (p-value < 0.001). The *stx2d* AE-STEC EH3320/SES3090 strain gave similar results to those previously obtained [30]. Although all three *stx2d* AE-STEC were also significantly more lethal compared to the PBS group at the log4 concentration (p-value < 0.001) with ca. 40% death at 96 HPI (Figure 3; Table S1), this was not the case for the *stx1a* AE-STEC and at least for one EPEC (Figures 2 and 4; Table S1). Conversely, the results with the lowest concentrations (log1 to log3) were much more heterogeneous, even within the same virulotype (Figures 2–4; Table S1), and were not further analyzed. Therefore, the hazard ratios (HRs) and the confidence intervals 95% (HR-95%) were statistically analyzed only for the log5 and log6 concentrations.

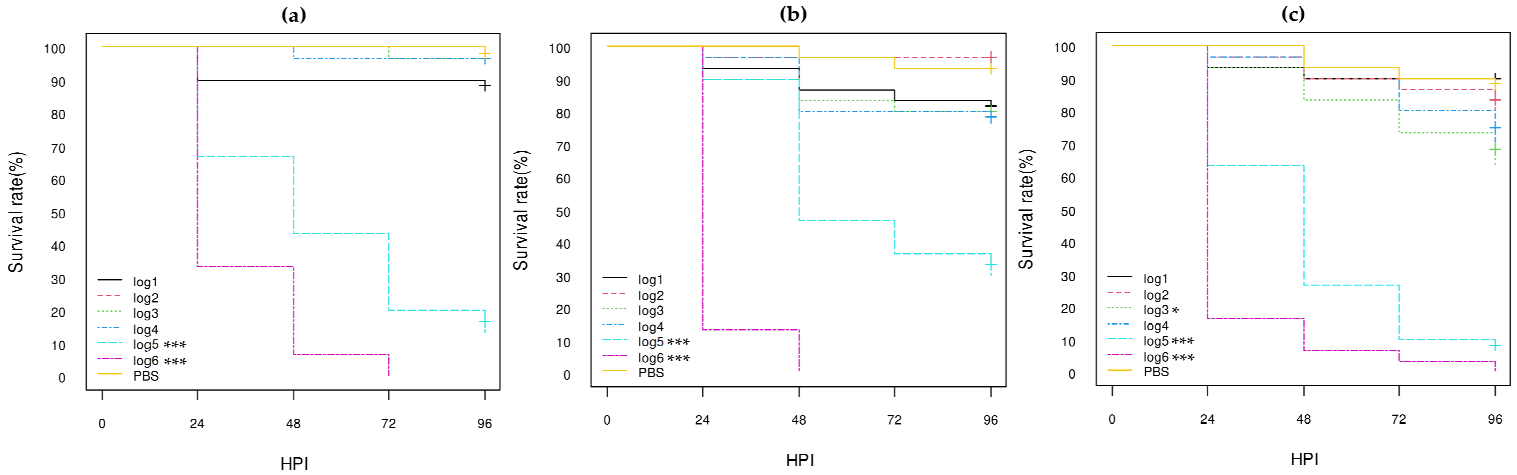

Figure 2. Kaplan–Meier survival curves of *stx1a* AE-STEC O80:H2 strains: (**a**) SES5320 strain (pS88--); (**b**) SES5363 strain (pS88++); (**c**) EH2282 strain (pS88++). HPI = Hour Post-Inoculation. * *p*-value statistically significant at the threshold 0.05. *** *p*-value statistically significant at the threshold 0.001. pS88++: pS88 plasmid carrying the *etsC*/*iucC* genes. pS88--: pS88 plasmid not carrying the *etsC*/*iucC* genes.

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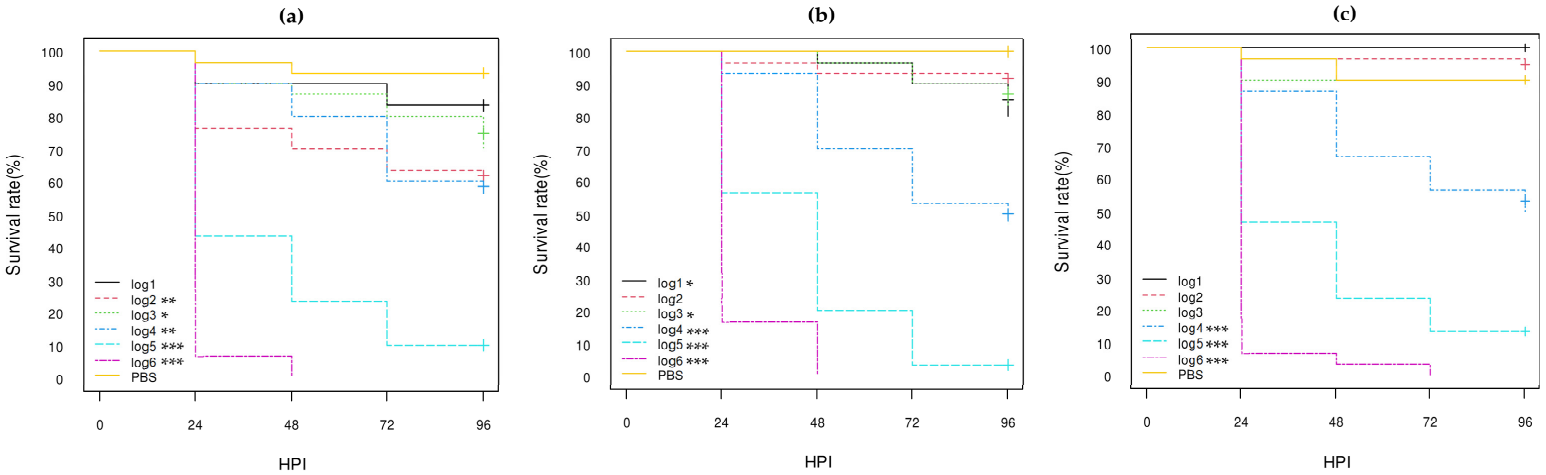

Figure 3. Kaplan–Meier survival curves of *stx2d* AE-STEC O80:H2 strains: (**a**) EH3160 strain (pS88++); (**b**) EH3307/SES2959 strain (pS88--); (**c**) EH3320/SES3090 strain (pS88++). HPI = Hour Post-Inoculation. * *p*-value statistically significant at the threshold 0.05. ** *p*-value statistically significant at the threshold 0.01. *** *p*-value statistically significant at the threshold 0.001. pS88++: pS88 plasmid carrying the *etsC*/*iucC* genes. pS88--: pS88 plasmid not carrying the *etsC*/*iucC* genes.

At the highest bacterial concentration (log6), almost all larvae were dead at 96 HPI regardless of the virulotype (Figures 2–4). However, the *stx2d* AE-STEC strains significantly killed more larvae and more quickly than the EPEC (Figures 3 and 4) with all larvae dead at 72 HPI while a few larvae were still alive at 96 HPI with the EPEC. The HR-95% of the *stx2d* AE-STEC vs. EPEC strains was statistically significant: between 0.44 and 0.88 (p-value < 0.01) (Table S2). Conversely, the results with *stx1a* vs. *stx2d* AE-STEC and with *stx1a* AE-STEC vs. EPEC were not significantly different (Figures 2 and 3; Table S2).

At the log5 concentration, the *stx2d* AE-STEC strains significantly killed more larvae and more quickly than the EPEC and the *stx1a* AE-STEC, with a survival rate lower than 20% at 96 HPI vs. 10–40% for the different *stx1a* AE-STEC and EPEC strains (Figures 2–4). The HC-95% of the *stx1a* AE-STEC vs. *stx2d* AE-STEC was higher than 1 (between 1.17 and 2.19 with a p-value < 0.01), while the HC-95% of the *stx2d* AE-STEC vs. EPEC was lower than 1 (between 0.40 and 0.83 with a p-value < 0.01) (Table S2). Conversely, the results of the *stx1a* AE-STEC vs. EPEC were not significant, similar to the log6 concentration.

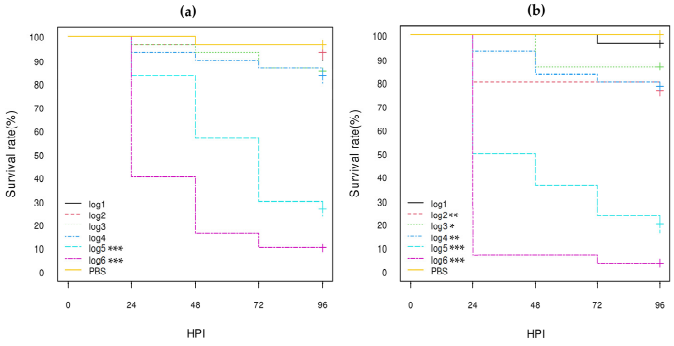

Figure 4. Kaplan–Meier survival curves of EPEC O80:H2 strains: (**a**) EH3308/SES2973 strain (pS88--); (**b**) EH3322/SES3122 strain (pS88++). HPI = Hour Post-Inoculation. * p-value statistically significant at the threshold 0.05. ** p-value statistically significant at the threshold 0.01. *** p-value statistically significant at the threshold 0.001. pS88++: pS88 plasmid carrying the *etsC*/*iucC* genes. pS88--: pS88 plasmid not carrying the *etsC*/*iucC* genes.

Since some results could be due to intra-group variations as consequence of different genetic backgrounds, the results of the three *stx2d* AE-STEC, the three *stx1a* AE-STEC, and the two EPEC were intra-virulotype compared (Table S2). Only the results of the two EPEC strains were statistically different at the log6 concentration. The EPEC EH3322/SES3122 strain harboring a pS88 plasmid carrying the *etsC* and *iucC* genes (pS88++) killed more larvae and more rapidly than the EH3308/SES2973 strain whose pS88 plasmid does not carry these two genes (pS88--) (Figure 4). The HR-95% of the EPEC EH3308/SES2973 pS88-- strain vs. the EPEC EH3322/SES3122 pS88++ strain was statistically significant: between 1.09 and 3.23 (p-value < 0.05) (Table S2).

3.3.3. Comparison of the Role of the pS88 Plasmid and of the STX2d Phage

According to the results described above, the *etsC*/*iucC*-positive pS88 plasmid may play a role in the pathogenicity of some *E. coli* O80:H2 strains in *G. mellonella* larvae. The virulence of one *etsC*/*iucC*-positive pS88 plasmid-conjugated *E. coli* K12 DH10B strain was therefore compared with the virulence of one STX2d phage-transduced *E. coli* K12 DH10B, of the *E. coli* K12 DH10B recipient, and of the AE-STEC O80:H2 EH2282 and EH3160 plasmid and phage donor strains (Table 1).

The log-rank analysis of the Kaplan–Meier curves confirmed that the acquisition by conjugation of the *etsC*/*iucC*-positive pS88 plasmid significantly increased the lethality of

the *E. coli* K12 DH10B recipient strain (ca. 25% at 96 HPI), both at log5 (p-value < 0.05) and log6 (p-value < 0.01) concentrations (Figure 5; Table S1). However, the HR-95% of the transconjugant vs. the *E. coli* K12 DH10B recipient strain was not statistically significant, neither at log6 nor at log5 (Table S3). Conversely, the HR-95% of the transconjugant vs. the *stx1a* AE-STEC EH2282 donor strain was highly significant (between 5.74 and 29.37) at log6 (p-value < 0.001) and between 4.32 and 23.82 at log5 (p-value < 0.001) concentrations (Table S3). Nevertheless, the HR-95% of the *E. coli* K12 DH10B recipient strain vs. the *stx1a* AE-STEC EH2282 donor strain was even more highly significant: between 12.78 and 153.50 at log6 (p-value < 0.001) and between 10.57 and 586.30 at log5 (p-value < 0.001) concentrations (Table S3).

The log-rank analysis of the Kaplan–Meier curves also confirmed that the acquisition by transduction of the STX2d phage significantly increased the lethality of the *E. coli* K12 DH10B recipient strain, at log4 (p-value < 0.01), log 5 (p-value < 0.001), and log 6 (p-value < 0.001) concentrations. The lethality of the transduced *E. coli* K12 DH10B strain was very similar to the lethality of the *stx2d* AE-STEC EH3160 donor strain (Figure 6; Table S1): less than 20% of the larvae inoculated with the log5 concentration survived at 96 HPI with both transduced and donor strains while all larvae were dead at 72 HPI and at 48 HPI, respectively, at the log6 concentration (Figure 6). Moreover, the HR-95% of the STX2d-transduced *E. coli* K12 DH10B and of the *stx2d* AE-STEC EH3160 donor strains vs. the *E. coli* K12 DH10B recipient strain were similar and also highly significant with HR-95% values between ca. 8 and more than 400 at log5 and between ca. 17 and more than 350 at log6 (Table S3). Conversely, the HR-95% of the STX2d-transduced *E. coli* K12 DH10B vs. the *stx2d* AE-STEC EH3160 donor strains was not statistically significant (Table S3).

3.3.4. Comparison with Other *E. coli* O80 Serotypes

In addition to AE-STEC and EPEC O80:H2, the virulence in *G. mellonella* larvae of three pS88 plasmid-negative, non-AE-STEC, non-EPEC *E. coli* O80:H6 and O80:H45 strains isolated from healthy cattle (Tables 1 and 3) was assessed for comparison with all *E. coli* O80:H2 strains. The control *E. coli* O80:H26 strain was also included in this comparison.

According to the log-rank analysis of the Kaplan–Meier curves (Figure 7; Table S1), the log5 (p-value < 0.001) and log6 (p-value < 0.001) concentrations of all three *E. coli* O80:H6 and O80:H45 strains gave significantly different results from the PBS group results with less than 30% and 10% survival at 96 HPI, respectively, while the results with the lower concentrations (log1 to log4) were not consistently statistically significant with more than 70% survival at 96 HPI (Figure 7; Table S1). The results of the *E. coli* O80:H26 strain were similar to the log5 and log6 concentrations, but the log4 concentration was also statistically significant (p-value < 0.001) (Figure 7; Table S1). Moreover, the HR-95% ratios between these three O80 serotypes were not statistically significant with the exception of the HR-95% of the *E. coli* O80:H6 strain vs. the *E. coli* O80:H26 strain that was between 1.11 and 3.12 (p-value < 0.05) at the log6 concentration (Table S4).

As for the comparison between the results of *E. coli* O80:H6, O80:H26, and O80:H45 strains on the one hand and the *stx1a* AE_STEC, *stx2d* AE-STEC, and EPEC O80:H2 on the other hand, some statistically significant differences were observed, especially between the *E. coli* O80:H6 strain and the AE-STEC O80:H2 strains (Table S4). For instance, the HR-95% ratios of the *E. coli* O80:H6 strain vs. the *stx1a* and *stx2d* AE-STEC O80:H2 strains were statistically significant at log6: between 0.41 and 0.98 (p-value < 0.05) and between 0.31 and 0.76 (p-value < 0.01), respectively. At log5, the HR-95% of the *E. coli* O80:H6 strain vs. the *stx2d* AE-STEC O80:H2 strains was also statistically significant: between 0.38 and 0.93 (p-value < 0.05). In addition, the HR-95% of the *E. coli* O80:H45 strain vs. the *stx2d* AE-STEC O80:H2 strains was statistically significant at log6 (between 0.49 and 0.97; p-value < 0.05), but not at log5 (Table S4). Conversely, the HR-95% ratios of any *E. coli* O80:non-H2 strain vs. the EPEC O80:H2 strains were not statistically significant (Table S4).

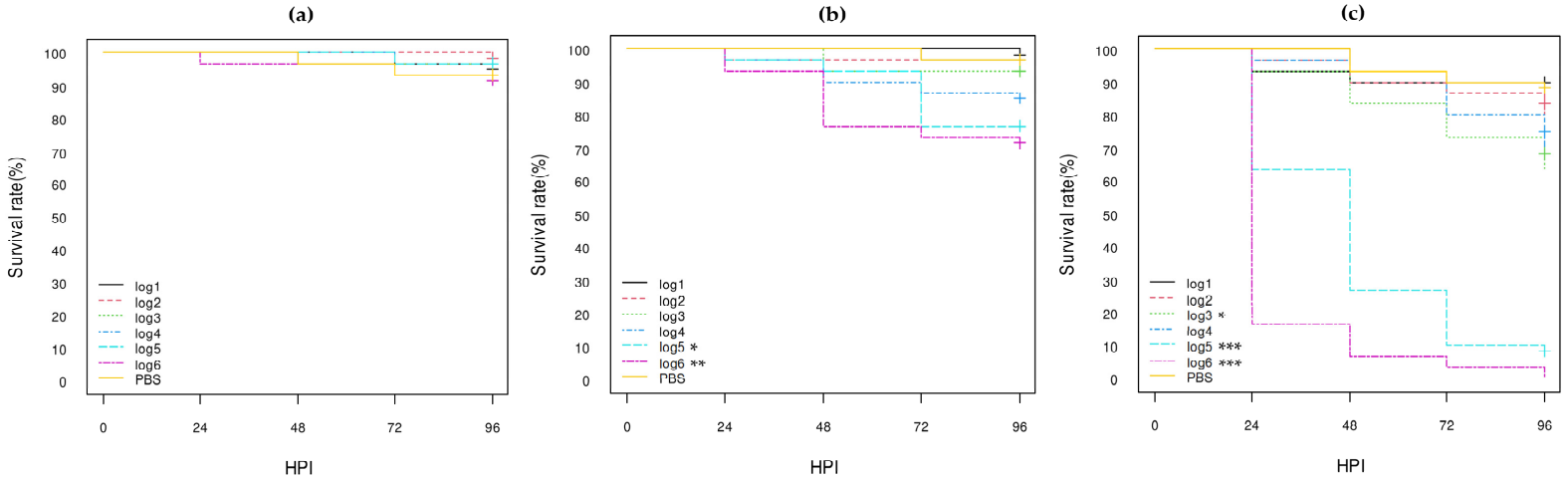

Figure 5. Kaplan–Meier survival curves of: (**a**) K12-DH10B recipient strain; (**b**) K12-DH10B pS88 plasmid-conjugated strain (pS88++); (**c**) *stx1a* AE-STEC O80:H2 EH2282 pS88 plasmid donor strain (pS88++). HPI = Hour Post-Inoculation. * *p*-value statistically significant at the threshold 0.05. ** *p*-value statistically significant at the threshold 0.01. *** *p*-value statistically significant at the threshold 0.001. pS88++: pS88 plasmid carrying the *etsC*/*iucC* genes.

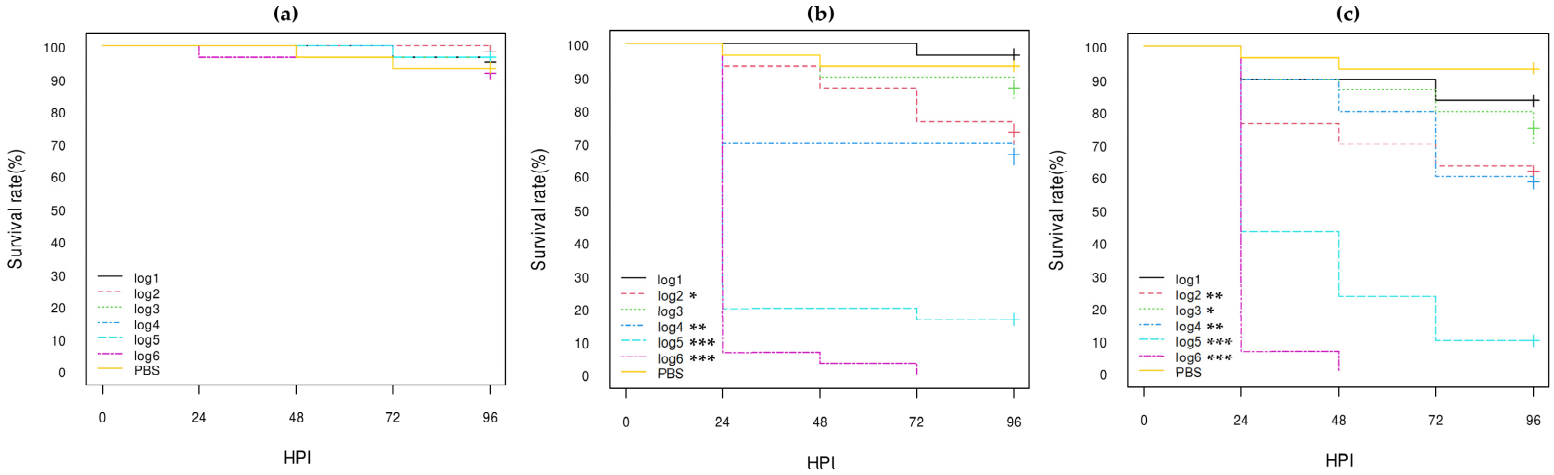

Figure 6. Kaplan–Meier survival curves of: (**a**) K12 DH10B recipient strain; (**b**) K12 DH10B STX2d phage-transduced strain; (**c**) *stx2d* AE-STEC O80:H2 EH3160 STX2d phage donor strain. HPI = Hour Post-Inoculation. * *p*-value statistically significant at the threshold 0.05. ** *p*-value statistically significant at the threshold 0.01. *** *p*-value statistically significant at the threshold 0.001.

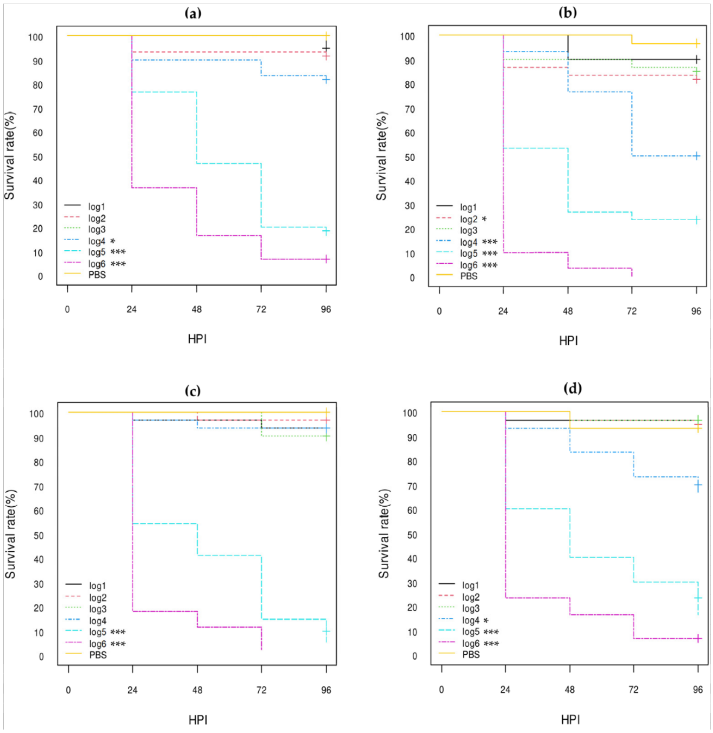

Figure 7. Kaplan–Meier survival curves of *E. coli* O80:non-H2 strains: (**a**) serotype O80:H6 SES6039 strain; (**b**) serotype O80:H26 (EH3161 strain); (**c**) serotype O80:H45 SES5725 strain; (**d**) serotype O80:H45 SES6156 strain. HPI = Hour Post-Inoculation. * *p*-value statistically significant at the threshold 0.05. *** *p*-value statistically significant at the threshold 0.001.

4. Discussion

The emerging AE-STEC O80:H2 are recognized as a multiple hybrid pathotype responsible for enteritis, hemolytic–uremic syndrome, and bacteremia or septicemia in humans and/or young calves [13,15,21]. They indeed harbor not only phage-located *stx* genes with a large majority of *stx2d* gene and/or LEE pathogenicity island-located genes, but also pS88-like ColV plasmids carrying invasiveness-encoding genes [15,21,22]. However, the respective role(s) of the Stx toxins, AE lesion, and pS88-encoded properties in their pathogenicity have not been confirmed by in vivo studies yet. Following the 3R policy (Replacement, Reduction and Refinement) [23], insects have been recommended and have been used for some years as a first step for in vivo testing of pathogenic bacterial species [24,25]. Thus, the main purpose of this study was to assess larvae of the *G. mellonella* moth [24,25,27] as a model to study and compare the virulence of different AE-STEC and EPEC O80:H2 isolated from calves in Belgium [21], and the respective roles of the pS88-like ColV plasmid and STX2d phage.

According to the statistical analysis results, all eight calf *stx1a* AE-STEC, *stx2d* AE-STEC, and EPEC O80:H2 were pathogenic for *G. mellonella* larvae at log5 and log6 concentrations compared to the PBS group (Figures 2–4; Table S1). They were also more pathogenic than the *E. coli* K12 DH5α and DH10B strains, but less than the *E. coli* O78:H4 strain (Figures 1, 5 and 6; Table S1). Moreover, all three *stx2d* AE-STEC were already lethal

for larvae at log4 and more rapidly and intensively at log5 and log6 than the *stx1a* AE-STEC and EPEC (Figures 2–4; Tables S1 and S2).

The reason for the *stx2d* AE-STEC being more virulent to *G. mellonella* larvae may be related to the production of the Stx2d toxin. Indeed, the lethality rate of larvae with STX2d phage-transduced *E. coli* K12 DH10B strain was significantly higher compared to the *E. coli* K12 DH10B recipient strain and was similar to the AE-STEC O80:H2 EH3160 phage donor strain (Figure 6; Table S3), as previously reported with the STX2d phage-transduced *E. coli* K12 DH5α strain [30]. The HR-95% ratios of the STX2d phage-transduced *E. coli* K12 DH10B strain and of the EH3160 phage donor strains were also similar and significantly higher when compared with the *E. coli* K12 DH10B recipient strain. These results confirmed that the Stx2d toxin plays an important role in the pathogenicity of *stx2d* AE-STEC O80:H2 in *G. mellonella* larvae, as previously suggested [30].

Although the HR-95% between *stx1a* AE-STEC and *stx2d* AE-STEC was statistically significant at log5, a role for the Stx1a toxin in the lethality of larvae cannot be totally excluded, since the difference was not statistically significant at log6 (Table S2). The reasons for these conflicting results may be several: (i) low numbers of CFUs of one *stx1a* AE-STEC actually inoculated, especially at log5 (3×10^4 CFU instead of 10^5 CFU after back-titration; data not shown); (ii) slower multiplication of *stx1a* AE-STEC compared to *stx2d* AE-STEC as a consequence of different general genetic backgrounds of the strains; (iii) delay in the production of the Stx1a toxin compared to the Stx2d toxin; (iv) less efficacious action of the Stx1a toxin in *G. mellonella* larvae compared to the Stx2d toxin. Whatever the actual reason, counting the number of CFUs of *stx1a* AE-STEC and *stx2d* AE-STEC in dead larvae, testing STX1a phage transductant, and/or following the expression of the *stx1a* and *stx2d* genes may answer these questions.

Two other specific properties of the AE-STEC and EPEC O80:H2 are the production of the AE lesion and the invasiveness properties encoded by pS88-like plasmid-located genes [15,18,19,21]. The role of the T3SS responsible for the development of the AE lesion was not assessed in his study but is indeed partially responsible for the lethality of the human EPEC E2348/69 (serotype O127:H6) in *G. mellonella* larvae compared to a mutant in one of the encoding genes, as observed by others [31]. However, the situation seems different for AE-STEC O157:H7 [32,33], possibly because the Stx toxins play a more important role ([30], this study).

As far as the pS88 plasmids are concerned, a role of the *etsC*/*iucC*-positive pS88 plasmid can be hypothesized, according to the statistical analysis of the pathogenicity in *G. mellonella* larvae of the two EPEC O80:H2 (Figure 4; Tables S1 and S2). Indeed, the EPEC EH3322/SES3122 strain harboring one *etsC*/*iucC*-positive pS88 plasmid (Tables 1 and 3) was almost twice as lethal as the EPEC EH3308/SES2973 strain harboring a pS88 plasmid not carrying these two genes at log6 (Table S2). However, the *etsC*/*iucC*-positive pS88 plasmid transconjugant was not statistically more lethal than the *E. coli* K12 DH10B recipient strain, although the HR-95% lower values were borderline at both log6 (between 0.92 and 12.60) and log5 (between 0.95 and 63.07) concentrations (Table S3). Nevertheless, this transconjugant was far from being as lethal as the *stx1a* AE-STEC O80:H2 plasmid donor strain (Figure 5; Tables S1 and S3). The *etsC* gene is a member of one operon coding for an ABC transporter system while the *iucC* gene is a member of an operon encoding the aerobactin siderophore [43]. Although both *ets* and *iuc* genes are markers of ExPEC, especially of Avian Pathogenic *E. coli* (APEC) and of NMEC, the contribution of the *ets* genes in the pathogenicity of *E. coli* in larvae of *G. mellonella* is still unknown, while contradictory results have been obtained for the *iuc* genes [19,44]. Comparison with the results obtained with one *etsC*/*iucC*-negative pS88 plasmid transconjugant would represent a first step in the understanding of their role.

The role of the pS88 plasmids could actually depend more on the general genetic background of the *E. coli* tested than on the pS88 plasmid virulotype. For instance, the difference observed between the two EPEC O80:H2 strains was not observed between the three *stx2d* AE-STEC or between the three *stx1a* AE-STEC (Tables S1 and S2), possibly

because the Stx toxins were also here more important virulence factors in *G. mellonella* larvae than the properties encoded by the pS88 plasmid. Moreover, the great majority of AE-STEC and EPEC O80:H2 harboring one *etsC*/*iucC*-positive pS88 plasmid group together in a single nucleotide polymorphism-based phylogenetic tree were negative for the *iha* and *cma* chromosomal genes (Table 3), and vice versa [21]. Nevertheless, no role for the *cma* and *iha* genes in the pathogenicity of *E. coli* O80:H2 in this intrahemocoelic inoculation model of larvae of *G. mellonella* can be proposed at this stage. Indeed, the *cma* gene codes for a colicin degrading the glycan chain of the murein precursor of other *E. coli* cells [45] while the *iha* gene encodes an outer membrane protein, conferring adherence to epithelial cells in culture [46]. Moreover, the *iha* gene is located on the pathogenicity island SPLE1, and its absence can also mean the absence of other genes located on SPLE1 [46,47].

Another striking observation was the human EPEC O127:H6 that was already lethal for *G. mellonella* larvae at a concentration of 5×10^3 CFU, like the *stx2d* AE-STEC O80:H2, while not harboring any *stx* genes or pS88 plasmid [31]. Therefore, testing different pS88 plasmid-cured *E. coli* O80:H2 strains would also help to determine its actual role in *G. mellonella* larvae, as already published in a mammalian model with the S88 NMEC strain. The S88 strain cured of the pS88 plasmid loses almost all virulence in a neonatal rat model of infection while reintroduction of the plasmid restores full virulence [19]. The role of any bacterial property can indeed also depend on the animal model. Testing the S88 strain and its plasmid-cured derivative would also help to confirm *G. mellonella* larvae as an in vivo model to study the virulence of different *E. coli* pathotypes.

Still another example of the importance of the genetic background of the strains was the *E. coli* O78:H4 control strain that harbors an *etsC*/*iucC*-positive pS88 plasmid (Tables 1 and 3). This *E. coli* O78:H4 strain, however, had the highest lethality and pathogenicity to *G. mellonella* larvae in spite of being neither AE-STEC nor EPEC (Figure 1; Table S1). Septicemia-associated *E. coli* serotype O78 are frequently isolated from mammals and poultry and one of the important virulence factors is the production of the O78 lipopolysaccharide that displayed anti-complement properties in chickens [4,48]. Since *G. mellonella* larvae have an innate immune system similar to mammals and birds, including the presence of complement-like proteins [25–29], any *E. coli* property conferring enhanced resistance to complement may also increase its virulence in *G. mellonella* larvae.

Another reason could be the availability of and the access to iron in *G. mellonella* larvae and the challenge model. To the authors' knowledge, there are no data published about the former in *G. mellonella*. About the latter, APEC and NMEC produce several iron-chelation systems, including two encoded by pS88-located genes, the salmochelin (*iroBCDEN* genes) and the aerobactin (*iuc* and *iutA* genes). Although they both can contribute to the virulence of APEC in poultry [44,49], neither is important for the pathogenicity of NMEC strain S88 in the neonatal rat model [19]. Nevertheless, the situation may be different in *G. mellonella* larvae, since the EPEC O80:H2 strain harboring an *iucC*-positive pS88 plasmid was statistically more lethal for *G. mellonella* larvae than the EPEC strain harboring an *iucC*-negative pS88 plasmid (Figure 4; Tables S1 and S2).

The role of other genes located on plasmids or on the chromosome, including on pathogenicity islands and/or phages, in the virulence of different *E. coli* strains is beyond any doubt [1,50] but was here illustrated by the results of the four pS88-negative *E. coli* O80:H6, O80:H26, and O80:H45 strains in *G. mellonella* larvae. These four *E. coli* O80:non-H2 strains were indeed statistically significantly lethal for larvae at log5 and log6 compared to the PBS group, like all the *E. coli* O80:H2 strains (Figures 2–4 and 7; Table S1). Nevertheless, the *E. coli* O80:H26 strain was already statistically significantly lethal for the larvae at the log4 concentration, as previously reported [30], and like the *stx2d* AE-STEC O80:H2, killed half of the larvae at 96 HPI vs. less than 25% for the other three O80:non-H2 strains (Figure 7; Table S1).

The *E. coli* O80:H26 actually appeared to be the most lethal and the *E. coli* O80:H6 the least lethal of the *E. coli* O80:non-H2 serotypes for *G. mellonella* larvae. The *E. coli* O80:H26 was, for instance, statistically more lethal than the *E. coli* O80:H6 to *G. mellonella* larvae

with the HR-95% of the *E. coli* O80:H6 vs. the *E. coli* O80:H26 statistically significantly > 1 (Table S4). Moreover, the HR-95% ratios of all AE-STEC and EPEC O80:H2 vs. the *E. coli* O80:H26 strains were not statistically significant (Table S4). Conversely, the HR-95% of the *stx2d* and *stx1a* AE-STEC O80:H2 vs. the *E. coli* O80:H6 strain were significantly < 1 at the log5 and/or log6 concentrations (Table S4), while the HR-95% of the *stx2d* but not of the *stx1a* AE-STEC O80:H2 vs. the *E. coli* O80:H45 strains was significantly < 1 but only at the log6 concentration (Table S4).

All these results confirmed the necessary role of the general genetic background in the virulence of *E. coli* strains to *G. mellonella* larvae. Unfortunately, at this stage, no correlation between the general virulotypes of these four *E. coli* O80:non-H2 strains could be made, although some putative virulence genes related to ExEPEC strains (Table 3) were detected. It is however striking that none of these genes could be detected in the *E. coli* O80:H26 strain (Table 3) that was the most virulent of the *E. coli* O80:non-H2 strains. This represented either our shortage of knowledge on the actual role of several genes of *E. coli* in virulence in *G. mellonella* larvae, or the current limits of the Virulence Finder tool.

5. Conclusions

The general conclusions of this study in *G. mellonella* larvae are that: (i) not only the AE-STEC and EPEC O80:H2 but also different *E. coli* O80:non-H2 strains are lethal at high concentrations (log5 and log6 CFU); (ii) the pS88 plasmids, especially the *etsC*/*iucC*-positive pS88 plasmids, are partly responsible for the lethality of the EPEC O80:H2; (iii) the Stx2d toxins are entirely responsible for the lethality of the *stx2d* AE-STEC O80:H2; and (iv) the identity of the virulence factor(s) responsible for the lethality of the *E. coli* O80:non-H2 strains is unknown at this stage.

Identification of these different virulence factors and understanding their respective role(s) are beyond the purpose of this study but could be the goals of future studies with different mutants engineered by, for instance, plasmid curing and allelic exchanges and/or with an in vivo imaging system using bioluminescence or fluorescence microscopy strains [19,31–33,51–53]. In parallel, comparative in vivo studies with mammalian and avian models are needed to further assess insects, especially larvae of the *G. mellonella* moth, as an in vivo challenge model to study and elucidate bacterial virulence.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci10070420/s1, Table S1: Log-rank analysis results of the lethality rates of all *E. coli* O80 and non-O80 strains tested in *G. mellonella* larvae according to the inoculated concentration (log1 to log6 CFU) vs. the PBS injected larvae; Table S2: Interpretation of hazard ratios (HRs) and confidence intervals 95% (HR-95%) in the comparison of the log6 and log5 concentrations of *E. coli* O80:H2 strains tested in *G. mellonella* larvae according to the pathotype (*stx1a* AE-STEC, *stx2d* AE-STEC, EPEC) and to the detection (++) or not (--) of the pS88 plasmid-located *etsC* and *iucC* genes; Table S3: Interpretation of hazard ratios (HRs) and confidence intervals 95% (HR-95%) in the comparison of the log6 and log5 concentrations of laboratory *E. coli* K12 DH10B, *E. coli* O80:H2, pS88 plasmid DH10B transconjugant, and STX2d phage DH10B transductant strains tested in *G. mellonella* larvae; Table S4: Interpretation of hazard ratios (HRs) and confidence intervals 95% (HR-95%) in the comparison of the log6 and log5 concentrations of *E. coli* O80:H2 and O80:non-H2 strains tested in *G. mellonella* larvae.

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References

1. Kaper, J.B.; Nataro, J.P.; Mobley, H.L. Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* **2004**, *2*, 123–140. [CrossRef] [PubMed]
2. Russo, T.A.; Johnson, J.R. Proposal for a New Inclusive Designation for Extraintestinal Pathogenic Isolates of *Escherichia coli*: ExPEC. *J. Infect. Dis.* **2000**, *181*, 1753–1754. [CrossRef] [PubMed]
3. Tozzoli, R.; Scheutz, F. Diarrheagenic *Escherichia coli* Infections in Humans. In *Pathogenic Escherichia coli: Molecular and Cellular Microbiology*; Morabito, S., Ed.; Caister Academic Press: Norfolk, UK, 2014; pp. 1–18; ISBN 978-1-908230-37-9.
4. Mainil, J.G.; Fairbrother, M.J. Pathogenic *Escherichia coli* in Domestic Mammals and Birds. In *Pahogenic Escherichia coli: Molecular and Cellular Microbiology*; Morabito, S., Ed.; Caister Academic Press: Norfolk, UK, 2014; pp. 19–43; ISBN 978-1-908230-37-9.
5. Borgatta, B.; Kmet-Lunaček, N.; Rello, J.E. *coli* O104:H4 Outbreak and Haemolytic-Uraemic Syndrome. *Med. Intensiv.* **2012**, *36*, 576–583. [CrossRef] [PubMed]
6. Piérard, D.; De Greve, H.; Haesebrouck, F.; Mainil, J. O157:H7 and O104:H4 Vero/Shiga Toxin-Producing *Escherichia coli* Outbreaks: Respective Role of Cattle and Humans. *Vet. Res.* **2012**, *43*, 13. [CrossRef]
7. EFSA BIOHAZ Panel; Koutsoumanis, K.; Allende, A.; Alvarez-Ordóñez, A.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Herman, L.; Hilbert, F.; et al. Pathogenicity Assessment of Shiga Toxin-Producing *Escherichia coli* (STEC) and the Public Health Risk Posed by Contamination of Food with STEC. *EFSA J.* **2020**, *18*, 5967. [CrossRef]
8. Bai, X.; Fu, S.; Zhang, J.; Fan, R.; Xu, Y.; Sun, H.; He, X.; Xu, J.; Xiong, Y. Identification and Pathogenomic Analysis of an *Escherichia coli* Strain Producing a Novel Shiga Toxin 2 Subtype. *Sci. Rep.* **2018**, *8*, 6756. [CrossRef]
9. Gill, A.; Dussault, F.; McMahon, T.; Petronella, N.; Wang, X.; Cebelinski, E.; Scheutz, F.; Weedmark, K.; Blais, B.; Carrillo, C. Characterization of Atypical Shiga Toxin Gene Sequences and Description of Stx2j, a New Subtype. *J. Clin. Microbiol.* **2022**, *60*, e0222921. [CrossRef]
10. Martin, C.C.; Svanevik, C.S.; Lunestad, B.T.; Sekse, C.; Johannessen, G.S. Isolation and Characterisation of Shiga Toxin-Producing *Escherichia coli* from Norwegian Bivalves. *Food Microbiol.* **2019**, *84*, 103268. [CrossRef]
11. Yang, X.; Bai, X.; Zhang, J.; Sun, H.; Fu, S.; Fan, R.; He, X.; Scheutz, F.; Matussek, A.; Xiong, Y. *Escherichia coli* Strains Producing a Novel Shiga Toxin 2 Subtype Circulate in China. *Int. J. Med. Microbiol.* **2020**, *310*, 151377. [CrossRef]
12. Karmali, M.A.; Mascarenhas, M.; Shen, S.; Ziebell, K.; Johnson, S.; Reid-Smith, R.; Isaac-Renton, J.; Clark, C.; Rahn, K.; Kaper, J.B. Association of Genomic O Island 122 of *Escherichia coli* EDL 933 with Verocytotoxin-Producing *Escherichia coli* Seropathotypes That Are Linked to Epidemic and/or Serious Disease. *J. Clin. Microbiol.* **2003**, *41*, 4930–4940. [CrossRef]
13. Soysal, N.; Mariani-Kurkdjian, P.; Smail, Y.; Liguori, S.; Gouali, M.; Loukiadis, E.; Fach, P.; Bruyand, M.; Blanco, J.; Bidet, P.; et al. Enterohemorrhagic *Escherichia coli* Hybrid Pathotype O80:H2 as a New Therapeutic Challenge. *Emerg. Infect. Dis.* **2016**, *22*, 1604–1612. [CrossRef] [PubMed]
14. Nüesch-Inderbinen, M.; Morach, M.; Cernela, N.; Althaus, D.; Jost, M.; Mäusezahl, M.; Bloomberg, G.; Stephan, R. Serotypes and Virulence Profiles of Shiga Toxin-Producing *Escherichia coli* Strains Isolated during 2017 from Human Infections in Switzerland. *Int. J. Med. Microbiol.* **2018**, *308*, 933–939. [CrossRef] [PubMed]
15. Cointe, A.; Bizot, E.; Delannoy, S.; Fach, P.; Bidet, P.; Birgy, A.; Weill, F.-X.; Lefèvre, S.; Mariani-Kurkdjian, P.; Bonacorsi, S. Emergence of New ST301 Shiga Toxin-Producing *Escherichia coli* Clones Harboring Extra-Intestinal Virulence Traits in Europe. *Toxins* **2021**, *13*, 686. [CrossRef] [PubMed]

16. Gigliucci, F.; van Hoek, A.H.A.M.; Chiani, P.; Knijn, A.; Minelli, F.; Scavia, G.; Franz, E.; Morabito, S.; Michelacci, V. Genomic Characterization of HlyF-Positive Shiga Toxin-Producing *Escherichia coli*, Italy and the Netherlands, 2000–2019. *Emerg. Infect. Dis.* **2021**, *27*, 853–861. [CrossRef] [PubMed]
17. Wijnsma, K.L.; Schijvens, A.M.; Rossen, J.W.A.; Kooistra-Smid, A.M.D.; Schreuder, M.F.; van de Kar, N.C.A.J. Unusual Severe Case of Hemolytic Uremic Syndrome Due to Shiga Toxin 2d-Producing *E. coli* O80:H2. *Pediatr. Nephrol.* **2017**, *32*, 1263–1268. [CrossRef]
18. De Rauw, K.; Thiry, D.; Caljon, B.; Saulmont, M.; Mainil, J.; Piérard, D. Characteristics of Shiga toxin Producing- and Enteropathogenic *Escherichia coli* of the Emerging Serotype O80:H2 Isolated from Humans and Diarrheic Calves in Belgium. *Clin. Microbiol. Infect.* **2019**, *25*, 111.e5–111.e8. [CrossRef]
19. Peigne, C.; Bidet, P.; Mahjoub-Messai, F.; Plainvert, C.; Barbe, V.; Médigue, C.; Frapy, E.; Nassif, X.; Denamur, E.; Bingen, E.; et al. The Plasmid of *Escherichia coli* Strain S88 (O45:K1:H7) That Causes Neonatal Meningitis Is Closely Related to Avian Pathogenic *E. coli* Plasmids and Is Associated with High-Level Bacteremia in a Neonatal Rat Meningitis Model. *Infect. Immun.* **2009**, *77*, 2272–2284. [CrossRef]
20. Thiry, D.; Saulmont, M.; Takaki, S.; De Rauw, K.; Duprez, J.-N.; Iguchi, A.; Piérard, D.; Mainil, J.G. Enteropathogenic *Escherichia coli* O80:H2 in Young Calves with Diarrhea, Belgium. *Emerg. Infect. Dis.* **2017**, *23*, 2093–2095. [CrossRef]
21. Habets, A.; Crombé, F.; Nakamura, K.; Guérin, V.; De Rauw, K.; Piérard, D.; Saulmont, M.; Hayashi, T.; Mainil, J.G.; Thiry, D. Genetic Characterization of Shigatoxigenic and Enteropathogenic *Escherichia coli* O80:H2 from Diarrhoeic and Septicaemic Calves and Relatedness to Human Shigatoxigenic *E. coli* O80:H2. *J. Appl. Microbiol.* **2021**, *130*, 258–264. [CrossRef]
22. Cointe, A.; Birgy, A.; Mariani-Kurkdjian, P.; Liguori, S.; Courroux, C.; Blanco, J.; Delannoy, S.; Fach, P.; Loukiadis, E.; Bidet, P.; et al. Emerging Multidrug-Resistant Hybrid Pathotype Shiga Toxin-Producing *Escherichia coli* O80 and Related Strains of Clonal Complex 165, Europe. *Emerg. Infect. Dis.* **2018**, *24*, 2262–2269. [CrossRef]
23. Lewis, D.I. Animal Experimentation: Implementation and Application of the 3Rs. *Emerg. Top Life Sci.* **2019**, *3*, 675–679. [CrossRef]
24. Harnish, J.M.; Link, N.; Yamamoto, S. *Drosophila* as a Model for Infectious Diseases. *Int. J. Mol. Sci.* **2021**, *22*, 2724. [CrossRef] [PubMed]
25. Ahlawat, S.; Sharma, K.K. Lepidopteran Insects: Emerging Model Organisms to Study Infection by Enteropathogens. *Folia Microbiol.* **2023**, *68*, 181–196. [CrossRef] [PubMed]
26. Cutuli, M.A.; Petronio Petronio, G.; Vergalito, F.; Magnifico, I.; Pietrangelo, L.; Venditti, N.; Di Marco, R. *Galleria mellonella* as a Consolidated in vivo Model Hosts: New Developments in Antibacterial Strategies and Novel Drug Testing. *Virulence* **2019**, *10*, 527–541. [CrossRef] [PubMed]
27. Pereira, M.F.; Rossi, C.C.; da Silva, G.C.; Rosa, J.N.; Bazzolli, D.M.S. *Galleria mellonella* as an Infection Model: An In-Depth Look at why it Works and Practical Considerations for Successful Application. *Pathog. Dis.* **2020**, *78*, ftaa056. [CrossRef] [PubMed]
28. Antoine, C.; Laforêt, F.; Blasdel, B.; Fall, A.; Duprez, J.-N.; Mainil, J.; Delcenserie, V.; Thiry, D. In vitro Characterization and in vivo Efficacy Assessment in *Galleria mellonella* Larvae of Newly Isolated Bacteriophages against *Escherichia coli* K1. *Viruses* **2021**, *13*, 2005. [CrossRef] [PubMed]
29. Wojda, I.; Staniec, B.; Sułek, M.; Kordaczuk, J. The Greater Wax Moth *Galleria mellonella*: Biology and Use in Immune Studies. *Pathog. Dis.* **2020**, *78*, ftaa057. [CrossRef]
30. Habets, A.; Antoine, C.; Wagemans, J.; Vermeersch, M.; Laforêt, F.; Diderich, J.; Lavigne, R.; Mainil, J.; Thiry, D. Impact of Shiga-Toxin Encoding Gene Transduction from O80:H2 Shiga Toxigenic *Escherichia coli* (STEC) on Non-STEC Strains. *Sci. Rep.* **2022**, *12*, 21587. [CrossRef]
31. Chen, R.Y.; Keddie, B.A. The *Galleria mellonella*-Enteropathogenic *Escherichia coli* Model System: Characterization of Pathogen Virulence and Insect Immune Responses. *J. Insect. Sci.* **2021**, *21*, 7. [CrossRef]
32. Morgan, J.K.; Ortiz, J.A.; Riordan, J.T. The Role for TolA in Enterohemorrhagic *Escherichia coli* Pathogenesis and Virulence Gene Transcription. *Microb. Pathog.* **2014**, *77*, 42–52. [CrossRef]
33. De la Cruz, M.A.; Morgan, J.K.; Ares, M.A.; Yanez-Santos, J.A.; Riordan, J.T.; Giron, J.A. The Two-Component Sytem CpxRA Negatively Regulates the Locus of Enterocyte Effacement of Enterohemorrhagic *Escherichia coli* Involving σ^{32} and Lon Protease. *Front. Cell. Infect. Microbiol.* **2016**, *6*, 11. [CrossRef] [PubMed]
34. Ikeda, R.; Nakamura, K.; Saulmont, M.; Habets, A.; Duprez, J.-N.; Korsak, N.; Hayashi, T.; Thiry, D.; Mainil, J.G. *Escherichia coli* O80 in Healthy Cattle: Absence of Shigatoxigenic and Enteropathogenic *E. coli* O80:H2 and (Phylo) Genomics of Non-Clonal Complex 165 *E. coli* O80. *Microorganisms* **2023**, *11*, 230. [CrossRef] [PubMed]
35. Liu, D.; Reeves, P.R. *Escherichia coli* K12 Regains Its O Antigen. *Microbiology* **1994**, *140 Pt 1*, 49–57. [CrossRef] [PubMed]
36. Gilson, L.; Mahanty, H.K.; Kolter, R. Four Plasmid Genes Are Required for Colicin V Synthesis, Export, and Immunity. *J. Bacteriol.* **1987**, *169*, 2466–2470. [CrossRef]
37. Bauer, A.W.; Kirby, W.M.; Sherris, J.C.; Turck, M. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. *Am. J. Clin. Pathol.* **1966**, *45*, 493–496. [CrossRef]
38. Pohl, P. Introduction à la Plasmidologie: Données actuelles sur sa Signification en Thérapeutique et en Pathologie. *J. Pharm. Belg.* **1981**, *36*, 165–174.
39. Brosius, J.; Holy, A. Regulation of Ribosomal RNA Promoters with a Synthetic Lac Operator. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 6929–6933. [CrossRef]

40. Iguchi, A.; Iyoda, S.; Seto, K.; Morita-Ishihara, T.; Scheutz, F.; Ohnishi, M. Pathogenic *E. coli* Working Group in Japan *Escherichia coli* O-Genotyping PCR: A Comprehensive and Practical Platform for Molecular O Serogrouping. *J. Clin. Microbiol.* **2015**, *53*, 2427–2432. [CrossRef]
41. Banjo, M.; Iguchi, A.; Seto, K.; Kikuchi, T.; Harada, T.; Scheutz, F.; Iyoda, S. Pathogenic *E. coli* Working Group in Japan *Escherichia coli* H-Genotyping PCR: A Complete and Practical Platform for Molecular H Typing. *J. Clin. Microbiol.* **2018**, *56*, e00190-18. [CrossRef]
42. Vandekerchove, D.; Vandemaele, F.; Adriaensen, C.; Zaleska, M.; Hernalsteens, J.-P.; De Baets, L.; Butaye, P.; Van Immerseel, F.; Wattiau, P.; Laevens, H.; et al. Virulence-Associated Traits in Avian *Escherichia coli*: Comparison between Isolates from Colibacillosis-Affected and Clinically Healthy Layer Flocks. *Vet. Microbiol.* **2005**, *108*, 75–87. [CrossRef]
43. Johnson, T.J.; Siek, K.E.; Johnson, S.J.; Nolan, L.K. DNA Sequence of a ColV Plasmid and Prevalence of Selected Plasmid-Encoded Virulence genes among Avian *Escherichia coli* Strains. *J. Bacteriol.* **2006**, *188*, 755–758. [CrossRef] [PubMed]
44. Ling, J.; Pan, H.; Gao, Q.; Xiong, L.; Zhou, Y.; Zhang, D.; Gao, S.; Liu, X. Aerobactin Synthesis Genes *iucA* and *iucC* Contribute to the Pathogenicity of Avian Pathogenic *Escherichia coli* O2 Strain E058. *PLoS ONE* **2013**, *8*, e57794. [CrossRef] [PubMed]
45. Braun, V.; Helbig, S.; Patzer, S.I.; Pramanik, A.; Römer, C. Import and Export of Bacterial Protein Toxins. *Int. J. Med. Microbiol.* **2015**, *305*, 238–242. [CrossRef] [PubMed]
46. Tarr, P.I.; Bilge, S.S.; Vary, J.C., Jr.; Jelacic, S.; Habeeb, R.L.; Ward, T.R.; Baylor, M.R.; Besser, T.E. Iha: A Novel *Escherichia coli* O157:H7 Adherence-conferring Molecule Encoded on a Recently Acquired Chromosomal Island of Conserved Structure. *Infect. Immun.* **2000**, *68*, 1400–1407. [CrossRef]
47. Schmidt, H.; Hensel, M. Pathogenicity Islands in Bacterial Pathogenesis. *Clin. Microbiol. Rev.* **2004**, *17*, 14–56. [CrossRef]
48. Mellata, M.; Dho-Moulin, M.; Dozois, C.M.; Curtiss, R.; Brown, P.K.; Arné, P.; Brée, A.; Desautels, C.; Fairbrother, J.M. Role of Virulence Factors in Resistance of Avian Pathogenic *Escherichia coli* to Serum and in Pathogenicity. *Infect. Immun.* **2003**, *71*, 536–540. [CrossRef]
49. Caza, M.; Lépine, F.; Milot, S.; Dozois, C.M. Specific Roles of the *iroBCDEN* Genes in Virulence of an Avian Pathogenic *Escherichia coli* O78 Strain and in Production of Salmochelins. *Infect. Immun.* **2008**, *76*, 3539–3549. [CrossRef]
50. Gyles, C.; Boerlin, P. Horizontally Transferred Genetic Elements and their Role in Pathogenesis of Bacterial Disease. *Vet. Pathol.* **2014**, *51*, 328–340. [CrossRef]
51. Datsenko, K.A.; Wanner, B.L. One-Step Inactivation of Chromosomal Genes in *Escherichia coli* K-12 Using PCR Products. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 6640–6645. [CrossRef]
52. Hutchens, M.; Luker, G.D. Applications of Bioluminescence Imaging to the Study of Infectious Diseases. *Cell. Microbiol.* **2007**, *9*, 2315–2322. [CrossRef]
53. Witcomb, L.A.; Czupryna, J.; Francis, K.P.; Frankel, G.; Taylor, P.W. Non-Invasive Three-Dimensional Imaging of *Escherichia coli* K1 Infection Using Diffuse Light Imaging Tomography Combined with Micro-Computed Tomography. *Methods* **2017**, *127*, 62–68. [CrossRef] [PubMed]

GENERAL DISCUSSION

GENERAL DISCUSSION

The AE-STEC serotype O80:H2 is a HUS-associated STEC serotype that has been emerging since the early 2010s and is now endemic in several western European countries (Bruyand et al., 2019; Soysal et al., 2016; Soleau et al., 2024). AE-STEC serotype O80:H2 was previously considered as a "minor" STEC serotype, much less frequent than the classical major serotypes, such as AE-STEC O26:H11 and O157:H7. Emergence of minor STEC and, sometimes hybrid pathotypes occurs from time to time. For instance, the Aggregative-STEC serotype, O104:H4 caused a major outbreak in Germany in 2011, resulting in numerous deaths due to HUS (Beutin and Martin, 2012: Ibarra et al., 2013). Today, AE-STEC serotype O80:H2 represents one of the at least top five serotypes most frequently associated with HUS in Europe, although no outbreak has been caused by this serotype yet (EFSA, 2021; 2022; 2023; 2024). In this regard, O80:H2 could be classified as seropathotype groups A or B based on the classification method by Karmali and collaborators because it has been associated with HUS and epidemic disease in European countries (Karmali et al., 2003). However, the amount of epidemiological and experimental data accumulated worldwide for serotype O80:H2 remains overwhelmingly lower than that available for other serotypes belonging to seropathotype groups A and B, such as O157:H7, O26:H11, O103:H2, O111:NM, O121:H19, and O145:NM. Therefore, although O80:H2 fulfills the criteria for classification within seropathotype groups A or B according to the Karmali classification, its global epidemiological weight and impact are considered to be still lower than those of the classical major (AE-)STEC serotypes. Although AE-STEC O80:H2 have also been isolated in other continents and countries, indicating that the presence of O80:H2 is not restricted to European countries (Mainil et al., 2025; Patel et al., 2018; Yoshida et al., 2024), its prevalence and incidence would not be comparable to those observed in Western Europe to date.

EPEC and some major human AE-STEC serotypes are also associated with diarrhea in animals, such as young (< 3 months old) calves (Mainil and Fairbrother 2014; Fakih et al., 2017). The O80:H2 serotype is no exception, with several cases in young calves presenting diarrhoea and, although more rarely septicaemia in Belgium (Thiry et al.,

2017; Habets et al., 2021). Other than from diarrheic calves in Belgium, a very small number of AE-STEC and EPEC O80:H2 have been also isolated from cattle and their feces: healthy adult cattle from one farm in France and in Spain and from healthy calves in several farms in Switzerland (Bernier Gosselin et al., 2024; Blanco et al., 2004; Soleau et al., 2024). Considering this background, the potential public health risk for future outbreaks with AE-STEC serotype O80:H2 should not be overlooked.

In European countries where the frequency of AE-STEC O80:H2-associated infection occurrence is higher than in other regions, several studies have been conducted to compare strains from different sources (humans, calves, adult cattle, and others) and to identify the source of contamination of humans (Blanco et al., 2004; Cointe et al., 2018; Fierz et al., 2017; Gigliucci et al., 2021; Habets et al., 2021; Soleau et al., 2024; Soysal et al., 2016; Thiry et al., 2018).

One of the most frequent contamination sources of the major AE-STEC serotypes is ruminants. Among them, cattle have been recognized as a major contamination source of humans with AE-STEC, with up to approximately 80% of healthy cattle shedding AE-STEC in their faeces (Menge, 2020b). Despite these facts, no association with cattle or with bovine food consumption could be identified during the different epidemiological studies performed on the reported human cases of AE-STEC O80:H2 infections (ANSES, 2023; Bruyand et al., 2019; Ingelbeen et al., 2019). Moreover, most attempts to isolate O80:H2 from healthy cattle remain unsuccessful to date (Thiry et al., 2018; Habets et al., 2020; Ikeda et al., 2023a; Ikeda et al., 2025), with only a few sporadic exceptions (Bernier Gosselin et al., 2024; Blanco et al., 2004; Soysal et al., 2016; Cointe et al., 2018; Soleau et al., 2024).

The major problem to isolate AE-STEC that can be present in low numbers in ruminant healthy carriers is that, apart from the CT-SMAC medium (Sorbitol-MacConkey Agar supplemented with Cefixim and Tellurite) for the serotype O157:H7, few selective growth media or specific methodology have been established (Hiramatsu et al., 2002; Verhaegen et al., 2015), especially to specifically isolate and study minor serotypes that are associated with human infections. This is a major barrier in terms of infection control. Therefore, STUDIES 1 and 2 aimed to identify healthy adult cattle in slaughterhouses

and farms as potential contamination source of AE-STEC and EPEC O80:H2 using different non-specific and specific methods for their isolation and identification.

In STUDY 1, 15% of the total enrichment broths of adult cattle faeces sampled at slaughterhouses and in farms tested positive for the O80 antigen PCR, and a total of 835 colonies were subsequently recovered from the different (semi)-selective agar media: CHROMagar™ STEC which contains potassium tellurite (2.5 µg/ml) and cefixim, Chromocult Coliform ES, MacConkey and EnteroHemolysin blood agar plate. Of these, only 16 colonies were identified as *E. coli* O80 by PCR, all of which were recovered from Chromocult Coliform ES, MacConkey and EnteroHemolysin blood agar plates. Even though some candidate *E. coli* O80 colonies were recovered from Enterohemolysin blood agar plates, only *E. coli* O80:H45 and O80:H6 were identified, that are neither EPEC nor STEC and belong to a different sequence type than O80:H2 (ST301).

Therefore, a more specific methodology was assessed. Major (AE)-STEC are known to be highly resistant to the bacteriotoxic tellure Te^{++} ion (Lewis et al., 2018; Kerangart et al., 2016), mainly due to the presence of a *ter* resistance operon containing the *terZ, A, B, C, D, E*, and *F* genes (Taylor et al., 2002). Due to this property, K_2TeO_3 has been widely used to detect (AE)-STEC O157 and major non-O157 serotypes (Gouali et al., 2013; Tzschoppe et al., 2012; Hirvonen et al., 2012; Tillman et al. 2012), like in CHROMagar™ STEC agar plates. However, several other AE-STEC serotypes associated with human infection are known to be unable to grow on K_2TeO_3-supplemented agar plates (Orth et al., 2007; Sato et al., 2007). Indeed, not only no PCR O80-positive colony was obtained from TeK Chromocult Coliform ES agar in this STUDY 1, but also numbers of growing colonies were lower than on other agar plates (3% in slautgher house vs 1% in farm). Hence, it could assume that the *ter* resistance operon was absent in *E. coli* O80:H2 and STUDY 2 was carried out to clarify this assumption.

In addition, many studies on AE-STEC O80:H2 focused on their virulence and antibiotic resistance properties (Cointe et al., 2018; Gigliucci et al., 2021; Nüesch-Inderbinen et al., 2018; Rodwell et al., 2021), but their biochemical properties are largely uncharacterized. In 2022, AE-STEC O80:H2 from humans were reported as being 'non-fermentative to melibiose' (Bizot et al., 2022b) due to deletion of the melibiose *mel* operon

that is replaced by the insertion of a specific 70-bp-long DNA sequence (*70mel*). Consequently, 52 previously studied AE-STEC and EPEC O80:H2 isolated from calves and humans in Belgium (Habets et al., 2021) were characterized for non-melibiose fermentation and for tellurite resistance properties, hoping to develop an alternative selective and specific procedure based on these two properties to isolate and identify *E. coli* O80:H2 from healthy adult cattle.

In STUDY 2, the *70mel* sequence, but not the *mel* operon was detected in the genomes of all 52 strains analyzed, with the 21 calf AE-STEC and EPEC strains phenotypically tested forming colonies that were non-fermentative on melibiose-supplemented MacConkey agar plates. This finding was the confirmation that calf AE-STEC and EPEC O80:H2 are non-melibiose fermenters as well as human strains, as Bizot and colleagues reported (Bizot et al., 2022b). In contrast, studies on tellurite resistance give different results depending on the belonging of the strains to the main sub-lineages L1/SL1.1 or SL1.2, as defined by Habets and collaborators (Habets et al., 2021): in L1/SL1.1, most strains possessed *ter*-type 1 operon and ten of the 11 calf strains (mostly EPEC) phenotypically tested in K_2TeO_3-supplemented broth have high MIC (>128 µg/ml), while in L1/SL1.2, strains do not possess the *ter*-type 1 operon and the nine strains (mostly *stx2d* AE-STEC) tested have low MIC (1–2 µg/ml). The presence of the *ter* resistance operon directly or indirectly contributes to the growth of the *ter*-type 1-positive *stx1a* AE-STEC (MIC >256 µg/ml) in 2.0 µg/ml K_2TeO_3-supplemented enrichment broth even at low inoculation concentrations. Conversely, under the same conditions, the growth of the *ter*-negative low MIC (2 µg/ml) EPEC could not be detected. However, despite these results, AE-STEC and EPEC O80:H2 could be not isolated in the tests on slaughterhouse faecal samples conducted in STUDY 2.

Isolation of *E. coli* O80:H2 from healthy cattle has still not been achieved in Belgium between 2017 and 2025 study periods (Thiry et al., 2018; Habets et al., 2021; Ikeda et al., 2023a; Ikeda et al., 2025) and only very rarely in other countries (Blanco et al., 2004; Soleau et al., 2024). Interestingly, AE-STEC O80:H2 was isolated and identified from healthy adult cattle in France in 2024 in one farm located in one of the areas marked in previous epidemiological studies as one high incidence area for AE-STEC O80:H2-related infections (Soleau et al., 2024; Soysal et al., 2016). Given that

most of the cases in which bovine AE-STEC O80:H2 has been identified in farms (Bernier Gossen et al., 2024; Blanco et al., 2004; Soleau et al., 2024) and that the general tendency for major AE-STEC serotype detection rates in slaughterhouses to be around 10%, greater emphasis should need to be placed on investigating the farm environment and their surroundings, along with analyzing more fecal samples in large slaughterhouses (Bibbal et al., 2015; Blanco et al., 2004; Browne et al., 2021; McCabe et al., 2019; Soleau et al., 2024). In addition, surveys should also be performed in veal calf farms that are frequent in Western European countries, but are not often included in STEC studies. For instance, no survey has been performed in Belgium, since the study Bardiau and collaborators in 2010 with prevalence of (AE-)STEC and EPEC of ca. 12% (Bardiau et al., 2010).

Nevertheless, besides the absence of any specific procedure, the rarity of positive results could also be due to ecological issues. For instance, the colonization site of AE-STEC and EPEC O80:H2 in adult cattle could be the small intestine as hypothesized based on a previous study in calves (Habets et al., 2020), rather than the rectum where we collected samples. Alternatively, their concentration in the rectum and in feces would not exceed the detection limit even after enrichment bacterial growth making isolation impossible. In addition, unlike major AE-STEC serotypes, the origin of AE-STEC and EPEC O80:H2 may not be healthy adult cattle. This includes the possibility that domestic animals other than cattle, such as poutry and swine, or environmental factors around the farms, without forgetting wildlife, may be the actual source of human contamination.

It is also important to use a combination of selective agar media and methodologies in future surveys, based on the following two considerations: (i) the deletion of the *mel* operon is likely to be effective for identifying AE-STEC and EPEC O80:H2. However, the inability to ferment melibiose is not specific to the serotype O80:H2, as this phenotype—caused by the *70mel* DNA insertion—is also observed in other *E. coli* serotypes, such as O45:H2, O55:H9, and O186:H2, all belonging to Sequence Type (ST) 301, as well as in other Gram-negative species like *Escherichia albertii* (Bizot et al., 2022b); (ii) the CHROMagar™ STEC is widely used as a selective medium for AE-STEC and can still help in helping to isolate *E. coli* O80:H2 although no positive result was obtained in previous studies, including STUDY 1 (Thiry et al., 2018; Habets et al., 2020; Jenkins et al., 2020). In a case report on HUS-associated AE-STEC O55:H9 (ST301)

indeed, a French group reports that O55:H9 and O80:H2 serotypes form distinct colony morphologies on CHROMagar™ STEC (Cointe et al., 2022). Combining these findings together may improve the rate of identification of *E. coli* O80:H2 from contamination source(s). However, specific protocols for isolating and identifying AE-STEC and EPEC O80:H2 from sources have yet to be established. In conclusion, continuing research on different techniques and improved isolation strategies, based on agar-dependent and agar-independent procedures such as immunomagnetic beads (IMB) (Tillman et al., 2012), is essential for establishing a methodology to identify the reservoir and contamination way of AE-STEC and EPEC O80:H2.

The identification of the infection origin of AE-STEC and/or EPEC O80:H2 also requires a deeper understanding of their molecular characteristics, particularly their genomic structures. However, the available genome information on the serotype O80:H2 is scarce compared to major serotypes. Hence, STUDY 3 employed a whole genome-based approach to (i) investigate the phylogenetic population structure, gene distribution and virulence profiles of human and calf Belgian AE-STEC and EPEC O80:H2 strains; and (ii) to precisely and completely characterize the mobile genetic elements, especially LEE pathogenicity islands, Stx phages, and pS88 and other plasmids of two calf *stx2f* AE-STEC isolates after long read sequencing methodology.

At first, the phylogenetic population structure of 52 calf and human AE-STEC and EPEC O80:H2 (Habets et al., 2021) was expanded and re-analyzed with additional genomes of 77 strains, for a total of 127 calf and human AE-STEC and EPEC isolated between 2008 and 2024 and two calf AE-STEC isolated in 1987. The results confirm that the population of Belgian AE-STEC and EPEC O80:H2 strains from humans and calves has a stable phylogenetic structure at the SNP level throughout the study period with two lineages (L) and four sub-lineages (SL).

Indeed, 96% of the 129 AE-STEC and EPEC O80:H2 belong to L1/SL1.1 and L1/SL1.2. Furthermore, 46 of the 59 calf EPEC were classified in L1/SL1.1 whereas 51 of the 70 calf and human AE-STEC belonged to L1/SL1.2. The tendency confirms that L1/SL1.1 is essentially an EPEC-associated lineage while L1/SL1.2 is more *stx2d* AE-STEC-associated. At the same time, no additional isolate was classified in L2 (two calf

AE-STEC from 1987), L1/SL1.3 (one human AE-STEC), and L1/SL1.4 (two human AE-STEC). Similarly, a core genome-based analysis of Belgian, British, French and Swiss calf and human AE-STEC strains by a French group confirms the classification of AE-STEC O80:H2 in three consistent sub-lineages, further supporting a conserved phylogenetic structure (Soleau et al., 2024).

One of the features of the AE-STEC and EPEC O80:H2 is the conserved virulence gene composition. Indeed, *eaeξ* gene coding for Intimin adhesin, LEE-located Type 3 Secretion System (T3SS)-encoding genes (*espA*, *B*, *F*, and *tir*), and non-LEE-located TS3 effector-encoding genes were detected at high frequency in most strains not only in STUDY 3, but also in other studies carried out in France and UK (Cointe et al., 2018; Rodwell et al., 2021). This conserved virulence gene profile among the O80:H2 serotype implies that genetic similarities in pathogenicity factor profiles of the O80:H2 strains is not limited to Belgium, but is present in strains of different origins and countries. Exceptionally, one human strain, EH4328 isolated in 2024 was different by the absence *eaeξ* gene and LEE-located genes. However, this strain is an *stx2d* AE-STEC belonging to the L1/SL1.2 and still possess non-LEE-encoded effectors (*nleA*, *nleB*, and *nleC*). This observation indicates that the strain likely shares a common genetic backbone with other LEE-positive AE-STEC O80:H2 and therefore, may have originally possessed the LEE pathogenicity island.

Previous research identified LEE-negative STEC in other serotypes isolated from humans and occasionally from ruminants. To the author's knowledge, LEE-negative STEC have not been reported among the major AE-STEC serotypes; however, such strains have been found in O113:H21 and O91:H21 isolated from HUS, O91:H14 from gastrointestinal infection, and the aggegrative-STEC O104:H4 responsible for the outbreak in Germany (Ito et al., 1990; Lucchesi et al., 2006; Mellmann et al., 2011; Paton et al., 1999; Rodwell et al., 2024; Zaheri et al., 2020). Accordingly, a different genetic profile from LEE-positive STEC strains has also been identified in such LEE-negative STEC with, for instance a higher prevalence of other putative adherence factor-encoding genes, such as *saa* (autoagglutinating adhesin) (Lucchesi et al., 2006; Zaheri et al. 2020). Although the fact that this LEE-negative STEC O80:H2 identified in this STUDY 3 possesses neither the *saa* gene, nor any specific adherence-encoding genes, the existence

of this human isolate implies the possibility of emergence of strains with different virulence profiles compared to classical AE-STEC O80:H2 strains and may provide clues to mechanisms in the pathogenicity adaptation of this serotype.

Stx-converting phages and virulence plasmids are mobile genetic elements also essential for the virulence of AE-STEC ad EPEC in general. Of the latter, the pS88 plasmid is especially specific to the serotype O80:H2 (Soysal et al., 2016). This pS88 plasmid was originally identified in NMEC and is also highly identical to the ColicinV-related plasmid identified in APEC strain (Johnson et al., 2006a; Johnson et al., 2006b; Peigne et al., 2009). What pS88 plasmids make O80:H2 unique is that it possesses putative virulence genes and gene clusters generally found in ExPEC strains and coding for avian hemolysin (*hlyF*), iron-uptake and acquisition systems (*iutA-iucA/B/C/D*, and *iroB/C/E/D/N*), outer membrane protein (*ompTp*), putative secretion system type 1 (*etsA/B/C*), and resistance to the bactericidal activity of the complement (*iss*). Conversely, two of these gene clusters, *ets* and *iutA-iuc* were detected in only ca. 50% of the 129 AE-STEC and EPEC O80:H2. At first, their presence could be associated with the virulotypes since 76% of calf AE-STEC and 83% of human AE-STEC were positive compared to only 20% of calf EPEC strains. Nevertheless, the 12 calf EPEC and different non-*stx2d* AE-STEC that are positive for both *ets* and *iutA*/*iuc* gene clusters are classified in L1/SL1.2 along with the majority of *stx2d* AE-STEC, while the two *stx2d* AE-STEC that are negative for both gene clusters are classified in L1/SL1.1 along with the majority of EPEC and of non-*stx2d* AE-STEC (**see Fig.2 STUDY 3**). This indicates that the presence or absence of these two genes is actually more closely associated with sub-lineage distribution than with the virulotypes of AE-STEC and EPEC O80:H2.

In sharp contrast to the *ets* and *iutA/iuc* gene clusters, detection rates of both *cma* and *iha* genes was highly associated with L1/ SL1.1 strains and not with L1/SL1.2 strains. The *iha* gene coding for an “IrgA homologue adhesin” is associated with initial adherence and iron-uptake, which also may be required for AE-STEC and EPEC as virulence properties (Blanco Crivelli et al., 2018; Colello et al., 2009; Tarr et al., 2000). Conversely, the *cma* and *iha* genes may have been lost by L1/SL1.2 AE-STEC and EPEC because they would not be essential for their virulence. Indeed, if cattle are one of the contamination sources of AE-STEC O80:H2 to humans, the reduced detection of the *cma*

and *iha* genes in AE-STEC strains might result from: (i) selective pressures during multiple stages of transmission (e.g., from other reservoirs to the cattle intestine, then to humans), and (ii) external interventions such as antimicrobial treatments during calf-rearing which may have contributed to the loss of these virulence genes (Bernier Gosselin et al., 2024).

Genome analysis using hybrid assemblies constructed by complementary short and long read sequences of two bovine *stx2f* AE-STEC O80:H2 confirmed that not only the *ets* and *iuc/iutA* gene clusters of strains SES0108 belonging to L1/SL1.2 and the *cma* gene of strain SES0057 belonging to L1/SL1.1 were located on their respective pS88 plasmids, while the *iha* gene of strain SES0057 of L1/SL1.1 was located on a chromosome-inserted pathogenicity island called SpLE-1 (Sakai prophage-like element 1), along with the *ter*-type 1 operon. In STUDY 2 indeed, genetic typing revealed that the *ter*-type 1 resistance operon was detected in L1/SL1.1 strains, but not in L1/SL1.2 strains. This situation may be due to a genetic change during evolution of the serotype O80:H2, such as site-specific excision of the SpLE-1, which would result in the loss of the *ter*-resistance operon along with the *iha* gene (Bielaszewska et al., 2011).

In the two calf *stx2f* AE-STEC strains, the Stx2f phages are duplicated with one copy inserted within the *ssrA* tmRNA locus, a common insertion site of Stx phages. The second copy of the Stx2f phages are located upstream in the bacterial genomes at the height of the *thrW* tRNA locus, but are actually integrated in another prophage in a manner called the “prophage-in-prophage” integration pattern, similarly to a previous report concerning Stx2a phages in STEC O145:H28 (Nakamura et al., 2021). This pattern has potential implications for virulence accumulation and integration opportunities between distinct STEC serotypes and Stx phages, as suggested by Nakamura and collaborators.

While a highly conserved region (>99.5%) from the integrase-encoding gene through to lysis-related genes is present in both Stx2f phage genomes, the other two regions, mainly containing phage morphogenesis-encoding genes do not show high identity (>70%, and 70%-99%). This difference is also observed when comparing these two Stx2f phages with Stx2f phages of other STEC serotypes. The entire genome of the

Stx2f phage of O80:H2 strain SES0108 (L1/SL1.2) is also highly identical (>99.5%) to Stx2f phages of STEC O63:H3 and O145:H34. Notably, all three Stx2f phages carry *cdtA/B* genes which are frequently reported in *E. coli* and *E. albertii* isolated from humans and (wild) bird species. Furthermore, it has been also reported that *cdt*-carrying Stx2f phage are often integrated into the *ssrA* locus (Carter et al., 2023; Crombé et al., 2024).

On the other hand, even if they are integrated, directly or indirectly, into the same *ssrA* and *thrW* loci, the presence of the two genome regions of the Stx2f phages in strain SES0057 (L1/SL1.1) with (much) lower identity (>70%, and 70%-99%) to the Stx2f phages of strain SES0108 and of other human-associated STEC serotypes implies that the Stx2f phages are structurally variable. Interestingly enough, these two regions are highly identical (>99.7%) to another prophage present elsewhere in the SES0057 genome and also with prophage sequences present in the "gang of seven" major AE-STEC serotypes. Given the microbial diversity in the calf intestines, it is plausible that the Stx2f phage in SES0057 underwent recombination event(s) with prophages transferred from other STEC serotypes, during co-infection for instance. This hypothesis may be supported by a retrospective study in which a French research group reported that a dual infection involving STEC O80 and O26 in HUS cases associated with raw milk camembert cheese (Espié et al., 2008). Although a very few *stx2f* AE-STEC O80:H2 have been described since 2011 including in humans (Mainil et al., 2025), their frequency remains very low. In the recent review about AE-STEC and EPEC O80:H2, Mainil and colleagues further suggest that deletion or replacement of Stx phages has occurred frequently in AE-STEC O80:H2. Although still a matter of speculation, Stx2f phages may have been excluded because their contribution to virulence during human infection is lower than that of other Stx subtypes such as Stx2a and Stx2d.

Deletion or replacement study of Stx phages has been also conducted on other AE-STEC serotypes. For instance, Bielaszewska and collaborators suggest that bidirectional transfers between AE-STEC and EPEC serotype O26 occurred through acquisition and/or loss of *stx* genes and Stx phages based on several lines of evidence: *stx2* gene loss *in vitro*, successful transduction of EPEC with Stx2 phages, shared core genome between human AE-STEC and EPEC isolates (Bielaszewska et al., 2007). Core genomes of AE-STEC and EPEC O80:H2 are closely related at the SNP level. In addition, successful *in vitro*

transduction of Stx2d phages into K12 laboratory *E. coli* strains and into an *E. coli* O80:H26 strain has been demonstrated (Habets et al., 2022), indicating that at least Stx2d phages can be horizontally transferred within the O80 lineage. Given the close phylogenetic relationship between AE-STEC and EPEC O80:H2, it is therefore plausible that bidirectional transfers of Stx phages could also occur between these two pathotypes.

Additionally, the distribution of Stx subtypes differed between the sub-lineages: while L1/SL1.2 strains were predominantly *stx2d*-positive, L1/SL1.1 AE-STEC strains mostly carried *stx2a* or *stx1a* and rarely *stx2d*. Although a chronological genotype shift from *stx1* to *stx2* was previously reported for STEC O26 (Zhang et al., 2000), such a pattern may not be applicable in O80:H2. Instead, the observed distribution is more likely to reflect the independent acquisition and maintenance of distinct Stx phages in each (sub-)lineage, as suggested by Mainil and collaborators, shaped by separate evolutionary events (Mainil et al., 2025).

To conclude, subtle differences in the genetic profiles of the Stx-converting phages and of the pS88 plasmids were observed between strains belonging not only to different lineages, but also sometimes to the same lineage. Although the presence and role of other plasmids was not investigated in STUDY 3 (with the exception of the two calf *stx2f* AE-STEC), the actual impact of these differences in the genetic profiles and factors may be key to the diverse pathogenicity of the AE-STEC and EPEC O80:H2.

The most frequently identified Shiga toxins (Stx) in severe human infections are Stx2, especially the Stx2d subtype. Concerning this observation, AE-STEC O80:H2 are no exception, indicating that Stx2d probably plays a major role in their virulence, at least in human clinical cases (Cointe et al., 2018; Mariani-Kurkdjian et al., 2014 Soysal et al., 2016; Wijnsma et al., 2017). However, how about their actual relative role compared to the other virulence-associated properties, like the LEE region and the pS88 plasmids, *in vivo*? In STUDY 4, the role of the Stx2d and of two types of pS88 plasmids were assessed in the moth *Galleria mellonella* larval model.

The *stx2d* AE-STEC and Stx2d-transduced K12DH10B strains have the statistically significant highest lethality compared to the Stx1a AE-STEC and EPEC strains suggesting that the Stx2d is a major virulence factor in this model. Although

the *stx1a* AE-STEC strains also displayed a clear and rapid lethality at high concentrations, the results are not statistically different from those of the EPEC strains, under the conditions of STUDY 4. The contribution of Stx1a to virulence in this model, if any, is therefore impossible to assess.

Since the K12 DH10B strain transduced with the Stx2d phage exhibited a lethality level comparable to that of the donor *stx2d* AE-STEC, the next logical step would be to evaluate the virulence of a *stx2d* deletion mutant derived from the wild-type O80:H2 strain. This approach would help to clarify whether the presence of *stx2d* is necessary for the virulence phenotype observed in larvae of *G. mellonella* and thereby confirm its causal role. Although the presence of the receptor for Stx in this insect model has not been demonstrated to date, it remains possible that alternative mechanisms could allow Stx2d to exert its cytotoxicity in this model. While the activation of Stx2d by elastase in humans is established (Melton-Celsa et al., 2015), whether *G. mellonella* larvae produce proteases capable of similar activation also remains unknown. Interestingly, elastase-like activity has been detected in homogenates of *Galleria* larvae, including in uninfected controls (Andrejko et al., 2013). Therefore, it should not be excluded that endogenous proteases may contribute to Stx2d activation when larvae are infected with *stx2d* AE-STEC strains.

Another question is the level of expression of the Stx in *G. mellonella* larvae. The role in virulence of Stx1 and Stx2 has been assessed using different animal models and their respective levels of virulence depend on the animal species. For instance, Stx1 has higher virulence level than Stx2 using rabbits as a model, but the results are opposite in the case of mice (Shimizu et al., 2007; Tesh et al., 1993; Fujii et al., 2001). However, whether the insect model shows differential sensitivity to Stx1 and Stx2 compared to the mammal host and whether host-pathogen interaction which has been observed in EPEC, is exerted in distinct Stx subtypes of AE-STEC O80:H2, remains unknown (Chen and Keddie, 2021; Leuko and Raivio, 2012). Therefore, it is possible that the expression levels of Stx1a and Stx2d are not fully reproduced in the *in vivo G. mellonella* larval model.

The tested EPEC strains O80:H2 are of relatively low virulence in *G. mellonella* larvae compared to AE-STEC, though some significant difference was observed between the two EPEC strains harboring two different pS88 plasmids. Indeed, the EPEC strain

harbouring one *etsC* / *iucC*-positive pS88 plasmid is significantly more lethal. Conversely, the *E. coli* laboratory K12-DH10B strain acquiring the pS88 plasmid harbouring the *etsC* and *iucC* by conjugation is considerably less lethal than the two EPEC strains. The pathogenicity of EPEC using *G. mellonella* larvae has already been assessed with the human-specific typical EPEC E2348/69 strain (O127:H6) and it has been suggested that besides the presence of the LEE, other virulence factors increase the survival rate of EPEC in the hemocoele (Chen and Keddie, 2021). Of these other virulence factors, the ExPEC virulence genes present on the pS88 plasmids do not however represent any major virulence factors of EPEC, and probably AE-STEC O80:H2, but may still play a supporting role in modulating or enhancing their overall virulence.

The next step is the assessment of the different pS88-associated genes in the pathogenicity of AE-STEC and EPEC O80:H2. For example, one EPEC strain harboring the *ets* and *iuc/iut* genes on the pS88 plasmid shows higher lethality compared to the other EPEC strain whose pS88 plasmid does not carry those gene sets. The *ets* genes form one operon coding for an ABC transporter system, while the *iuc/iut* genes form another operon encoding the aerobactin siderophore (Peigne et al., 2009). Although contradictory results have been obtained for the *iuc*/*iut* genes *in vitro* or *in vivo* study (Peigne et al., 2009; Ling et al., 2013), the actual contribution of the *ets* genes in the pathogenicity of *E. coli* is still unknown.

The role in the virulence of the AE-STEC and EPEC O80:H2 of the *iss* gene that contributes to increase the serum survival which allows *E. coli* to evade innate immune responses in host serum and typically observed in ExPEC, is more difficult to analyze. Indeed, the *iss* gene is also identified in the *eae*-*stx*-pS88 plasmid-negative *E. coli*, like the *E. coli* O80:H45 and O80:H6 strains identified in STUDY 1 (Ikeda et al., 2023a; Pfaff-McDonough et al., 2000; Johnson et al., 2002; Wilczyński et al., 2022). Although they may be regarded as non-pathogenic (Ikeda et al., 2023a), the results of STUDY 4 however show that the lethality rate of *G. mellonella* larvae injected with O80:H45 and O80:H6 strains is by no means lower than that of *stx1a* AE-STEC and of EPEC O80:H2, though not as high as that of *stx2d* AE-STEC. Conversely, the *E. coli* K12 strains, also harboring the *iss* gene, show no or very little lethality in larvae of *G. mellonella.* Besides the contribution of other putative virulence-associated genes of the different *E. coli* O80, the

levels of expression of the different pS88-associated genes in *G. mellonella* larvae have not been studied in STUDY 4.

Accordingly, these levels of expression may also depend on the bacterial strain and on the host. For instance, an *ex vivo* study using *E. coli* serotype O45:K1:H7 strains responsible for urinary tract infections (UTI) in young infants, reports that three pS88-associated gene clusters or genes (*etsC*, *iucC*, and *iss*) are up-regulated in chicken serum, whereas no similar changes are observed in human serum or urine (Lemaître et al., 2012). Additionally, Peigne and collaborators suggested that disruption of the salmochelin receptor gene (*iroN*) in the pS88 plasmid reduced bacterial load in blood of a new-born mouse model, whereas deletion of ferric aerobactin receptor-encoding gene (*iut*) or the Sit system (*sit*) alone had no clear effect (Peigne et al., 2009). To clarify the contribution and the role of each virulence-associated gene more precisely, further studies must be performed to (i) assess the virulence of mutant strains, including allelic variants, in genes present on the chromosome and the pS88 plasmid in different infection models, including other models than insects; (ii) measure each gene expression levels, for instance by RT-qPCR to elucidate their dynamics in these different infection models; and (iii) perform histopathological analyses, which would represent a valuable complementary approach to determine whether the observed differences in lethality are associated with bacterial dissemination, preferential tissue localization, or specific tissue damage.

Finally, the somatic antigen O80 may also contribute to the virulence in the *G. mellonella* larvae, like for other somatic antigens. Indeed, though it was inoculated only for comparison, the *E. coli* O78:H4 strain that possesses neither *stx* genes nor *eae* gene, but well the nine pS88 plasmid-associated genes (*cia*, *cvaA*, *hlyF*, *ompTp*, *iroN*, *iss*, *sitA, etcC,* and *iucC*), has the highest lethality rate of the tested 13 *E. coli*. Generally, O78 is a major serotype of invasive *E. coli*, especially of Avian Pathogenic *E. coli* (APEC) and one of the most important virulence factors of APEC O78 via the anti-complement role of the lipopolysaccharide (Mainil and Fairbrother, 2014; Mellata et al., 2003). Even though differences in sugar composition have not been described, molecular typing of O antigens suggests that structural differences may exist between the O78 and O80 antigens (Iguchi et al., 2015). Therefore, a precise comparison of these two O antigens would be of interest. Since *G. mellonella* larvae have innate immunity similar to that of mammals

and birds, including the presence of complement-like proteins, the presence of this O78 lipopolysaccharide certainly increases the virulence of this strain in larvae (Antoine et al., 2021; Cutuli et al., 2019). At this stage, the role, if any of the O80 somatic antigen is however unknown.

CONCLUSIONS

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Despite a frequent association with HUS in Europe, the actual reservoir and source of contamination of humans of AE-STEC and EPEC O80:H2 remain unidentified (Habets et al., 2020; Thiry et al., 2018 + STUDY 1 and STUDY 2). Here, the following three-step hypothesis 'AE-STEC serotype O80:H2 originates from non-European regions through multiple evolutionary stages based on previous and latest findings including this thesis STUDIES' is proposed.

Studying more than 400 genomes of *E. coli* O80:H2, Mainil and colleagues (Mainil et al., 2025) describe three main lineages (L1, L2, L3). The earliest-diverging lineage (L1) comprises five EPEC O80:H2 strains, all of them harboring the *eaeρ* gene subtype that is not genetically close to *eaeξ* and lacking both the pS88 plasmid and the SpLE-like element (Mainil et al., 2025). What is more, four of them were isolated in non-European countries: South Asia (three strains), and South America (one strain). Cointe and collaborators (Cointe et al., 2021) made an interesting hypothesis that O80:H2 may have emerged from an atypical EPEC non-O80:H2 serotype sharing core genome through O-antigen switching. However, the identification of *eaeρ*-positive, pS88-negative EPEC O80:H2 strains in non-European regions cannot rule out that the EPEC O80:H2 serotype itself may have existed early in the evolutionary history of the current clones of AE-STEC and EPEC O80:H2 strains, which are *eaeξ*- and pS88-positive and are frequent in Western European countries. Moreover, the *eaeρ*-subtype is not genetically close to *eaeξ*. Taken together with the phylogenetic findings by Mainil and colleagues, a three-step scenario may be considered:

Step I. In the past time, a primitive EPEC O80:H2 harboring the *eaeρ* subtype emerged and entered European countries.

Step II. EPEC O80:H2 harboring the *eaeρ* subtype lost their LEE region and became *eae*-negative strains. The event of losing LEE region is practical since one *eae*-negative STEC O80:H2 have also been identified in Belgium (**STUDY 3**).

Step III. *eae*-negative *E. coli* O80:H2 acquired a novel LEE region with the *eaeξ* gene

and the SpLE1 through interaction with e.g. '*eaeξ*- and SpLE1-positive EPEC strains' belonging to other serotype(s) or other pathogenic *E. coli* strains (Kusumoto et al., 2014; Nakamura et al., 2023; Shepard et al., 2012; Vu-Khac et al., 2007).

It has indeed been reported that SpLE1 may confer a selective advantage to more efficiently colonize the intestinal tract in pigs, based on a study using an AE-STEC O157:H7 strain in swine intestines (Kusumoto et al., 2014; Yin et al., 2009). To the author's knowledge, although similar *in vivo* report in cattle is currently lacking, it is tempting to speculate that SpLE1 could also enhance colonization of the intestinal tract in cattle. This finding may explain why the majority of L1/SL1.1 strains that are identified as calf EPEC possess SpLE1 carrying the *iha* gene and the *ter* operon. Conversely, the L1/SL1.2 strains whose majority possess neither *iha* gene, nor the *ter* operon are identified as human AE-STEC. We may therefore hypothesize that the L1/SL1.2 strains represent a sub-lineage that lost the SpLE1 and becoming more suited for human infections. From the results of Mainil and colleagues (Mainil et al., 2025), it is also possible that Step II and Step III occurred in South- or North America before the *eae*□-SpLE1-positive clone entered Europe. Alternatively, Step II and Step III may have occurred in parallel in Europe and both American continents.

Emergence and spreading of the current AE-STEC and EPEC O80:H2 in Europe after 2010s

After the gain and loss of SpLE1, the acquisition of the pS88 plasmid and of different Stx phages is believed to have contributed to the emergence of the current AE-STEC O80:H2 clone (L1 in Habet et al., 2021 and STUDY 3; L3 in Mainil et al., 2025), which is now widespread in Western European countries. Several observations may support this scenario:

(i) After the acquisition of the original complete pS88 plasmid, a different type of the pS88 plasmid emerged in one of the sub-lineages. Thereafter, L1/SL1.2 strains retain a complete form of the original pS88 plasmid, while L1/SL1.1 strains possess the incomplete pS88 plasmid without the *ets* and *iutA/iuc* gene clusters. Alternatively, the original acquired pS88 plasmid could be the incomplete form followed by acquisition of the *ets* and *iutA/iuc* gene clusters. Either way, evolutionary adaptation shaped two types

of pS88 depending on the lineage and the preferential host, just like for SpLE1;

(ii) Given that O80:H2 AE-STEC strains carrying Stx1a and Stx2d have also been isolated before and after 2000s, it is difficult to identify which Stx phage was first acquired by this serotype. Possibly, the acquisition events of different Stx phages (Stx1a, 2a, 2d, and 2f) occurred independently through different hosts, e.g. the bovine intestinal tract, where major AE-STEC Stx subtypes circulate more frequently. Accordingly, the current distribution of Stx subtypes among O80:H2 AE-STEC may reflect adaptation shaped by lineage-specific and host-associated processes. Although two Stx2f-positive O80:H2 strains were isolated from calves in Belgium in 2010 and 2011, to our knowledge, no similar isolates from cattle have been reported since then, at least in European countries.

In summary, the evolutionary development of AE-STEC and EPEC O80:H2 has been shaped by multiple events of gaining and losing key virulence factors, such as the LEE pathogenicity island, Stx phages, pS88 and other plasmids, and SpLE1 prophage-like element. This complexity cannot be explained by a single-host model, but instead supports a multi-host transmission route, not only between cattle and humans, but also possibly swine and birds (Kindle et al., 2019b; Peigne et al., 2009; van Hoek et al., 2019; Vu-Khac et al., 2007). Most of the AE-STEC and EPEC O80:H2 strains currently isolated belong to the latest-diverging lineage L3 (Mainil et al., 2025). This may indicate that ancestral types of O80:H2 are less commonly found in Europe today, making it harder to trace. Although the epidemiological trigger for the emergence of these clones remains unclear, infrequent detection of the ancestral types could be one of the reasons that their actual reservoir has not been identified yet.

In the future, the serotype O80:H2 may be expected to diversify even further and novel clone to emerge that might cause large outbreaks. Although O80:H2 variants that are neither EPEC, nor STEC, and lack the pS88 plasmids have not yet been reported, the frequent emergence of hybrid *Escherichia coli* pathotypes, together with the close core genome relatedness, such as between O80:H2 and the porcine ETEC O149:H10 lineage, suggests that atypical O80:H2 carrying distinct virulence-associated gene combinations could plausibly arise or may have already arisen, but have remained undetected so far (Hritzo et al., 2025; Nakamura et al., 2023; Shepard et al., 2012).

In addition, recent reports suggest that O80:non-H2 strains could potentially acquire virulence factors (Habets et al., 2023; Ikeda et al., 2023a), indicating the need for continued monitoring of not only O80:H2, but also O80: non-H2, e.g., O80:H6, O80:H26 and O80:H45 in the future. To prevent a global threat similar to the O157:H7 outbreaks since the 1980s or the O104:H4 outbreak in 2011, comprehensive investigations must be carried out for further comprehension, such as:

(i) Epidemiological surveillance of cattle, swine, poultry, and their farms along with surrounding environments in European and in non-European countries;

(ii) Development of reliable, sensitive and specific methods for isolation and identification;

(iii) Whole-genome sequencing and pathogenicity evaluation both inside and outside intestinal environments.

REFERENCE LIST

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Abe, A., Kenny, B., Stein, M., Finlay, B.B., 1997. Characterization of two virule nce proteins secreted by rabbit enteropathogenic *Escherichia coli*, EspA and Esp B, whose maximal expression is sensitive to host body temperature. *Infect Immu n* 65, 3547–3555. https://doi.org/10.1128/iai.65.9.3547-3555.1997

Adu-Bobie, J., Frankel, G., Bain, C., Goncalves, A.G., Trabulsi, L.R., Douce, G., Knutton, S., Dougan, G., 1998. Detection of intimins alpha, beta, gamma, and delta, four intimin derivatives expressed by attaching and effacing microbial pathogens. *J Clin Microbiol* 36, 662–668. https://doi.org/10.1128/JCM.36.3.662-668.1998

Afset, J.E., Bruant, G., Brousseau, R., Harel, J., Anderssen, E., Bevanger, L., Bergh, K., 2006. Identification of virulence genes linked with diarrhea due to atypical enteropathogenic *Escherichia coli* by DNA microarray analysis and PCR. *J Clin Microbiol* 44, 3703–3711. https://doi.org/10.1128/JCM.00429-06

Ahlawat, S., Sharma, K.K., 2023. Lepidopteran insects: emerging model organism s to study infection by enteropathogens. *Folia Microbiol (Praha)* 68, 181–196. ht tps://doi.org/10.1007/s12223-022-01014-y

Ahmed, S.A., Awosika, J., Baldwin, C., Bishop-Lilly, K.A., Biswas, B., Broomall, S., Chain, P.S.G., Chertkov, O., Chokoshvili, O., Coyne, S., Davenport, K., Dett er, J.C., Dorman, W., Erkkila, T.H., Folster, J.P., Frey, K.G., George, M., Gleasn er, C., Henry, M., Hill, K.K., Hubbard, K., Insalaco, J., Johnson, S., Kitzmiller, A., Krepps, M., Lo, C.-C., Luu, T., McNew, L.A., Minogue, T., Munk, C.A., Os borne, B., Patel, M., Reitenga, K.G., Rosenzweig, C.N., Shea, A., Shen, X., Stro ckbine, N., Tarr, C., Teshima, H., van Gieson, E., Verratti, K., Wolcott, M., Xie, G., Sozhamannan, S., Gibbons, H.S., Threat Characterization Consortium, 2012. Genomic comparison of *Escherichia coli* O104:H4 isolates from 2009 and 2011 r eveals plasmid, and prophage heterogeneity, including shiga toxin encoding phage stx2. *PLoS One* 7, e48228. https://doi.org/10.1371/journal.pone.0048228

Aldick, T., Bielaszewska, M., Zhang, W., Brockmeyer, J., Schmidt, H., Friedrich, A.W., Kim, K.S., Schmidt, M.A., Karch, H., 2007. Hemolysin from Shiga toxin-negative *Escherichia coli* O26 strains injures microvascular endothelium. *Microbes Infect* 9, 282–290. https://doi.org/10.1016/j.micinf.2006.12.001

Alhadlaq, M.A., Aljurayyad, O.I., Almansour, A., Al-Akeel, S.I., Alzahrani, K.O., Alsalman, S.A., Yahya, R., Al-Hindi, R.R., Hakami, M.A., Alshahrani, S.D., Alhumeed, N.A., Al Moneea, A.M., Al-Seghayer, M.S., AlHarbi, A.L., Al-Reshoodi, F.M., Alajel, S., 2024. Overview of pathogenic *Escherichia coli*, with a focus on Shiga toxin-producing serotypes, global outbreaks (1982-2024) and food safety criteria. *Gut Pathog* 16, 57. https://doi.org/10.1186/s13099-024-00641-9

Al-Marri, T., Al-Marri, A., Al-Zanbaqi, R., Ajmi, A.A., Fayez, M., 2021. Multidrug resistance, biofilm formation, and virulence genes of *Escherichia coli* from backyard poultry farms. *Vet World* 14, 2869–2877. https://doi.org/10.14202/vetworld.2021.2869-2877

Alotaibi, K., Khan, A.A., 2023. Prevalence and molecular characterization of Shiga toxin-producing *Escherichia coli* from food and clinical samples. *Pathogens* 12, 1302. https://doi.org/10.3390/pathogens12111302

ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail, 2023), Avis relatif à la définition des souches pathogènes d'*Escherichia coli* productrices de Shiga-toxines, Saisine n° 2020-SA-0095, n°2016-SA-0121, 2010-SA-0031. https://www.anses.fr/fr/system/files/BIORISK2020SA0095.pdf

Andrejko, M., Zdybicka-Barabas, A., Cytryńska, M., 2014. Diverse effects of *Galleria mellonella* infection with entomopathogenic and clinical strains of *Pseudomonas aeruginosa*. Journal of Invertebrate Pathology 115, 14–25. https://doi.org/10.1016/j.jip.2013.10.006

Antoine, C., Laforêt, F., Blasdel, B., Fall, A., Duprez, J.-N., Mainil, J., Delcenserie, V., Thiry, D., 2021. In vitro characterization and in vivo efficacy assessment in *Galleria mellonella* larvae of newly isolated bacteriophages against *Escherichia coli* K1. *Viruses* 13, 2021. https://doi.org/10.3390/v13102005

Ardissino, G., Capone, V., Tedeschi, S., Porcaro, L., Cugno, M., 2022. Complement system as a new target for hematopoietic stem cell transplantation-related thrombotic microangiopathy. *Pharmaceuticals (Basel)* 15, 845. https://doi.org/10.3390/ph15070845

Auvray, F., Bièche-Terrier, C., Um, M.M., Dupouy, V., Nzuzi, N., David, L., Allais, L., Drouet, M., Oswald, E., Bibbal, D., Brugère, H., 2023. Prevalence and characterization of the seven major serotypes of Shiga toxin-producing *Escherichia coli* (STEC) in veal calves slaughtered in France. *Vet Microbiol* 282, 109754. https://doi.org/10.1016/j.vetmic.2023.109754

Awad, W.S., El-Sayed, A.A., Mohammed, F.F., Bakry, N.M., Abdou, N.-E.M.I., Kamel, M.S., 2020. Molecular characterization of pathogenic *Escherichia coli* isolated from diarrheic and in-contact cattle and buffalo calves. *Trop Anim Health Prod* 52, 3173–3185. https://doi.org/10.1007/s11250-020-02343-1

Bai, X., Fu, S., Zhang, J., Fan, R., Xu, Y., Sun, H., He, X., Xu, J., Xiong, Y., 2018. Identification and pathogenomic analysis of an *Escherichia coli* strain producing a novel Shiga toxin 2 subtype. *Sci Rep* 8, 6756. https://doi.org/10.1038/s41598-018-25233-x

Balali-Mood, M., Naseri, K., Tahergorabi, Z., Khazdair, M.R., Sadeghi, M., 2021. Toxic mechanisms of five heavy metals: mercury, lead, chromium, cadmium, and arsenic. *Front Pharmacol* 12, 643972. https://doi.org/10.3389/fphar.2021.643972

Banjo, M., Iguchi, A., Seto, K., Kikuchi, T., Harada, T., Scheutz, F., Iyoda, S., Pathogenic *E. coli* Working Group in Japan, 2018. *Escherichia coli* H-genotyping PCR: a complete and practical platform for molecular H typing. *J Clin Microbiol* 56, e00190-18. https://doi.org/10.1128/JCM.00190-18

Bardiau, M., Labrozzo, S., Mainil, J.G., 2009. Putative adhesins of enteropathogenic and enterohemorrhagic *Escherichia coli* of serogroup O26 isolated from humans and cattle. *J Clin Microbiol* 47, 2090–2096. https://doi.org/10.1128/JCM.02048-08

Bardiau, M., Szalo, M., Mainil, J.G., 2010. Initial adherence of EPEC, EHEC and VTEC to host cells. *Vet. Res*. 41, 57. https://doi.org/10.1051/vetres/2010029

Bardiau, M., Muylaert, A., Duprez, J.-N., Labrozzo, S., Mainil, J.G., 2010. Prevalence, molecular typing, and antibiotic sensitivity of enteropathogenic, enterohaemorrhagic, and verotoxigenic *Escherichia coli* isolated from veal calves. Tijdschr Diergeneeskd 135, 554–558.

Barer, M.R., 2012. Morphology and nature of micro-organisms, in: *Medical Microbiology*. Elsevier, pp. 9–23. https://doi.org/10.1016/B978-0-7020-4089-4.00017-2

Basavaraju, M., Gunashree, B.S., 2023. *Escherichia coli*: an overview of main characteristics, in: Starčič Erjavec, M. (Ed.), *Escherichia Coli* - Old and New Insights. *IntechOpen*. https://doi.org/10.5772/intechopen.105508

Bauwens, A., Betz, J., Meisen, I., Kemper, B., Karch, H., Müthing, J., 2013. Facing glycosphingolipid-Shiga toxin interaction: dire straits for endothelial cells of the human vasculature. *Cell Mol Life Sci* 70, 425–457. https://doi.org/10.1007/s00018-012-1060-z

Beghain, J., Bridier-Nahmias, A., Le Nagard, H., Denamur, E., Clermont, O., 2018. ClermonTyping: an easy-to-use and accurate in silico method for *Escherichia* genus strain phylotyping. *Microbial Genomics* 4. https://doi.org/10.1099/mgen.0.000192

Ben Zakour, N.L., Alsheikh-Hussain, A.S., Ashcroft, M.M., Khanh Nhu, N.T., Roberts, L.W., Stanton-Cook, M., Schembri, M.A., Beatson, S.A., 2016. Sequential acquisition of virulence and fluoroquinolone resistance has shaped the evolution of *Escherichia coli* ST131. *mBio* 7, e00347-00316. https://doi.org/10.1128/mBio.00347-16

Beraldo, L.G., Borges, C.A., Maluta, R.P., Cardozo, M.V., de Ávila, F.A., 2023. Molecular analysis of enteropathogenic *Escherichia coli* (EPEC) isolates from healthy food-producing animals and humans with diarrhoea. *Zoonoses Public Health* 70, 117–124. https://doi.org/10.1111/zph.13007

Bernier Gosselin, V., Fernandez, J.E., Ollagnier, C., Morel, I., Siegenthaler, R., Collaud, A., Meylan, M., Perreten, V., 2024. Whole-Genome sequencing-based antimicrobial resistance and shedding dynamics of *Escherichia coli* isolated from calves before and after antimicrobial group treatments. *Microbiol Spectr* 12, e0321423. https://doi.org/10.1128/spectrum.03214-23

Bettelheim, K.A., 2000. Role of non-O157 VTEC. *Symp Ser Soc Appl Microbiol* 38S-50S. https://doi.org/10.1111/j.1365-2672.2000.tb05331.x

Beutin, L., 1999. *Escherichia coli* as a pathogen in dogs and cats. Vet Res 30, 285–298.

Beutin, L., Fach, P., 2014. Detection of Shiga toxin-producing *Escherichia coli* fr om nonhuman sources and strain typing. *Microbiol Spectr* 2. https://doi.org/10.112 8/microbiolspec.EHEC-0001-2013

Beutin, L., Krause, G., Zimmermann, S., Kaulfuss, S., Gleier, K., 2004. Characte rization of Shiga toxin-producing *Escherichia coli* strains isolated from human pa tients in Germany over a 3-year period. *J Clin Microbiol* 42, 1099–1108. https:// doi.org/10.1128/JCM.42.3.1099-1108.2004

Beutin, L., Martin, A., 2012. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. *J Food Prot* 75, 408–418. https://doi.org/10.4315/0362-028X.JFP-11-452

Beutin, L., Miko, A., Krause, G., Pries, K., Haby, S., Steege, K., Albrecht, N., 2007. Identification of human-pathogenic strains of Shiga toxin-producing *Escherichia coli* from food by a combination of serotyping and molecular typing of Shiga toxin genes. *Appl Environ Microbiol* 73, 4769–4775. https://doi.org/10.1128/AEM.00873-07

Beutin, L., Prada, J., Zimmermann, S., Stephan, R., Orskov, I., Orskov, F., 1988. Enterohemolysin, a new type of hemolysin produced by some strains of enteropathogenic *E. coli* (EPEC). *Zentralbl Bakteriol Mikrobiol Hyg A* 267, 576–588. https://doi.org/10.1016/s0176-6724(88)80042-7

Bibbal, D., Loukiadis, E., Kérourédan, M., Ferré, F., Dilasser, F., Peytavin de Garam, C., Cartier, P., Oswald, E., Gay, E., Auvray, F., Brugère, H., 2015. Prevalence of carriage of Shiga toxin-producing *Escherichia coli* serotypes O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 among slaughtered adult cattle in France. Appl Environ Microbiol 81, 1397–1405. https://doi.org/10.1128/AEM.03315-14

Bibbal, D., Loukiadis, E., Kérourédan, M., Peytavin de Garam, C., Ferré, F., Cartier, P., Gay, E., Oswald, E., Auvray, F., Brugère, H., 2014. Intimin gene (*eae*) subtype-based real-time PCR strategy for specific detection of Shiga toxin-producing *Escherichia coli* serotypes O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 in cattle feces. *Appl Environ Microbiol* 80, 1177–1184. https://doi.org/10.1128/AEM.03161-13

Bielaszewska, M., Aldick, T., Bauwens, A., Karch, H., 2014. Hemolysin of enter ohemorrhagic *Escherichia coli*: structure, transport, biological activity and putative role in virulence. *Int J Med Microbiol* 304, 521–529. https://doi.org/10.1016/j.ijm m.2014.05.005

Bielaszewska, M., Middendorf, B., Tarr, P.I., Zhang, W., Prager, R., Aldick, T., Dobrindt, U., Karch, H., Mellmann, A., 2011. Chromosomal instability in enteroh aemorrhagic *Escherichia coli* O157:H7: impact on adherence, tellurite resistance a nd colony phenotype. *Molecular Microbiology* 79, 1024–1044. https://doi.org/10.11 11/j.1365-2958.2010.07499.x

Bielaszewska, M., Prager, R., Köck, R., Mellmann, A., Zhang, W., Tschäpe, H., Tarr, P.I., Karch, H., 2007. Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Appl Environ Microbiol* 73, 3144–3150. https://doi.org/10.1128/AEM.02937-06

Bielaszewska, M., Stoewe, F., Fruth, A., Zhang, W., Prager, R., Brockmeyer, J., Mellmann, A., Karch, H., Friedrich, A.W., 2009. Shiga toxin, cytolethal distending toxin, and hemolysin repertoires in clinical *Escherichia coli* O91 isolates. *J Clin Microbiol* 47, 2061–2066. https://doi.org/10.1128/JCM.00201-09

Bizot, E., Cointe, A., Bidet, P., Mariani-Kurkdjian, P., Hobson, C.A., Courroux, C., Liguori, S., Bridier-Nahmias, A., Magnan, M., Merimèche, M., Caméléna, F., Berçot, B., Weill, F.-X., Lefèvre, S., Bonacorsi, S., Birgy, A., 2022a. Azithromycin resistance in Shiga toxin-producing *Escherichia coli* in france between 2004 and 2020 and detection of *mef*(C)-*mph*(G) genes. *Antimicrob Agents Chemother* 66, e0194921. https://doi.org/10.1128/AAC.01949-21

Bizot, E., Cointe, A., Smadja, N., Sergentet, D., Lefèvre, S., Weill, F.-X., Levy, C., Cohen, R., Mariani-Kurkdjian, P., Bonacorsi, S., 2022b. Improved molecular diagnosis and culture of the emerging heteropathotype enterohemorrhagic *Escherichia coli* O80:H2 using its non-melibiose-fermenting and antibiotic-resistance properties. *J Clin Microbiol* 60, e0153021. https://doi.org/10.1128/JCM.01530-21

Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J.V., 2015. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 13, 42–51. https://doi.org/10.1038/nrmicro3380

Blanco Crivelli, X., Bonino, M.P., Von Wernich Castillo, P., Navarro, A., Degregorio, O., Bentancor, A., 2018. Detection and characterization of enteropathogenic and Shiga toxin-producing *Escherichia coli* strains in rattus spp. from Buenos Aires. *Front Microbiol* 9, 199. https://doi.org/10.3389/fmicb.2018.00199

Blanco, M., Blanco, J.E., Mora, A., Dahbi, G., Alonso, M.P., González, E.A., Bernárdez, M.I., Blanco, J., 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (*eae*-xi). *J Clin Microbiol* 42, 645–651. https://doi.org/10.1128/JCM.42.2.645-651.2004

Blanco, M., Schumacher, S., Tasara, T., Zweifel, C., Blanco, J.E., Dahbi, G., Blanco, J., Stephan, R., 2005. Serotypes, intimin variants and other virulence factors of *eae* positive *Escherichia coli* strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (*eae*-η2). *BMC Microbiol* 5, 23. https://doi.org/10.1186/1471-2180-5-23

Bonanno, L., Loukiadis, E., Mariani-Kurkdjian, P., Oswald, E., Garnier, L., Michel, V., Auvray, F., 2015. Diversity of Shiga toxin-producing *Escherichia coli* (STEC) O26:H11 strains examined via *stx* subtypes and insertion sites of Stx and EspK Bacteriophages. *Appl Environ Microbiol* 81, 3712–3721. https://doi.org/10.1128/AEM.00077-15

Bray, J., 1945. Isolation of antigenically homogeneous strains of *Bact. coli neapolitanum* from summer diarrhœa of infants. *J. Patho*l. 57, 239–247. https://doi.org/10.1002/path.1700570210

Brett, K.N., Ramachandran, V., Hornitzky, M.A., Bettelheim, K.A., Walker, M.J., Djordjevic, S.P., 2003. *stx1c* is the most common Shiga toxin 1 subtype among Shiga toxin-producing *Escherichia coli* isolates from sheep but not among isolates from cattle. *J Clin Microbiol* 41, 926–936. https://doi.org/10.1128/JCM.41.3.926-936.2003

Briffa, J., Sinagra, E., Blundell, R., 2020. Heavy metal pollution in the environm ent and their toxicological effects on humans. *Heliyon* 6, e04691. https://doi.org/1 0.1016/j.heliyon.2020.e04691

Browne, A.S., Midwinter, A.C., Withers, H., Cookson, A.L., Biggs, P.J., Marshall, J.C., Benschop, J., Hathaway, S., Rogers, L., Nisa, S., Hranac, C.R., Winkleman, T., French, N.P., 2021. Transmission dynamics of Shiga toxin-producing *Escherichia coli* in New Zealand cattle from farm to slaughter. *Appl Environ Microbiol* 87, e02907-20. https://doi.org/10.1128/AEM.02907-20

Bruyand, M., Mariani-Kurkdjian, P., Le Hello, S., King, L.-A., Van Cauteren, D., Lefevre, S., Gouali, M., Jourdan-da Silva, N., Mailles, A., Donguy, M.-P., Loukia dis, E., Sergentet-Thevenot, D., Loirat, C., Bonacorsi, S., Weill, F.-X., De Valk, H., Réseau hospitalier de surveillance du SHU pédiatrique, 2019. Paediatric ha emolytic uraemic syndrome related to Shiga toxin-producing *Escherichia coli*, an overview of 10 years of surveillance in France, 2007 to 2016. *Euro Surveill* 24, 1800068. https://doi.org/10.2807/1560-7917.ES.2019.24.8.1800068

Bush, K., Bradford, P.A., 2016. β-lactams and β-lactamase inhibitors: an overview. *Cold Spring Harb Perspect Med* 6, a025247. https://doi.org/10.1101/cshperspect.a025247

Buvens, G., De Gheldre, Y., Dediste, A., De Moreau, A.-I., Mascart, G., Simon, A., Allemeersch, D., Scheutz, F., Lauwers, S., Piérard, D., 2012. Incidence and v irulence determinants of verocytotoxin-producing *Escherichia coli* infections in the Brussels-Capital region, Belgium, in 2008–2010. *J Clin Microbio*l 50, 1336–1345. https://doi.org/10.1128/JCM.05317-11

Buvens, G., Lauwers, S., Piérard, D., 2010. Prevalence of subtilase cytotoxin in verocytotoxin-producing *Escherichia coli* isolated from humans and raw meats in

Belgium. *Eur J Clin Microbiol Infect Dis* 29, 1395–1399. https://doi.org/10.1007/s10096-010-1014-z

Calderón, I.L., Arenas, F.A., Pérez, J.M., Fuentes, D.E., Araya, M.A., Saavedra, C.P., Tantaleán, J.C., Pichuantes, S.E., Youderian, P.A., Vásquez, C.C., 2006. Catalases are NAD(P)H-dependent tellurite reductases. *PLoS ONE* 1, e70. https://doi.org/10.1371/journal.pone.0000070

Calderwood, S.B., Acheson, D.W.K., Keusch, G.T., Brarrett, T.J., Griffin, P.M., Strockbine, N.A., Swaminathan, B., Kaper, J.B., Levine, M.M., Kaplan, B.S., Karch, H., O'Brien, A.D., Obrig, T.G., Takeda, Y., Tarr, P.I., Wachsmuth, I.K., 1996. Proposed new nomenclature for SLT (VT) family. *ASM* News 62, 118–119.

Calderwood, S.B., Mekalanos, J.J., 1990. Genetic analysis of regulation and structure of Shiga-like toxin I in *Escherichia Coli*, in: Pohland, A.E., Dowell, V.R., Richard, J.L., Cole, R.J., Eklund, M.W., Green, S.S., Norred, W.P., Potter, M.E. (Eds.), *Microbial Toxins in Foods and Feeds*. Springer US, Boston, MA, pp. 57–64. https://doi.org/10.1007/978-1-4613-0663-4_5

Campbell, A., 1994. Comparative molecular biology of lambdoid phages. *Annu Rev Microbiol* 48, 193–222. https://doi.org/10.1146/annurev.mi.48.100194.001205

Campos, L.C., Franzolin, M.R., Trabulsi, L.R., 2004. Diarrheagenic *Escherichia coli* categories among the traditional enteropathogenic *E. coli* O serogroups: a review. *Mem. Inst. Oswaldo Cruz* 99, 545–552. https://doi.org/10.1590/S0074-02762004000600001

Carter, M.Q., Quiñones, B., He, X., Pham, A., Carychao, D., Cooley, M.B., Lo, C.-C., Chain, P.S.G., Lindsey, R.L., Bono, J.L., 2023. Genomic and phenotypic characterization of Shiga toxin-producing *Escherichia albertii* strains isolated from wild birds in a major agricultural region in California. *Microorganisms* 11, 2803. https://doi.org/10.3390/microorganisms11112803

Castellani, A., Chalmers, A.J., 1919. *Manual of tropical medicine*. Baillière, Tindall and Cox, London. https://doi.org/10.5962/bhl.title.84653

Centers for Disease Control and Prevention (CDC), 2018. National Shiga toxin-pr oducing *Escherichia coli* (STEC) surveillance annual report, 2016. CDC, Atlanta, GA, USA. Published 16 April 2018. Available at: https://www.cdc.gov/ecoli/surv2 016/index.html.

Cervantes, C., Ji, G., Ramírez, J.L., Silver, S., 1994. Resistance to arsenic compounds in microorganisms. *FEMS Microbiol Rev* 15, 355–367. https://doi.org/10.1111/j.1574-6976.1994.tb00145.x

Chark, D., Nutikka, A., Trusevych, N., Kuzmina, J., Lingwood, C., 2004. Differential carbohydrate epitope recognition of globotriaosyl ceramide by verotoxins and a monoclonal antibody. *Eur J Biochem* 271, 405–417. https://doi.org/10.1046/j.1432-1033.2003.03941.x

Chasteen, T.G., Fuentes, D.E., Tantaleán, J.C., Vásquez, C.C., 2009. Tellurite: history, oxidative stress, and molecular mechanisms of resistance. *FEMS Microbiol Rev* 33, 820–832. https://doi.org/10.1111/j.1574-6976.2009.00177.x

Chatterjee, A., Caballero-Franco, C., Bakker, D., Totten, S., Jardim, A., 2015. Pore-forming activity of the *Escherichia coli* Type III secretion system protein EspD. *J Biol Chem* 290, 25579–25594. https://doi.org/10.1074/jbc.M115.648204

Chen, R.Y., Keddie, B.A., 2021. The *Galleria mellonella*-enteropathogenic *Escherichia coli* model system: characterization of pathogen virulence and insect immune responses. *J Insect Sci* 21, 7. https://doi.org/10.1093/jisesa/ieab046

Chérier, D., Patin, D., Blanot, D., Touzé, T., Barreteau, H., 2021. The biology of colicin M and its orthologs. *Antibiotics (Basel)* 10, 1109. https://doi.org/10.3390/a ntibiotics10091109

Chhabra, S., Taksande, A.B., Munjewar, P., 2024. The penicillin pioneer: Alexand er Fleming's journey to a medical breakthrough. *Cureus* 16, e65179. https://doi.or g/10.7759/cureus.65179

Ching, J.C.Y., Jones, N.L., Ceponis, P.J.M., Karmali, M.A., Sherman, P.M., 2002. *Escherichia coli* Shiga-like toxins induce apoptosis and cleavage of poly (ADP-ri

bose) polymerase via in vitro activation of caspases. *Infect Immun* 70, 4669–467 7. https://doi.org/10.1128/IAI.70.8.4669-4677.2002

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and de tection of new phylo-groups. *Environ Microbiol Rep* 5, 58–65. https://doi.org/10.1 111/1758-2229.12019

Clermont, O., Dixit, O.V.A., Vangchhia, B., Condamine, B., Dion, S., Bridier-Nahmias, A., Denamur, E., Gordon, D., 2019. Characterization and rapid identification of phylogroup G in *Escherichia coli*, a lineage with high virulence and antibiotic resistance potential. *Environmental Microbiology* 21, 3107–3117. https://doi.org/10.1111/1462-2920.14713

Cointe, A., Birgy, A., Bridier-Nahmias, A., Mariani-Kurkdjian, P., Walewski, V., Lévy, C., Cohen, R., Fach, P., Delannoy, S., Bidet, P., Bonacorsi, S., 2020a. *Escherichia coli* O80 hybrid pathotype strains producing Shiga toxin and ESBL: molecular characterization and potential therapeutic options. *J Antimicrob Chemother* 75, 537–542. https://doi.org/10.1093/jac/dkz484

Cointe, A., Birgy, A., Mariani-Kurkdjian, P., Liguori, S., Courroux, C., Blanco, J., Delannoy, S., Fach, P., Loukiadis, E., Bidet, P., Bonacorsi, S., 2018. Emerging multidrug-resistant hybrid pathotype Shiga toxin-producing *Escherichia coli* O80 and Related Strains of Clonal Complex 165, Europe. *Emerg Infect Dis* 24, 2262–2269. https://doi.org/10.3201/eid2412.180272

Cointe, A., Birgy, A., Pascault, A., Louillet, F., Dufougeray, A., Mariani-Kurkdjian, P., Bonacorsi, S., 2020b. Be aware of Shiga toxin 2f-producing *Escherichia coli*: case report and false-negative results with certain rapid molecular panels. *Diagn Microbiol Infect Dis* 98, 115177. https://doi.org/10.1016/j.diagmicrobio.2020.115177

Cointe, A., Bizot, E., Delannoy, S., Fach, P., Bidet, P., Birgy, A., Weill, F.-X., Lefèvre, S., Mariani-Kurkdjian, P., Bonacorsi, S., 2021. Emergence of new ST301 Shiga toxin-producing *Escherichia coli* clones harboring extra-intestinal virulence traits in Europe. *Toxins (Basel)* 13, 686. https://doi.org/10.3390/toxins13100686

Colello, R., Krüger, A., Velez, M.V., Del Canto, F., Etcheverría, A.I., Vidal, R., Padola, N.L., 2019. Identification and detection of *iha* subtypes in LEE-negative Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from humans, cattle and food. *Heliyon* 5, e03015. https://doi.org/10.1016/j.heliyon.2019.e03015

Cornick, N.A., Helgerson, A.F., Mai, V., Ritchie, J.M., Acheson, D.W.K., 2006. In vivo transduction of an Stx-encoding phage in ruminants. *Appl Environ Microbiol* 72, 5086–5088. https://doi.org/10.1128/AEM.00157-06

Crombé, F., van Hoek, A.H.A.M., Nailis, H., Auvray, F., Janssen, T., Piérard, D., 2024. Intestinal carriage of two distinct *stx2f*-carrying *Escherichia coli* strains by a child with uncomplicated diarrhea. *Pathogens* 13, 1002. https://doi.org/10.3390/pathogens13111002

Cunningham, N., Jenkins, C., Williams, S., Garner, J., Eggen, B., Douglas, A., Potter, T., Wilson, A., Leonardi, G., Larkin, L., Hopkins, S., 2024. An outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with contaminated lettuce and the cascading risks from climate change, the United Kingdom, August to September 2022. *Euro Surveill* 29, 2400161. https://doi.org/10.2807/1560-7917.ES.2024.29.36.2400161

Cutuli, M.A., Petronio Petronio, G., Vergalito, F., Magnifico, I., Pietrangelo, L., Venditti, N., Di Marco, R., 2019. *Galleria mellonella* as a consolidated in vivo model hosts: new developments in antibacterial strategies and novel drug testing. *Virulence* 10, 527–541. https://doi.org/10.1080/21505594.2019.1621649

De Greve, H., Qizhi, C., Deboeck, F., Hernalsteens, J.P., 2002. The Shiga-toxin VT2-encoding bacteriophage varphi297 integrates at a distinct position in the *Escherichia coli* genome. *Biochim Biophys Acta* 1579, 196–202. https://doi.org/10.1016/s0167-4781(02)00539-0

De Rauw, K., Jacobs, S., Piérard, D., 2018. Twenty-seven years of screening for Shiga toxin-producing *Escherichia coli* in a university hospital. Brussels, Belgium, 1987-2014. *PLoS One* 13, e0199968. https://doi.org/10.1371/journal.pone.0199968

De Rauw, K., Thiry, D., Caljon, B., Saulmont, M., Mainil, J., Piérard, D., 2019. Characteristics of Shiga toxin-producing and enteropathogenic *Escherichia coli* of the emerging serotype O80:H2 isolated from humans and diarrhoeic calves in Belgium. *Clin Microbiol Infect* 25, 111.e5-111.e8. https://doi.org/10.1016/j.cmi.2018.07.023

Dean, P., Kenny, B., 2009. The effector repertoire of enteropathogenic *E. coli*: ga nging up on the host cell. *Curr Opin Microbiol* 12, 101–109. https://doi.org/10.10 16/j.mib.2008.11.006

Dean, P., Maresca, M., Schüller, S., Phillips, A.D., Kenny, B., 2006. Potent diarrheagenic mechanism mediated by the cooperative action of three enteropathogenic *Escherichia coli* injected effector proteins. *Proc Natl Acad Sci USA* 103, 1876–1881. https://doi.org/10.1073/pnas.0509451103

Deborah Chen, H., Frankel, G., 2005. Enteropathogenic *Escherichia coli*: unravell ing pathogenesis. *FEMS Microbiol Rev* 29, 83–98. https://doi.org/10.1016/j.femsre. 2004.07.002

Den Bakker, H.C., Moreno Switt, A.I., Cummings, C.A., Hoelzer, K., Degoricija, L., Rodriguez-Rivera, L.D., Wright, E.M., Fang, R., Davis, M., Root, T., Schoonmaker-Bopp, D., Musser, K.A., Villamil, E., Waechter, H., Kornstein, L., Furtado, M.R., Wiedmann, M., 2011. A Whole-Genome Single Nucleotide Polymorphism-based approach to trace and identify outbreaks linked to a common *Salmonella enterica subsp. enterica* Serovar Montevideo pulsed-field gel electrophoresis type. *Appl Environ Microbiol* 77, 8648–8655. https://doi.org/10.1128/AEM.06538-11

Den Ouden, A., Greig, D.R., Rodwell, E.V., Tripodo, F., Olonade, I., Swift, C., J enkins, C., 2023. *Escherichia coli* encoding Shiga toxin subtype Stx2f causing hu man infections in England, 2015–2022. *J. Med. Microbiol*. 72, 001707. https://doi. org/10.1099/jmm.0.001707

Deng, W., Puente, J.L., Gruenheid, S., Li, Y., Vallance, B.A., Vázquez, A., Barb a, J., Ibarra, J.A., O'Donnell, P., Metalnikov, P., Ashman, K., Lee, S., Goode, D., Pawson, T., Finlay, B.B., 2004. Dissecting virulence: systematic and functional a

nalyses of a pathogenicity island. *Proc Natl Acad Sci U S A* 101, 3597–3602.https://doi.org/10.1073/pnas.0400326101

Deng, W., Yu, H.B., Li, Y., Finlay, B.B., 2015. SepD/SepL-dependent secretion signals of the type III secretion system translocator proteins in enteropathogenic *Escherichia coli*. *J Bacteriol* 197, 1263–1275. https://doi.org/10.1128/JB.02401-14

Devinney, R., Nisan, I., Ruschkowski, S., Rosenshine, I., Finlay, B.B., 2001. Tir tyrosine phosphorylation and pedestal formation are delayed in enteropathogenic *Escherichia coli sepZ*::Tn *phoA* mutant 30-5-1(3). *Infect Immun* 69, 559–563. https://doi.org/10.1128/IAI.69.1.559-563.2001

Díaz-Jiménez, D., García-Meniño, I., Herrera, A., García, V., López-Beceiro, A.M., Alonso, M.P., Blanco, J., Mora, A., 2020. Genomic characterization of *Escherichia coli* isolates belonging to a new hybrid aEPEC/ExPEC pathotype O153:H10-A-ST10 *eae*-beta1 occurred in meat, poultry, wildlife and human diarrheagenic samples. *Antibiotics (Basel)* 9, 192. https://doi.org/10.3390/antibiotics9040192

Dieckmann, R., Malorny, B., 2011. Rapid screening of epidemiologically important *Salmonella enterica subsp. enterica* serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl Environ Microbiol* 77, 4136–4146. https://doi.org/10.1128/AEM.02418-10

Do, V.H., Nguyen, V.S., Nguyen, S.H., Le, D.Q., Nguyen, T.T., Nguyen, C.H., Ho, T.H., Vo, N.S., Nguyen, T., Nguyen, H.A., Cao, M.D., 2024. PanKA: leveraging population pangenome to predict antibiotic resistance. *iScience* 27, 110623. https://doi.org/10.1016/j.isci.2024.110623

Dobrescu, L., 1983. New biological effect of edema disease principle (*Escherichia coli*-neurotoxin) and its use as an in vitro for this toxin. *Am J Vet Res* 44, 31–34.

Donnenberg, M.S., Whittam, T.S., 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. *J Clin Invest* 107, 539–548. https://doi.org/10.1172/JCI12404

Duneton, C., Kwon, T., Dossier, C., Baudouin, V., Fila, M., Mariani-Kurkdijan, P., Nel, I., Boyer, O., Hogan, J., 2024. IgG-immunoadsorptions and eculizumab combination in STEC-hemolytic and uremic syndrome pediatric patients with neurological involvement. *Pediatr Nephrol*. https://doi.org/10.1007/s00467-024-06418-1

Duriez, P., Clermont, O., Bonacorsi, S., Bingen, E., Chaventré, A., Elion, J., Picard, B., Denamur, E., 2001. Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. *Microbiology* 147, 1671–1676. https://doi.org/10.1099/00221287-147-6-1671

Dutta, T.K., Roychoudhury, P., Bandyopadhyay, S., Wani, S.A., Hussain, I., 2011. Detection & characterization of Shiga toxin-producing *Escherichia coli* (STEC) & enteropathogenic *Escherichia coli* (EPEC) in poultry birds with diarrhoea. *Indian J Med Res* 133, 541–545.

Dziva, F., Mahajan, A., Cameron, P., Currie, C., McKendrick, I.J., Wallis, T.S., Smith, D.G.E., Stevens, M.P., 2007. EspP, a Type V-secreted serine protease of enterohaemorrhagic *Escherichia coli* O157:H7, influences intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells. *FEMS Microbiol Lett* 271, 258–264. https://doi.org/10.1111/j.1574-6968.2007.00724.x

European Centre for Disease Prevention and Control (ECDC), 2025. STEC infection. In: Annual epidemiological report for 2023. Stockholm: ECDC. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/shiga-toxin-producing-escherichia-coli-annual-epidemiological-report-2023.pdf (accessed December 10, 2025).

EFSA Panel on Biological Hazards (EFSA BIOHAZ Panel), Koutsoumanis, K., A llende, A., Alvarez-Ordóñez, A., Bover-Cid, S., Chemaly, M., Davies, R., De Ces are, A., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Si mmons, M., Skandamis, P., Suffredini, E., Jenkins, C., Monteiro Pires, S., Morab ito, S., Niskanen, T., Scheutz, F., da Silva Felício, M.T., Messens, W., Bolton, D., 2020. Pathogenicity assessment of Shiga toxin-producing *Escherichia coli* (ST EC) and the public health risk posed by contamination of food with STEC. EFS A Journal 18. https://doi.org/10.2903/j.efsa.2020.5967

EFSA Panel on Biological Hazards (EFSA BIOHAZ Panel), Koutsoumanis, K., A llende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Sim mons, M., Skandamis, P., Suffredini, E., Jenkins, C., Malorny, B., Ribeiro Duarte, A.S., Torpdahl, M., da Silva Felício, M.T., Guerra, B., Rossi, M., Herman, L.,2 019. Whole genome sequencing and metagenomics for outbreak investigation, sou rce attribution and risk assessment of food-borne microorganisms. EFSA Journal 17. https://doi.org/10.2903/j.efsa.2019.5898

Elliott, S.J., Wainwright, L.A., McDaniel, T.K., Jarvis, K.G., Deng, Y.K., Lai, L.C., McNamara, B.P., Donnenberg, M.S., Kaper, J.B., 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol Microbiol* 28, 1–4. https://doi.org/10.1046/j.1365-2958.1998.00783.x

Elsayed, M.S.A.E., Eldsouky, S.M., Roshdy, T., Bayoume, A.M.A., Nasr, G.M., Salama, A.S.A., Akl, B.A., Hasan, A.S., Shahat, A.K., Khashaba, R.A., Abdelhalim, W.A., Nasr, H.E., Mohammed, L.A., Salah, A., 2021. Genetic and antimicrobial resistance profiles of non-O157 Shiga toxin-producing *Escherichia coli* from different sources in Egypt. *BMC Microbiol* 21, 257. https://doi.org/10.1186/s12866-021-02308-w

Escherich, T., 1885. Die Darmbakterien des Neugeborenen und Sauglings. *Fortschritte der Medizin*, 3, 515–522.

Espié, E., Grimont, F., Mariani-Kurkdjian, P., Bouvet, P., Haeghebaert, S., Filliol, I., Loirat, C., Decludt, B., Minh, N.N.T., Vaillant, V., de Valk, H., 2008. Surveillance of hemolytic uremic syndrome in children less than 15 years of age, a system to monitor O157 and non-O157 Shiga toxin-producing *Escherichia coli* infections in France, 1996-2006. *Pediatr Infect Dis J* 27, 595–601. https://doi.org/10.1097/INF.0b013e31816a062f

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2024. The European Union One Health 2023 Zoonoses rep ort. EFSA Journal 22. https://doi.org/10.2903/j.efsa.2024.9106

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2023. The European Union One Health 2022 Zoonoses Re port. EFSA Journal 21. https://doi.org/10.2903/j.efsa.2023.8442

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2022. The European Union One Health 2021 Zoonoses Re port. EFSA Journal 20. https://doi.org/10.2903/j.efsa.2022.7666

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2021a. The European Union One Health 2020 Zoonoses R eport. EFSA Journal 19. https://doi.org/10.2903/j.efsa.2021.6971

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2021b. The European Union One Health 2019 Zoonoses R eport. EFSA Journal 19. https://doi.org/10.2903/j.efsa.2021.6406

Ewing, W.H., Tanner, K.E., Tatum, H.W., 1955. A new serotype of *Escherichia coli* associated with infantile diarrhea. *Pub. Health Rep,* 107–114.

Fairbrother, J.M., Nadeau, E., 2006. *Escherichia coli*: on-farm contamination of animals. *Rev Sci Tech* 25, 555–569.

Fakih, I., Thiry, D., Duprez, J.-N., Saulmont, M., Iguchi, A., Piérard, D., Jouant, L., Daube, G., Ogura, Y., Hayashi, T., Taminiau, B., Mainil, J.G., 2017. Identification of Shiga toxin-producing (STEC) and enteropathogenic (EPEC) *Escherichia coli* in diarrhoeic calves and comparative genomics of O5 bovine and human STEC. *Vet Microbiol* 202, 16–22. https://doi.org/10.1016/j.vetmic.2016.02.017

Falkow, S., 1988. Molecular Koch's postulates applied to microbial pathogenicity. *Rev Infect Dis 10 Suppl* 2, S274-276. https://doi.org/10.1093/cid/10.supplement_2.s274

Farooq, S., Hussain, I., Mir, M.A., Bhat, M.A., Wani, S.A., 2009. Isolation of at ypical enteropathogenic *Escherichia coli* and Shiga toxin 1 and 2f-producing *Esc herichia coli* from avian species in India. *Lett. Appl. Microbiol.* https://doi.org/10. 1111/j.1472-765X.2009.02594.x

Fierz, L., Cernela, N., Hauser, E., Nüesch-Inderbinen, M., Stephan, R., 2017. Ch aracteristics of Shigatoxin-producing *Escherichia coli* strains isolated during 2010-2014 from human infections in Switzerland. *Front Microbiol* 8, 1471. https://doi.o rg/10.3389/fmicb.2017.01471

Filipiak, M., Łoś, J.M., Łoś, M., 2020. Efficiency of induction of Shiga-toxin lambdoid prophages in *Escherichia coli* due to oxidative and antibiotic stress depends on the combination of prophage and the bacterial strain. *J Appl Genet* 61, 131–140. https://doi.org/10.1007/s13353-019-00525-8

Fischer Walker, C.L., Perin, J., Aryee, M.J., Boschi-Pinto, C., Black, R.E., 2012. Diarrhea incidence in low- and middle-income countries in 1990 and 2010: a systematic review. *BMC Public Health* 12, 220. https://doi.org/10.1186/1471-2458-12-220

Frank, C., Werber, D., Cramer, J.P., Askar, M., Faber, M., An Der Heiden, M., Bernard, H., Fruth, A., Prager, R., Spode, A., Wadl, M., Zoufaly, A., Jordan, S., Kemper, M.J., Follin, P., Müller, L., King, L.A., Rosner, B., Buchholz, U., Stark, K., Krause, G., 2011. Epidemic profile of Shiga toxin–producing *Escherichia coli* O104:H4 outbreak in Germany. *N Engl J Med* 365, 1771–1780. https://doi.org/10.1056/NEJMoa1106483

Frankel, G., Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J.B., Knutton, S., 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol Microbiol* 30, 911–921. https://doi.org/10.1046/j.1365-2958.1998.01144.x

Franzin, F.M., Sircili, M.P., 2015. Locus of Enterocyte Effacement: a pathogenicity island involved in the virulence of enteropathogenic and enterohemorragic *Escherichia coli* subjected to a complex network of gene regulation. *BioMed Research International* 2015, 1–10. https://doi.org/10.1155/2015/534738

Freedman, S.B., Xie, J., Neufeld, M.S., Hamilton, W.L., Hartling, L., Tarr, P.I., Alberta Provincial Pediatric Enteric Infection Team (APPETITE), Nettel-Aguirre, A., Chuck, A., Lee, B., Johnson, D., Currie, G., Talbot, J., Jiang, J., Dickinson, J., Kellner, J., MacDonald, J., Svenson, L., Chui, L., Louie, M., Lavoie, M., Eltorki, M., Vanderkooi, O., Tellier, R., Ali, S., Drews, S., Graham, T., Pang, X.-L., 2016. Shiga Toxin-Producing *Escherichia*

coli Infection, Antibiotics, and Risk of Developing Hemolytic Uremic Syndrome: A Meta-analysis. *Clin Infect Dis* 62, 1251–1258. https://doi.org/10.1093/cid/ciw099

Friesema, I., van der Zwaluw, K., Schuurman, T., Kooistra-Smid, M., Franz, E., van Duynhoven, Y., van Pelt, W., 2014. Emergence of *Escherichia coli* encoding Shiga toxin 2f in human Shiga toxin-producing *E. coli* (STEC) infections in the Netherlands, January 2008 to December 2011. *Euro Surveill* 19, 26–32.

Friesema, I.H.M., Keijzer-Veen, M.G., Koppejan, M., Schipper, H.S., van Griethuysen, A.J., Heck, M.E.O.C., van Pelt, W., 2015. Hemolytic uremic syndrome associated with *Escherichia coli* O8:H19 and Shiga toxin 2f gene. *Emerg Infect Dis* 21, 168–169. https://doi.org/10.3201/eid2101.140515

Frippiat, T., Paindaveine, C., Duprez, J.-N., Delguste, C., Mainil, J., Art, T., 2021. Evaluation of the bactericidal effect of nebulized silver nanoparticles on common respiratory bacteria in horses- in vitro studies. *J Equine Vet Sci* 103, 103635. https://doi.org/10.1016/j.jevs.2021.103635

Fruth, A., Lang, C., Größl, T., Garn, T., Flieger, A., 2024. Genomic surveillance of STEC/EHEC infections in Germany 2020 to 2022 permits insight into virulence gene profiles and novel O-antigen gene clusters. *Int J Med Microbiol* 314, 151610. https://doi.org/10.1016/j.ijmm.2024.151610

Fujii, J., Kinoshita, Y., Yutsudo, T., Taniguchi, H., Obrig, T., Yoshida, S.I., 2001. Toxicity of Shiga toxin 1 in the central nervous system of rabbits. *Infect Immun* 69, 6545–6548. https://doi.org/10.1128/IAI.69.10.6545-6548.2001

Fujisawa, T., Sata, S., Aikawa, K., Takahashi, T., Yamai, S., Shimada, T., 2000. Modification of sorbitol MacConkey medium containing cefixim and tellurite for isolation of *Escherichia coli* O157:H7 from radish sprouts. *Appl Environ Microbiol* 66, 3117–3118. https://doi.org/10.1128/AEM.66.7.3117-3118.2000

Furevi, A., Ståhle, J., Muheim, C., Gkotzis, S., Udekwu, K.I., Daley, D.O., Wid malm, G., 2020. Structural analysis of the O-antigen polysaccharide from *Escheri chia coli* O188. *Carbohydrate Research* 498, 108051. https://doi.org/10.1016/j.carr es.2020.108051

Gallegos, K.M., Conrady, D.G., Karve, S.S., Gunasekera, T.S., Herr, A.B., Weiss, A.A., 2012. Shiga toxin binding to glycolipids and glycans. *PLoS One* 7, e30368. https://doi.org/10.1371/journal.pone.0030368

Gally, D.L., Naylor, S.W., Low, J.C., Gunn, G.J., Synge, B.A., Pearce, M.C., Donachie, W., Besser, T.E., 2003. Colonisation site of *E. coli* O157 in cattle. *Vet Rec* 152, 307.

Gao, X., Wang, X., Pham, T.H., Feuerbacher, L.A., Lubos, M.-L., Huang, M., Olsen, R., Mushegian, A., Slawson, C., Hardwidge, P.R., 2013. NleB, a bacterial effector with glycosyltransferase activity, targets GAPDH function to inhibit NF-κB activation. *Cell Host Microbe* 13, 87–99. https://doi.org/10.1016/j.chom.2012.11.010

Garcia-Graells, C., Berbers, B., Verhaegen, B., Vanneste, K., Marchal, K., Roosens, N.H.C., Botteldoorn, N., De Keersmaecker, S.C.J., 2020. First detection of a plasmid-located carbapenem resistant *bla*VIM-1 gene in *E. coli* isolated from meat products at retail in Belgium in 2015. *Int J Food Microbiol* 324, 108624. https://doi.org/10.1016/j.ijfoodmicro.2020.108624

Gardner, S.N., Slezak, T., Hall, B.G., 2015. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. *Bioinformatics* 31, 2877–2878. https://doi.org/10.1093/bioinformatics/btv271

Gasser, C., Gautier, E., Steck, A., Siebenmann, R.E., Oechslin, R., 1955. [Hemolytic-uremic syndrome: bilateral necrosis of the renal cortex in acute acquired hemolytic anemia]. *Schweiz Med Wochenschr* 85, 905–909.

Gaytán, M.O., Martínez-Santos, V.I., Soto, E., González-Pedrajo, B., 2016. Type Three Secretion System in attaching and effacing pathogens. *Front Cell Infect Microbiol* 6, 129. https://doi.org/10.3389/fcimb.2016.00129

Gigliucci, F., van Hoek, A.H.A.M., Chiani, P., Knijn, A., Minelli, F., Scavia, G., Franz, E., Morabito, S., Michelacci, V., 2021. Genomic characterization of *hlyF*-positive Shiga toxin-producing *Escherichia coli*, Italy and the Netherlands, 2000-2019. *Emerg Infect Dis* 27, 853–861. https://doi.org/10.3201/eid2703.203110

Gill, A., Dussault, F., McMahon, T., Petronella, N., Wang, X., Cebelinski, E., Scheutz, F., Weedmark, K., Blais, B., Carrillo, C., 2022. Characterization of atypical Shiga toxin gene sequences and description of Stx2j, a new subtype. *J Clin Microbiol* 60, e0222921. https://doi.org/10.1128/jcm.02229-21

Gilmour, M.W., Thomson, N.R., Sanders, M., Parkhill, J., Taylor, D.E., 2004. The complete nucleotide sequence of the resistance plasmid R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. *Plasmid* 52, 182–202. https://doi.org/10.1016/j.plasmid.2004.06.006

Girard, F., Dziva, F., van Diemen, P., Phillips, A.D., Stevens, M.P., Frankel, G., 2007. Adherence of enterohemorrhagic *Escherichia coli* O157, O26, and O111 strains to bovine intestinal explants ex vivo. *Appl Environ Microbiol* 73, 3084–3090. https://doi.org/10.1128/AEM.02893-06

Girón, J.A., Ho, A.S., Schoolnik, G.K., 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science* 254, 710–713. https://doi.org/10.1126/science.1683004

Gobin, M., Hawker, J., Cleary, P., Inns, T., Gardiner, D., Mikhail, A., McCormick, J., Elson, R., Ready, D., Dallman, T., Roddick, I., Hall, I., Willis, C., Crook, P., Godbole, G., Tubin-Delic, D., Oliver, I., 2018. National outbreak of Shiga toxin-producing *Escherichia coli* O157:H7 linked to mixed salad leaves, United Kingdom, 2016. *Euro Surveill* 23, 17–00197. https://doi.org/10.2807/1560-7917.ES.2018.23.18.17-00197

Gomes, T.A.T., Hernandes, R.T., Torres, A.G., Salvador, F.A., Guth, B.E.C., Vaz, T.M.I., Irino, K., Silva, R.M., Vieira, M.A.M., 2011. Adhesin-encoding genes fro m Shiga toxin-producing *Escherichia coli* are more prevalent in atypical than in typical enteropathogenic *E. coli*. *J Clin Microbiol* 49, 3334–3337. https://doi.org/10.1128/JCM.00779-11

Goosney, D.L., de Grado, M., Finlay, B.B., 1999. Putting *E. coli* on a pedestal: a unique system to study signal transduction and the actin cytoskeleton. *Trends Cell Biol* 9, 11–14. https://doi.org/10.1016/s0962-8924(98)01418-4

Gouali, M., Ruckly, C., Carle, I., Lejay-Collin, M., Weill, F.-X., 2013. Evaluation of CHROMagar STEC and STEC O104 chromogenic agar media for detection of Shiga toxin-producing *Escherichia coli* in stool specimens. *J Clin Microbiol* 51, 894–900. https://doi.org/10.1128/JCM.03121-12

Goullet, P., Picard, B., 1989. Comparative electrophoretic polymorphism of estera ses and other enzymes in *Escherichia coli*. *J Gen Microbiol* 135, 135–143. https: //doi.org/10.1099/00221287-135-1-135

Grande, L., Michelacci, V., Bondì, R., Gigliucci, F., Franz, E., Badouei, M.A., S chlager, S., Minelli, F., Tozzoli, R., Caprioli, A., Morabito, S., 2016. Whole-Gen ome characterization and strain comparison of VT2f-producing *Escherichia coli* C ausing Hemolytic Uremic Syndrome. *Emerg Infect Dis* 22, 2078–2086. https://doi. org/10.3201/eid2212.160017

Griffin, P.M., Olmstead, L.C., Petras, R.E., 1990. *Escherichia coli* O157:H7-associated colitis. A clinical and histological study of 11 cases. *Gastroenterology* 99, 142–149. https://doi.org/10.1016/0016-5085(90)91241-w

Habets, A., Antoine, C., Wagemans, J., Vermeersch, M., Laforêt, F., Diderich, J., Lavigne, R., Mainil, J., Thiry, D., 2022. Impact of Shiga-toxin encoding gene transduction from O80:H2 Shiga toxigenic *Escherichia coli* (STEC) on non-STEC strains. *Sci Rep* 12, 21587. https://doi.org/10.1038/s41598-022-26198-8

Habets, A., Crombé, F., Nakamura, K., Guérin, V., De Rauw, K., Piérard, D., Saulmont, M., Hayashi, T., Mainil, J.G., Thiry, D., 2021. Genetic characterization of Shigatoxigenic and enteropathogenic *Escherichia coli* O80:H2 from diarrhoeic and septicaemic calves and relatedness to human Shigatoxigenic *E. coli* O80:H2. *J. Appl. Microbiol*. 130, 258–264. https://doi.org/10.1111/jam.14759

Habets, A., Engelen, F., Duprez, J.-N., Devleesschauwer, B., Heyndrickx, M., De Zutter, L., Thiry, D., Cox, E., Mainil, J., 2020. Identification of Shigatoxigenic a nd enteropathogenic *Escherichia coli* serotypes in healthy young dairy calves in Belgium by Recto-Anal Mucosal Swabbing. *Vet Sci* 7, 167. https://doi.org/10.3390 /vetsci7040167

Hale, T.L., Oaks, E.V., Formal, S.B., 1985. Identification and antigenic characterization of virulence-associated, plasmid-coded proteins of *Shigella* spp. and enteroinvasive *Escherichia coli*. Infect Immun 50, 620–629. https://doi.org/10.1128/iai.50.3.620-629.1985

Hall, G., Kurosawa, S., Stearns-Kurosawa, D., 2017. Shiga toxin therapeutics: Beyond neutralization. *Toxins* 9, 291. https://doi.org/10.3390/toxins9090291

Hamabata, T., Sato, T., Takita, E., Matsui, T., Kawabata, T., Imaoka, T., Nakanishi, N., Tsukahara, T., Sawada, K., 2021. Shiga toxin 2eB-transgenic lettuce vaccine: N-glycosylation is important for protecting against porcine edema disease. *J Vet Med Sci* 83, 1708–1714. https://doi.org/10.1292/jvms.21-0240

Hambali, K.U., Eilu, E., Kumar, S., Afolabi, A.O., Tijani, N.A., Faseun, Y.O., O doki, M., Gechemba Mokaya, C., Makeri, D., Jakheng, S.P.E., Sankarapandian, V., Adeyemo, R.O., Adegboyega, T.T., Adebayo, I.A., Ntulume, I., Akinola, S.A., 2 024. Monitoring multi-drug resistant *Klebsiella pneumoniae* in kitagata hot spring, southwestern Uganda: A public health implication. *Infect Drug Resist* 17, 3325–3341. https://doi.org/10.2147/IDR.S472998

Harris, S.R., Feil, E.J., Holden, M.T.G., Quail, M.A., Nickerson, E.K., Chantratit a, N., Gardete, S., Tavares, A., Day, N., Lindsay, J.A., Edgeworth, J.D., De Len castre, H., Parkhill, J., Peacock, S.J., Bentley, S.D., 2010. Evolution of MRSA d uring hospital transmission and intercontinental spread. *Science* 327, 469–474. htt ps://doi.org/10.1126/science.1182395

Havelaar, A.H., Kirk, M.D., Torgerson, P.R., Gibb, H.J., Hald, T., Lake, R.J., Pra et, N., Bellinger, D.C., De Silva, N.R., Gargouri, N., Speybroeck, N., Cawthorne, A., Mathers, C., Stein, C., Angulo, F.J., Devleesschauwer, B., on behalf of Worl d Health Organization Foodborne Disease Burden Epidemiology Reference Group, 2015. World Health Organization global estimates and regional comparisons of th e burden of foodborne disease in 2010. *PLoS Med* 12, e1001923. https://doi.org/1 0.1371/journal.pmed.1001923

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C.G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M., Shinagawa, H., 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res* 8, 11–22. https://doi.org/10.1093/dnares/8.1.11

Helgerson, A.F., Sharma, V., Dow, A.M., Schroeder, R., Post, K., Cornick, N.A., 2006. Edema disease caused by a clone of *Escherichia coli* O147. *J Clin Microbiol* 44, 3074–3077. https://doi.org/10.1128/JCM.00617-06

Hernandes, R.T., Elias, W.P., Vieira, M.A.M., Gomes, T.A.T., 2009. An overview of atypical enteropathogenic *Escherichia coli*. *FEMS Microbiol Lett* 297, 137–149. https://doi.org/10.1111/j.1574-6968.2009.01664.x

Herold, S., Karch, H., Schmidt, H., 2004. Shiga toxin-encoding bacteriophages – genomes in motion. *Int. J. Med. Microbiol.* 294, 115–121. https://doi.org/10.1016/j.ijmm.2004.06.023

Hinenoya, A., 2021. [Molecular epidemiology of *Escherichia albertii*, emerging z oonotic enteropathogen]. *Nihon Saikingaku Zasshi* 76, 175–185. https://doi.org/10.3412/jsb.76.175

Hiramatsu, R., Matsumoto, M., Miwa, Y., Suzuki, Y., Saito, M., Miyazaki, Y., 2002. Characterization of Shiga toxin-producing *Escherichia coli* O26 strains and establishment of selective isolation media for these strains. *J Clin Microbiol* 40, 922–925. https://doi.org/10.1128/JCM.40.3.922-925.2002

Hirvonen, J.J., Siitonen, A., Kaukoranta, S.-S., 2012. Usability and performance of CHROMagar STEC medium in detection of Shiga toxin-producing *Escherichia coli* strains. *J Clin Microbiol 5*0, 3586–3590. https://doi.org/10.1128/JCM.01754-12

Hritzo, B.A., Michalski, J.M., Sikorski, M.J., Rasko, D.A., 2025. Complete genomes of hybrid *Escherichia coli* isolates containing virulence factors from enterotoxigenic and enteropathogenic pathovars. Microbiol Resour Announc 14, e00959-25. https://doi.org/10.1128/mra.00959-25

Hoey, D.E.E., Currie, C., Else, R.W., Nutikka, A., Lingwood, C.A., Gally, D.L., Smith, D.G.E., 2002. Expression of receptors for verotoxin 1 from *Escherichia c oli* O157 on bovine intestinal epithelium. *J Med Microbiol* 51, 143–149. https://doi.org/10.1099/0022-1317-51-2-143

Hofer, E., Cernela, N., Stephan, R., 2012. Shiga Toxin Subtypes Associated with Shiga toxin–producing *Escherichia coli* strains isolated from red deer, roe deer, chamois, and ibex. *Foodborne Pathog. Dis.*9, 792–795. https://doi.org/10.1089/fpd.2012.1156

Hughes, A.C., Zhang, Y., Bai, X., Xiong, Y., Wang, Y., Yang, X., Xu, Q., He, X., 2019. Structural and functional characterization of Stx2k, a new subtype of Shiga toxin 2. *Microorganisms* 8, 4. https://doi.org/10.3390/microorganisms8010004

Ibarra, C., Amaral, M.M., Palermo, M.S., 2013. Advances in pathogenesis and therapy of hemolytic uremic syndrome caused by Shiga toxin-2. *IUBMB Life* 65, 827–835. https://doi.org/10.1002/iub.1206

Iguchi, A., Iyoda, S., Seto, K., Morita-Ishihara, T., Scheutz, F., Ohnishi, M., Pathogenic *E. coli* Working Group in Japan, 2015. *Escherichia coli* O-genotyping PCR: a comprehensive and practical platform for molecular O serogrouping. *J Clin Microbiol* 53, 2427–2432. https://doi.org/10.1128/JCM.00321-15

Iizumi, Y., Sagara, H., Kabe, Y., Azuma, M., Kume, K., Ogawa, M., Nagai, T., Gillespie, P.G., Sasakawa, C., Handa, H., 2007. The enteropathogenic *E. coli* effector EspB facilitates microvillus effacing and antiphagocytosis by inhibiting myosin function. *Cell Host & Microbe* 2, 383–392. https://doi.org/10.1016/j.chom.2007.09.012

Ikeda, K., Ida, O., Kimoto, K., Takatorige, T., Nakanishi, N., Tatara, K., 1999. Effect of early fosfomycin treatment on prevention of hemolytic uremic syndrome accompanying *Escherichia coli* O157:H7 infection. Clin Nephrol 52, 357–362.

Ikeda, R., Laforêt, F., Antoine, C., Adachi, M., Nakamura, K., Habets, A., Kler, C., De Rauw, K., Hayashi, T., Mainil, J.G., Thiry, D., 2023a. Virulence of Shigatoxigenic and enteropathogenic *Escherichia coli* O80:H2 in *Galleria mellonella* larvae: comparison of the roles of the pS88 plasmids and STX2d Phage. *Vet Sci* 10, 420. https://doi.org/10.3390/vetsci10070420

Ikeda, R., Nakamura, K., Saulmont, M., Habets, A., Duprez, J.-N., Korsak, N., Hayashi, T., Thiry, D., Mainil, J.G., 2023b. *Escherichia coli* O80 in healthy cattle: Absence of Shigatoxigenic and enteropathogenic *E. coli* O80:H2 and (phylo) genomics of non-clonal complex 165 *E. coli* O80. *Microorganisms* 11, 230. https://doi.org/10.3390/microorganisms11020230

Ikeda, R., Nakamura, K., Korsak, N., Duprez, J.-N., Hayashi, T., Thiry, D., Mainil, J.G., 2025. Non-melibiose fermentation and tellurite resistance by Shigatoxigenic and enteropathogenic *Escherichia coli* O80:H2 from diseased calves: comparison with human Shigatoxigenic *E. coli* O80:H2. *Vet Sci* 12, 274. https://doi.org/10.3390/vetsci12030274

Imlay, J.A., 2003. Pathways of oxidative damage. *Annu Rev Microbiol* 57, 395–418. https://doi.org/10.1146/annurev.micro.57.030502.090938

Ingelbeen, B., Bruyand, M., Mariani-Kurkjian, P., Le Hello, S., Danis, K., Somm en, C., Bonacorsi, S., de Valk, H., 2018. Emerging Shiga-toxin-producing *Escherichia coli* serogroup O80 associated hemolytic and uremic syndrome in France, 2013-2016: Differences with other serogroups. *PLoS One* 13, e0207492. https://doi.org/10.1371/journal.pone.0207492

InVS (Institut de Veille Sanitaire). 2007. Epidémie d'infections à *E. coli* producteurs de Shiga-toxines non O157, liée à la consommation de camembert au lait cru, nord-ouest de la France, octobre-décembre 2005. Rapport d'investigation rédigé par l'Institut de veille sanitaire.https://www.santepubliquefrance.fr/regions/normandie/documents/rapport-synthese/2007/epidemie-d-infections-a-e.-coli-producteurs-de-shiga-toxines-non-0157-liee-a-la-consommation-de-camembert-au-lait-cru-nord-ouest-de-la-france-oct_

Ito, H., Terai, A., Kurazono, H., Takeda, Y., Nishibuchi, M., 1990. Cloning and nucleotide sequencing of Vero toxin 2 variant genes from *Escherichia coli* O91:H21 isolated from a patient with the hemolytic uremic syndrome. *Microb Pathog* 8, 47–60. https://doi.org/10.1016/0882-4010(90)90007-d

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B.B., Beeregowda, K.N., 2014. Toxicity, mechanism and health effects of some heavy metals. *Interdiscip Toxicol* 7, 60–72. https://doi.org/10.2478/intox-2014-0009

Jenkins, C., Perry, N.T., Godbole, G., Gharbia, S., 2020. Evaluation of chromoge nic selective agar (CHROMagar STEC) for the direct detection of Shiga toxin-pr oducing *Escherichia coli* from faecal specimens. *J Med Microbiol* 69, 487–491. h ttps://doi.org/10.1099/jmm.0.001136

Jensen, C.O., 1893. Ueber die Kälberruhr und deren Aetiologie. Mh.Tierheilk. 97–124.

Jerse, A.E., Yu, J., Tall, B.D., Kaper, J.B., 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci USA* 87, 7839–7843. https://doi.org/10.1073/pnas.87.20.7839

Jobling, M.G., Ritchie, D.A., 1988. The nucleotide sequence of a plasmid determinant for resistance to tellurium anions. *Gene* 66, 245–258. https://doi.org/10.1016/0378-1119(88)90361-7

Jobling, M.G., Ritchie, D.A., 1987. Genetic and physical analysis of plasmid genes expressing inducible resistance of tellurite in *Escherichia coli*. *Mol Gen Genet* 208, 288–293. https://doi.org/10.1007/BF00330455

Joensen, K.G., Tetzschner, A.M.M., Iguchi, A., Aarestrup, F.M., Scheutz, F., 2015. Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. *J Clin Microbiol* 53, 2410–2426. https://doi.org/10.1128/JCM.00008-15

Johnson, J.R., 1991. Virulence factors in *Escherichia coli* urinary tract infection. *Clin Microbiol Rev* 4, 80–128. https://doi.org/10.1128/CMR.4.1.80

Johnson, J.R., Stell, A.L., Scheutz, F., O'Bryan, T.T., Russo, T.A., Carlino, U.B., Fasching, C., Kavle, J., Van Dijk, L., Gaastra, W., 2000. Analysis of the F antig en-specific *papA* alleles of extraintestinal pathogenic *Escherichia coli* using a nov el multiplex PCR-based assay. *Infect Immun* 68, 1587–1599. https://doi.org/10.112 8/IAI.68.3.1587-1599.2000

Johnson, T.J., Giddings, C.W., Horne, S.M., Gibbs, P.S., Wooley, R.E., Skyberg, J., Olah, P., Kercher, R., Sherwood, J.S., Foley, S.L., Nolan, L.K., 2002. Locatio n of increased serum survival gene and selected virulence traits on a conjugative R plasmid in an avian *Escherichia coli* isolate. *Avian Dis* 46, 342–352. https://do i.org/10.1637/0005-2086(2002)046[0342:LOISSG]2.0.CO;2

Johnson, T.J., Johnson, S.J., Nolan, L.K., 2006a. Complete DNA sequence of a ColBM plasmid from avian pathogenic *Escherichia coli* suggests that it evolved f rom closely related ColV virulence plasmids. *J Bacteriol* 188, 5975–5983. https:// doi.org/10.1128/JB.00204-06

Johnson, T.J., Siek, K.E., Johnson, S.J., Nolan, L.K., 2006b. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. *J Bacteriol* 188, 745–758. https://doi.org/10.1128/J B.188.2.745-758.2006

Johnson, W.M., Pollard, D.R., Lior, H., Tyler, S.D., Rozee, K.R., 1990. Differentiation of genes coding for *Escherichia coli* verotoxin 2 and the verotoxin associated with porcine edema disease (VTe) by the polymerase chain reaction. *J Clin Microbiol* 28, 2351–2353. https://doi.org/10.1128/jcm.28.10.2351-2353.1990

Kaname, S., Ong, M.-L., Mathias, J., Gatta, F., Law, L., Wang, Y., 2024. Outco mes in patients with thrombotic microangiopathy associated with a trigger followi ng plasma exchange: A systematic literature review. *Transfus Apher Sci* 64, 1040 48. https://doi.org/10.1016/j.transci.2024.104048

Kaper, J.B., 1996. Defining EPEC. *Rev Microbiol* 27, 130–133.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2, 123–140. https://doi.org/10.1038/nrmicro818

Karmali, M.A., 1989. Infection by verocytotoxin-producing *Escherichia coli*. *Clin Microbiol Rev* 2, 15–38. https://doi.org/10.1128/CMR.2.1.15

Karmali, M.A., Mascarenhas, M., Shen, S., Ziebell, K., Johnson, S., Reid-Smith, R., Isaac-Renton, J., Clark, C., Rahn, K., Kaper, J.B., 2003. Association of geno

mic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Esc herichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J Clin Microbiol* 41, 4930–4940. https://doi.org/10.1128/JCM.41.11.4930-4940.2003

Karmali, M.A., Petric, M., Lim, C., Fleming, P.C., Arbus, G.S., Lior, H., 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. *J Infect Dis* 151, 775–782. https://doi.org/10.1093/infdis/151.5.775

Kauffmann, F., 1947. The serology of the *coli* group. *J Immunol* 57, 71–100.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D.J., Frey, E.A., Finlay, B.B., 1997. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91, 511–520. https://doi.org/10.1016/s0092-8674(00)80437-7

Kenny, B., Jepson, M., 2000. Targeting of an enteropathogenic *Escherichia coli* (EPEC) effector protein to host mitochondria. *Cell Microbiol* 2, 579–590. https://doi.org/10.1046/j.1462-5822.2000.00082.x

Kenny, B., Lai, L.C., Finlay, B.B., Donnenberg, M.S., 1996. EspA, a protein secreted by enteropathogenic *Escherichia coli*, is required to induce signals in epithelial cells. *Mol Microbiol* 20, 313–323. https://doi.org/10.1111/j.1365-2958.1996.tb02619.x

Kerangart, S., Douëllou, T., Delannoy, S., Fach, P., Beutin, L., Sergentet-Thévenot, D., Cournoyer, B., Loukiadis, E., 2016. Variable tellurite resistance profiles of clinically-relevant Shiga toxin-producing *Escherichia coli* (STEC) influence their recovery from foodstuffs. *Food Microbiol* 59, 32–42. https://doi.org/10.1016/j.fm.2016.05.005

Kindle, P., Nüesch-Inderbinen, M., Cernela, N., Stephan, R., 2019a. Detection, Isolation, and Characterization of Shiga Toxin-Producing *Escherichia coli* in Flour. *J Food Prot* 82, 164–167. https://doi.org/10.4315/0362-028X.JFP-18-256

Kindle, P., Zurfluh, K., Nüesch-Inderbinen, M., Von Ah, S., Sidler, X., Stephan, R., Kümmerlen, D., 2019b. Phenotypic and genotypic characteristics of *Escherichia coli* with non-susceptibility to quinolones isolated from environmental samples on pig farms. *Porc Health Manag* 5, 9. https://doi.org/10.1186/s40813-019-0116-y

Kinnula, S., Hemminki, K., Kotilainen, H., Ruotsalainen, E., Tarkka, E., Salmenlinna, S., Hallanvuo, S., Leinonen, E., Jukka, O., Rimhanen-Finne, R., 2018. Outbreak of multiple strains of non-O157 Shiga toxin-producing and enteropathogenic *Escherichia coli* associated with rocket salad, Finland, autumn 2016. *Euro Surveill* 23, 1700666. https://doi.org/10.2807/1560-7917.ES.2018.23.35.1700666

Knutton, S., Baldwin, T., Williams, P.H., McNeish, A.S., 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun* 57, 1290–1298. https://doi.org/10.1128/iai.57.4.1290-1298.1989

Koch, R., 2018. Die Ätiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte des Bacillus Anthracis (1876), in: Robert Koch, Klassische Texte Der Wissenschaft. Springer Berlin Heidelberg, Berlin, *Heidelberg*, pp. 19–43. https://doi.org/10.1007/978-3-662-56454-7_2

Koch, R., 1882. Die Ätiologie der Tuberkulose. Berliner klinishe Wochenschrift. 1882, 15, 221-230. https://doi.org/10.25646/5088

Konowalchuk, J., Speirs, J.I., Stavric, S., 1977. Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun* 18, 775–779. https://doi.org/10.1128/iai.18.3.775-779.1977

Krause, M., Barth, H., Schmidt, H., 2018. Toxins of locus of enterocyte effacement-negative Shiga toxin-producing *Escherichia coli*. *Toxins (Basel)* 10, 241. https://doi.org/10.3390/toxins10060241

Krüger, A., Lucchesi, P.M.A., 2015. Shiga toxins and *stx* phages: highly diverse entities. *Microbiology* 161, 451–462. https://doi.org/10.1099/mic.0.000003

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galán, J.E., Aizawa, S.I., 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* 280, 602–605. https://doi.org/10.1126/science.280.5363.602

Kusumoto, M., Fukamizu, D., Ogura, Y., Yoshida, E., Yamamoto, F., Iwata, T., Ooka, T., Akiba, M., Hayashi, T., 2014. Lineage-specific distribution of insertion sequence excision enhancer in enterotoxigenic *Escherichia coli* isolated from swine. *Appl Environ Microbiol* 80, 1394–1402. https://doi.org/10.1128/AEM.03696-13

Kyle, J.L., Cummings, C.A., Parker, C.T., Quiñones, B., Vatta, P., Newton, E., Huynh, S., Swimley, M., Degoricija, L., Barker, M., Fontanoz, S., Nguyen, K., Patel, R., Fang, R., Tebbs, R., Petrauskene, O., Furtado, M., Mandrell, R.E., 2012. *Escherichia coli* serotype O55:H7 diversity supports parallel acquisition of bacteriophage at Shiga toxin phage insertion sites during evolution of the O157:H7 lineage. *J Bacteriol* 194, 1885–1896. https://doi.org/10.1128/JB.00120-12

Lacher, D.W., Gangiredla, J., Patel, I., Elkins, C.A., Feng, P.C.H., 2016. Use of the *Escherichia coli* identification microarray for characterizing the health risks of Shiga toxin-producing *Escherichia coli* isolated from foods. *J Food Prot* 79, 1656–1662. https://doi.org/10.4315/0362-028X.JFP-16-176

Laidler, M.R., Tourdjman, M., Buser, G.L., Hostetler, T., Repp, K.K., Leman, R., Samadpour, M., Keene, W.E., 2013. *Escherichia coli* O157:H7 infections associated with consumption of locally grown strawberries contaminated by deer. *Clin Infect Dis* 57, 1129–1134. https://doi.org/10.1093/cid/cit468

Lampel, K.A., Formal†, S.B., Maurelli, A.T., 2018. A brief history of *Shigella*. *EcoSal Plus* 8, 10.1128/ecosalplus.ESP-0006–2017. https://doi.org/10.1128/ecosalplus.esp-0006-2017

Landy, A., Ross, W., 1977. Viral integration and excision: structure of the lambda *att* sites. *Science* 197, 1147–1160. https://doi.org/10.1126/science.331474

Lang, C., Hiller, M., Konrad, R., Fruth, A., Flieger, A., 2019. Whole-genome-based public health surveillance of less common Shiga toxin-producing *Escherichia coli* serovars and untypeable strains identifies four novel O genotypes. *J Clin Microbiol* 57, e00768-19. https://doi.org/10.1128/JCM.00768-19

Lee, J.B., Kim, S.K., Yoon, J.W., 2022. Pathophysiology of enteropathogenic *Esc herichia coli* during a host infection. *J Vet Sci* 23, e28. https://doi.org/10.4142/jvs. 21160

Lemaître, C., Bidet, P., Bingen, E., Bonacorsi, S., 2012. Transcriptional analysis of the *Escherichia coli* ColV-Ia plasmid pS88 during growth in human serum and urine. *BMC Microbiol* 12, 115. https://doi.org/10.1186/1471-2180-12-115

Lesage, A., 1897. Contribution à l'étude des entérites infantiles—sérodiagnostic des races de *Bacterium coli*. *Comptes Rendues des Societe Biologique (Paris)* 49, 900–1.

Leuko, S., Raivio, T.L., 2012. Mutations that impact the enteropathogenic *Escherichia coli* Cpx envelope stress response attenuate virulence in *Galleria mellonella*. *Infect Immun* 80, 3077–3085. https://doi.org/10.1128/IAI.00081-12

Levine, MyronM., Nalin, DavidR., Hornick, RichardB., Bergquist, ErickJ., Waterman, DanielH., Young, CharlesR., Sotman, S., Rowe, B., 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *The Lancet* 311, 1119–1122. https://doi.org/10.1016/S0140-6736(78)90299-4

Lewis, D.I., 2019. Animal experimentation: implementation and application of the 3Rs. *Emerg Top Life Sci* 3, 675–679. https://doi.org/10.1042/ETLS20190061

Lewis, G.L., Jorgensen, Q.R., Loy, J.D., Moxley, R.A., 2018. Tellurite resistance in Shiga toxin-producing *Escherichia coli*. *Curr Microbiol* 75, 752–759. https://do i.org/10.1007/s00284-018-1444-x

Lindsey, R.L., Prasad, A., Feldgarden, M., Gonzalez-Escalona, N., Kapsak, C., Klimke, W., Melton-Celsa, A., Smith, P., Souvorov, A., Truong, J., Scheutz, F., 2023. Identification and characterization of ten *Escherichia coli* strains encoding novel Shiga toxin 2 subtypes, Stx2n as well as Stx2j, Stx2m, and Stx2o, in the United States. *Microorganisms* 11, 2561. https://doi.org/10.3390/microorganisms11102561

Ling, J., Pan, H., Gao, Q., Xiong, L., Zhou, Y., Zhang, D., Gao, S., Liu, X., 20 13. Aerobactin synthesis genes *iucA* and *iucC* contribute to the pathogenicity of

avian pathogenic *Escherichia coli* O2 strain E058. *PLoS One* 8, e57794. https://doi.org/10.1371/journal.pone.0057794

Lingwood, C.A., 1996. Role of verotoxin receptors in pathogenesis. *Trends Microbiol* 4, 147–153. https://doi.org/10.1016/0966-842x(96)10017-2

Loukiadis, E., Nobe, R., Herold, S., Tramuta, C., Ogura, Y., Ooka, T., Morabito, S., Kérourédan, M., Brugère, H., Schmidt, H., Hayashi, T., Oswald, E., 2008. Distribution, functional expression, and genetic organization of Cif, a phage-encoded type III-secreted effector from enteropathogenic and enterohemorrhagic *Escherichia coli*. *J Bacteriol* 190, 275–285. https://doi.org/10.1128/JB.00844-07

Lucchesi, P.M.A., Krüger, A., Parma, A.E., 2006. Distribution of *saa* gene variants in verocytotoxigenic *Escherichia coli* isolated from cattle and food. *Res Microbiol* 157, 263–266. https://doi.org/10.1016/j.resmic.2005.07.002

Luna-Gierke, R.E., Griffin, P.M., Gould, L.H., Herman, K., Bopp, C.A., Strockbine, N., Mody, R.K., 2014. Outbreaks of non-O157 Shiga toxin-producing *Escherichia coli* infection: USA. *Epidemiol Infect* 142, 2270–2280. https://doi.org/10.1017/S0950268813003233

Magalhães, C.A., Rossato, S.S., Barbosa, A.S., Santos, T.O. dos, Elias, W.P., Sircili, M.P., Piazza, R.M.F., 2011. The ability of haemolysins expressed by atypical enteropathogenic *Escherichia coli* to bind to extracellular matrix components. *Mem Inst Oswaldo Cruz* 106, 146–152. https://doi.org/10.1590/s0074-02762011000200005

Mainil, G.J., Fairbrother, M.J., 2014. Pathogenic *Escherichia coli* in domestic Mammals and Birds, in: *Pahogenic* Escherichia Coli: *Molecular and Cellular Microbiology*. Caister Academic Press, Norfolk, UK, pp. 19–43.

Mainil, J., 2013. *Escherichia coli* virulence factors. *Vet Immunol Immunopathol* 152, 2–12. https://doi.org/10.1016/j.vetimm.2012.09.032

Mainil, J.G., Daube, G., 2005. Verotoxigenic *Escherichia coli* from animals, humans and foods: who's who? *J Appl Microbiol* 98, 1332–1344. https://doi.org/10.1111/j.1365-2672.2005.02653.x

Mainil, J.G., Nakamura, K., Ikeda, R., Crombé, F., Diderich, J., Saulmont, M., Piérard, D., Thiry, D., Hayashi, T., 2025. Emerging hybrid shigatoxigenic and enteropathogenic *Escherichia coli* serotype O80:H2 in humans and calves. *Clin Microbiol Rev* e0001125. https://doi.org/10.1128/cmr.00011-25

Makino, S., Asakura, H., Shirahata, T., Ikeda, T., Takeshi, K., Arai, K., Nagasawa, M., Abe, T., Sadamoto, T., 1999. Molecular epidemiological study of a mass outbreak caused by enteropathogenic *Escherichia coli* O157:H45. *Microbiology and Immunology* 43, 381–384. https://doi.org/10.1111/j.1348-0421.1999.tb02419.x

Mariani-Kurkdjian, P., Lemaître, C., Bidet, P., Perez, D., Boggini, L., Kwon, T., Bonacorsi, S., 2014. Haemolytic-uraemic syndrome with bacteraemia caused by a new hybrid *Escherichia coli* pathotype. *New Microbes New Infect* 2, 127–131. https://doi.org/10.1002/nmi2.49

McCabe, E., Burgess, C.M., Lawal, D., Whyte, P., Duffy, G., 2019. An investigation of shedding and super-shedding of Shiga toxigenic *Escherichia coli* O157 and *E. coli* O26 in cattle presented for slaughter in the Republic of Ireland. *Zoonoses Public Health* 66, 83–91. https://doi.org/10.1111/zph.12531

McDaniel, T.K., Jarvis, K.G., Donnenberg, M.S., Kaper, J.B., 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci U S A* 92, 1664–1668. https://doi.org/10.1073/pnas.92.5.1664

McDougall, F., Gordon, D., Robins-Browne, R., Bennett-Wood, V., Boardman, W. S.J., Graham, P.L., Power, M., 2023. Characterisation of typical enteropathogenic *Escherichia coli* (tEPEC) lineages and novel *bfpA* variants detected in Australian fruit bats (*Pteropus poliocephalus*). *Sci Total Environ* 902, 166336. https://doi.org/10.1016/j.scitotenv.2023.166336

McGann, P., Snesrud, E., Maybank, R., Corey, B., Ong, A.C., Clifford, R., Hinkle, M., Whitman, T., Lesho, E., Schaecher, K.E., 2016. *Escherichia coli* harboring *mcr-1* and $bla_{\text{CTX-M}}$ on a novel IncF plasmid: first report of *mcr-1* in the United States. *Antimicrob Agents Chemother* 60, 4420–4421. https://doi.org/10.1128/AAC.01103-16

McMahon, T., Clarke, S., Deschênes, M., Tapp, K., Blais, B., Gill, A., 2024. Real-time PCR primers and probes for the detection of Shiga toxin genes, including novel subtypes. *Int J Food Microbiol* 419, 110744. https://doi.org/10.1016/j.ijfoodmicro.2024.110744

Mellata, M., Dho-Moulin, M., Dozois, C.M., Curtiss, R., Brown, P.K., Arné, P., Brée, A., Desautels, C., Fairbrother, J.M., 2003. Role of virulence factors in resistance of avian pathogenic *Escherichia coli* to serum and in pathogenicity. *Infect Immun* 71, 536–540. https://doi.org/10.1128/IAI.71.1.536-540.2003

Mellies, J.L., Elliott, S.J., Sperandio, V., Donnenberg, M.S., Kaper, J.B., 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Mol Microbiol* 33, 296–306. https://doi.org/10.1046/j.1365-2958.1999.01473.x

Mellmann, A., Harmsen, D., Cummings, C.A., Zentz, E.B., Leopold, S.R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S.F., Henkhaus, J.K., Leopold, B., Bielaszewska, M., Prager, R., Brzoska, P.M., Moore, R.L., Gue nther, S., Rothberg, J.M., Karch, H., 2011. Prospective genomic characterization o f the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid nex t generation sequencing technology. *PLoS One* 6, e22751. https://doi.org/10.1371/j ournal.pone.0022751

Melton-Celsa, A.R., 2014. Shiga Toxin (Stx) Classification, Structure, and Function. *Microbiol Spectr* 2, EHEC-0024-2013. https://doi.org/10.1128/microbiolspec.EHEC-0024-2013

Melton-Celsa, A.R., O'Brien, A.D., Feng, P.C.H., 2015. Virulence Potential of Activatable Shiga Toxin 2d-Producing *Escherichia coli* Isolates from Fresh Produce. J Food Prot 78, 2085–2088. https://doi.org/10.4315/0362-028X.JFP-15-180

Meng, Q., Bai, Xiangning, Zhao, A., Lan, R., Du, H., Wang, T., Shi, C., Yuan, X., Bai, Xuemei, Ji, S., Jin, D., Yu, B., Wang, Y., Sun, H., Liu, K., Xu, J., Xiong, Y., 2014. Characterization of Shiga toxin-producing *Escherichia coli* isolated from healthy pigs in China. *BMC Microbiol* 14, 5. https://doi.org/10.1186/1471-2180-14-5

Menge, C., 2020a. Molecular biology of *Escherichia Coli* Shiga toxins' effects on mammalian cells. *Toxins (Basel)* 12, 345. https://doi.org/10.3390/toxins12050345

Menge, C., 2020b. The role of *Escherichia coli* Shiga toxins in STEC colonization of Cattle. *Toxins (Basel)* 12, 607. https://doi.org/10.3390/toxins12090607

Mercado, E.C., Gioffré, A., Rodríguez, S.M., Cataldi, A., Irino, K., Elizondo, A.M., Cipolla, A.L., Romano, M.I., Malena, R., Méndez, M.A., 2004. Non-O157 Shiga toxin-producing *Escherichia coli* isolated from diarrhoeic calves in Argentina. *J Vet Med B Infect Dis Vet Public Health* 51, 82–88. https://doi.org/10.1111/j.1439-0450.2004.00729.x

Meredith, T.C., Woodard, R.W., 2006. Characterization of *Escherichia coli* D-arabinose 5-phosphate isomerase encoded by *kpsF*: implications for group 2 capsule biosynthesis. *Biochem J* 395, 427–432. https://doi.org/10.1042/BJ20051828

Miko, A., Pries, K., Haby, S., Steege, K., Albrecht, N., Krause, G., Beutin, L., 2009. Assessment of Shiga toxin-producing *Escherichia coli* isolates from wildlife meat as potential pathogens for humans. *Appl Environ Microbiol* 75, 6462–6470. https://doi.org/10.1128/AEM.00904-09

Minnig, A., Gosselin, V.B., Griss, S., Schüpbach-Regula, G., Meylan, M., Schnidrig, G.-A., Thomann, B., 2025. Associations between animal health indicators, antimicrobial use, and antimicrobial resistance on Swiss dairy farms. Prev Vet Med 245, 106688. https://doi.org/10.1016/j.prevetmed.2025.106688

Mokhbatly, A.-A.A., Elsheikh, N., Ghazy, E.W., Elgamal, A.M., Hegazy, Y.M., Assar, D.H., 2022. Prevalence of Shiga toxin-producing *Escherichia coli* and *Salmonellae* and some associated hematologic and biochemical profile alterations in lambs. *Vet Res Forum* 13, 155–162. https://doi.org/10.30466/vrf.2020.124018.2907

Molina-Quiroz, R.C., Loyola, D.E., Muñoz-Villagrán, C.M., Quatrini, R., Vásquez, C.C., Pérez-Donoso, J.M., 2013. DNA, Cell wall and general oxidative damage underlie the tellurite/cefotaxime synergistic effect in *Escherichia coli*. *PLoS ONE* 8, e79499. https://doi.org/10.1371/journal.pone.0079499

Moura, R.A., Sircili, M.P., Leomil, L., Matté, M.H., Trabulsi, L.R., Elias, W.P., Irino, K., Pestana de Castro, A.F., 2009. Clonal relationship among atypical enteropathogenic *Escherichia coli* strains isolated from different animal species and humans. *Appl Environ Microbiol* 75, 7399–7408. https://doi.org/10.1128/AEM.00636-09

Mühlen, S., Dersch, P., 2020. Treatment strategies for infections with Shiga toxin -producing *Escherichia coli*. *Front. Cell. Infect. Microbiol*. 10, 169. https://doi.org /10.3389/fcimb.2020.00169

Myojin, S., Pak, K., Sako, M., Kobayashi, T., Takahashi, T., Sunagawa, T., Tsuboi, N., Ishikura, K., Kubota, Masaya, Kubota, Mitsuru, Igarashi, T., Morioka, I., Miyairi, I., 2022. Interventions for Shiga toxin-producing *Escherichia coli* gastroenteritis and risk of hemolytic uremic syndrome: A population-based matched case control study. PLoS One 17, e0263349. https://doi.org/10.1371/journal.pone.0263349

Nagao, I., Kawasaki, M., Goyama, T., Kim, H.J., Call, D.R., Ambrosini, Y.M., 2024. Enterohemorrhagic *Escherichia coli* (EHEC) disrupts intestinal barrier integrity in translational canine stem cell-derived monolayers. *Microbiol Spectr* 12, e0096124. https://doi.org/10.1128/spectrum.00961-24

Nakajima, H., Kiyokawa, N., Katagiri, Y.U., Taguchi, T., Suzuki, T., Sekino, T., Mimori, K., Ebata, T., Saito, M., Nakao, H., Takeda, T., Fujimoto, J., 2001. Kinetic analysis of binding between Shiga toxin and receptor glycolipid Gb3Cer by surface plasmon resonance. *J Biol Chem* 276, 42915–42922. https://doi.org/10.1074/jbc.M106015200

Nakamura, K., Ogura, Y., Gotoh, Y., Hayashi, T., 2021. Prophages integrating into prophages: a mechanism to accumulate type III secretion effector genes and duplicate Shiga toxin-encoding prophages in *Escherichia coli*. *PLoS Pathog* 17, e1009073. https://doi.org/10.1371/journal.ppat.1009073

Nakamura, K., Seto, K., Lee, K., Ooka, T., Gotoh, Y., Taniguchi, I., Ogura, Y., Mainil, J.G., Piérard, D., Harada, T., Etoh, Y., Ueda, S., Hamasaki, M., Isobe, J., Kimata, K., Narimatsu, H., Yatsuyanagi, J., Ohnishi, M., Iyoda, S., Hayashi, T., 2023. Global population structure, genomic diversity and carbohydrate fermentatio n characteristics of clonal complex 119 (CC119), an understudied Shiga toxin-pro

ducing *E. coli* (STEC) lineage including O165:H25 and O172:H25. *Microb Geno m* 9. https://doi.org/10.1099/mgen.0.000959

Nakamura, K., Taniguchi, I., Gotoh, Y., Isobe, J., Kimata, K., Igawa, Y., Kitahashi, T., Takahashi, Y., Nomoto, R., Iwabuchi, K., Morimoto, Y., Iyoda, S., Hayashi, T., 2024. Diversity of Shiga toxin transducing phages in *Escherichia coli* O145:H28 and the different Shiga toxin 2 production levels associated with short- or long-tailed phages. *Front Microbiol* 15, 1453887. https://doi.org/10.3389/fmicb.2024.1453887

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 11, 142–201. https://doi.org/10.1128/CMR.11.1.142

Nataro, J.P., Maher, K.O., Mackie, P., Kaper, J.B., 1987. Characterization of plasmids encoding the adherence factor of enteropathogenic *Escherichia coli*. *Infect Immun* 55, 2370–2377. https://doi.org/10.1128/iai.55.10.2370-2377.1987

Naylor, S.W., Low, J.C., Besser, T.E., Mahajan, A., Gunn, G.J., Pearce, M.C., M cKendrick, I.J., Smith, D.G.E., Gally, D.L., 2003. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infect Immun* 71, 1505–1512. https: //doi.org/10.1128/IAI.71.3.1505-1512.2003

Ndegwa, E., O'Brien, D., Matthew, K., Wang, Z., Kim, J., 2022. Shiga toxin subtypes, serogroups, phylogroups, RAPD genotypic diversity, and select virulence markers of Shiga-toxigenic *Escherichia coli* strains from goats in Mid-Atlantic US. *Microorganisms* 10, 1842. https://doi.org/10.3390/microorganisms10091842

Neter, E., Westphal, O., Luderitz, O., Gino, R.M., Gorzynski, E.A., 1955. Demonstration of antibodies against enteropathogenic *Escherichia coli* in sera of children of various ages. *Pediatrics* 16, 801–808.

Newton, H.J., Sloan, J., Bennett-Wood, V., Adams, L.M., Robins-Browne, R.M., Hartland, E.L., 2004. Contribution of long polar fimbriae to the virulence of rab bit-specific enteropathogenic *Escherichia coli*. *Infect Immun* 72, 1230–1239. https: //doi.org/10.1128/IAI.72.3.1230-1239.2004

Nguyen, T.T.H., Kikuchi, T., Tokunaga, T., Iyoda, S., Iguchi, A., 2021. Diversity of the tellurite resistance gene operon in *Escherichia coli*. *Front Microbiol* 12, 681175. https://doi.org/10.3389/fmicb.2021.681175

Nies, D.H., 1999. Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* 51, 730–750. https://doi.org/10.1007/s002530051457

Nüesch-Inderbinen, M., Barmettler, K., Stevens, M.J.A., Cernela, N., 2024. Shiga toxin-producing *Escherichia coli* isolated from hunted wild boar (*Sus scrofa*) in Switzerland. *Schweiz Arch Tierheilkd* 166, 131–140. https://doi.org/10.17236/sat00419

Nüesch-Inderbinen, M., Cernela, N., Wüthrich, D., Egli, A., Stephan, R., 2018. Genetic characterization of Shiga toxin-producing *Escherichia coli* belonging to the emerging hybrid pathotype O80:H2 isolated from humans 2010-2017 in Switzerland. *Int J Med Microbiol* 308, 534–538. https://doi.org/10.1016/j.ijmm.2018.05.007

O'Brien, A.D., Holmes, R.K., 1987. Shiga and Shiga-Like Toxins. *Microbio. Rev.* 51. https://doi.org/10.1128/mr.51.2.206-220.1987

Obrig, T.G., 2010. *Escherichia coli* Shiga toxin mechanisms of action in renal disease. *Toxins (Basel)* 2, 2769–2794. https://doi.org/10.3390/toxins2122769

Ochman, H., Selander, R.K., 1984. Standard reference strains of *Escherichia coli* from natural populations. *J Bacteriol* 157, 690–693. https://doi.org/10.1128/jb.157.2.690-693.1984

Ochoa, T.J., Contreras, C.A., 2011. Enteropathogenic *E. coli* (EPEC) infection in children. *Curr Opin Infect Dis* 24, 478–483. https://doi.org/10.1097/QCO.0b013e32834a8b8b

Octavia, S., Lan, R., 2010. Single Nucleotide Polymorphism typing of global *Salmonella enterica* serovar Typhi isolates by use of a hairpin primer real-time PCR assay. *J Clin Microbiol* 48, 3504–3509. https://doi.org/10.1128/JCM.00709-10

Ogura, Y., Ooka, T., Iguchi, A., Toh, H., Asadulghani, M., Oshima, K., Kodama, T., Abe, H., Nakayama, K., Kurokawa, K., Tobe, T., Hattori, M., Hayashi, T., 2009. Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157

enterohemorrhagic *Escherichia coli*. *Proc Natl Acad Sci U S A* 106, 17939–17944. https://doi.org/10.1073/pnas.0903585106

Ohara, T., Kojio, S., Taneike, I., Nakagawa, S., Gondaira, F., Tamura, Y., Gejyo, F., Zhang, H.-M., Yamamoto, T., 2002. Effects of azithromycin on shiga toxin production by *Escherichia coli* and subsequent host inflammatory response. Antimicrob Agents Chemother 46, 3478–3483. https://doi.org/10.1128/AAC.46.11.3478-3483.2002

Okubo, Y., Miyairi, I., Michihata, N., Morisaki, N., Kinoshita, N., Urayama, K.Y., Yasunaga, H., 2019. Recent Prescription Patterns for Children With Acute Infectious Diarrhea. J Pediatr Gastroenterol Nutr 68, 13–16. https://doi.org/10.1097/MPG.0000000000002115

Orskov, F., Orskov, I., 1992. *Escherichia coli* serotyping and disease in man and animals. *Can J Microbiol* 38, 699–704.

Orskov, I., Orskov, F., 1983. Serology of *Escherichia coli* fimbriae. *Prog Allergy* 33, 80–105.

Orskov, I., Orskov, F., Birch-Andersen, A., Kanamori, M., Svanborg-Edén, C., 1982. O, K, H and fimbrial antigens in *Escherichia coli* serotypes associated with pyelonephritis and cystitis. *Scand J Infect Dis Suppl* 33, 18–25.

Orth, D., Grif, K., Dierich, M.P., Würzner, R., 2007. Variability in tellurite resist ance and the *ter* gene cluster among Shiga toxin-producing *Escherichia coli* isola ted from humans, animals and food. *Research in Microbiology* 158, 105–111. htt ps://doi.org/10.1016/j.resmic.2006.10.007

Park, S.H., Byun, J.-H., Choi, S.-M., Lee, D.-G., Kim, S.-H., Kwon, J.-C., Park, C., Choi, J.-H., Yoo, J.-H., 2012. Molecular epidemiology of extended-spectrum β -lactamase-producing *Escherichia coli* in the community and hospital in Korea: e mergence of ST131 producing CTX-M-15. *BMC Infect Dis* 12, 149. https://doi.or g/10.1186/1471-2334-12-149

Parreira, V.R., Liao, J.H., Kim, S.-H., Gyles, C.L., 2008. A homolog of the O157 urease-encoding O island 48 is present in porcine O149:H10 enterotoxigenic *Escherichia coli*. *Vet Res* 39, 38. https://doi.org/10.1051/vetres:2008015

Patel, P.N., Lindsey, R.L., Garcia-Toledo, L., Rowe, L.A., Batra, D., Whitley, S. W., Drapeau, D., Stoneburg, D., Martin, H., Juieng, P., Loparev, V.N., Strockbine, N., 2018. High-Quality Whole-Genome sequences for 77 Shiga toxin-Producing *Escherichia coli* strains generated with PacBio sequencing. *Genome Announc* 6, e 00391-18. https://doi.org/10.1128/genomeA.00391-18

Paton, A.W., Manning, P.A., Woodrow, M.C., Paton, J.C., 1998. Translocated intimin receptors (Tir) of Shiga-toxigenic *Escherichia coli* isolates belonging to serogroups O26, O111, and O157 react with sera from patients with hemolytic-uremic syndrome and exhibit marked sequence heterogeneity. *Infect Immun* 66, 5580–5586. https://doi.org/10.1128/IAI.66.11.5580-5586.1998

Paton, J.C., Paton, A.W., 1998. Pathogenesis and diagnosis of Shiga toxin-produc ing *Escherichia coli* infections. Clin Microbiol Rev 11, 450–479. https://doi.org/1 0.1128/CMR.11.3.450

Paton, A.W., Srimanote, P., Woodrow, M.C., Paton, J.C., 2001. Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infect Immun* 69, 6999–7009. https://doi.org/10.1128/IAI.69.11.6999-7009.2001

Paton, A.W., Woodrow, M.C., Doyle, R.M., Lanser, J.A., Paton, J.C., 1999. Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. *J Clin Microbiol* 37, 3357–3361. https://doi.org/10.1128/JCM.37.10.3357-3361.1999

Pearce, M.E., Alikhan, N.-F., Dallman, T.J., Zhou, Z., Grant, K., Maiden, M.C.J., 2018. Comparative analysis of core genome MLST and SNP typing within a Eur opean *Salmonella* serovar Enteritidis outbreak. *Int J Food Microbiol* 274, 1–11. h ttps://doi.org/10.1016/j.ijfoodmicro.2018.02.023

Peigne, C., Bidet, P., Mahjoub-Messai, F., Plainvert, C., Barbe, V., Médigue, C., Frapy, E., Nassif, X., Denamur, E., Bingen, E., Bonacorsi, S., 2009. The plasmid of *Escherichia coli* strain S88 (O45:K1:H7) that causes neonatal meningitis is clo sely related to avian pathogenic *E. coli* plasmids and is associated with high-leve l bacteremia in a neonatal rat meningitis model. I*nfect Immun* 77, 2272–2284. htt ps://doi.org/10.1128/IAI.01333-08

Peng, W., Wang, Y., Fu, Y., Deng, Z., Lin, S., Liang, R., 2022. Characterization of the tellurite-resistance properties and identification of the core function genes for tellurite resistance in *Pseudomonas citronellolis* SJTE-3. *Microorganisms* 10, 95. https://doi.org/10.3390/microorganisms10010095

Pereira, M.F., Rossi, C.C., da Silva, G.C., Rosa, J.N., Bazzolli, D.M.S., 2020. *G alleria mellonella* as an infection model: an in-depth look at why it works and practical considerations for successful application. *Pathog Dis* 78, ftaa056. https:// doi.org/10.1093/femspd/ftaa056

Pérez, J.M., Calderón, I.L., Arenas, F.A., Fuentes, D.E., Pradenas, G.A., Fuentes, E.L., Sandoval, J.M., Castro, M.E., Elías, A.O., Vásquez, C.C., 2007. Bacterial toxicity of potassium tellurite: unveiling an ancient enigma. *PLoS ONE* 2, e211. https://doi.org/10.1371/journal.pone.0000211

Perna, N.T., Plunkett, G., Burland, V., Mau, B., Glasner, J.D., Rose, D.J., Mayhew, G.F., Evans, P.S., Gregor, J., Kirkpatrick, H.A., Pósfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E.J., Davis, N.W., Lim, A., Dimalanta, E.T., Potamousis, K.D., Apodaca, J., Anantharaman, T.S., Lin, J., Yen, G., Schwartz, D.C., Welch, R.A., Blattner, F.R., 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409, 529–533. https://doi.org/10.1038/35054089

Perrat, A., Branchu, P., Decors, A., Turci, S., Bayon-Auboyer, M.-H., Petit, G., Grosbois, V., Brugère, H., Auvray, F., Oswald, E., 2022. Wild boars as reservoir of highly virulent clone of hybrid Shiga toxigenic and enterotoxigenic *Escherichi a coli* responsible for edema disease, France. *Emerg Infect Dis* 28, 382–393. http s://doi.org/10.3201/eid2802.211491

Petty, N.K., Ben Zakour, N.L., Stanton-Cook, M., Skippington, E., Totsika, M., Forde, B.M., Phan, M.-D., Gomes Moriel, D., Peters, K.M., Davies, M., Rogers, B.A., Dougan, G., Rodriguez-Baño, J., Pascual, A., Pitout, J.D.D., Upton, M., Paterson, D.L., Walsh, T.R., Schembri, M.A., Beatson, S.A., 2014. Global dissemination of a multidrug resistant *Escherichia coli* clone. *Proc. Natl. Acad. Sci. U S A*. 111, 5694–5699. https://doi.org/10.1073/pnas.1322678111

Pfaff-McDonough, S.J., Horne, S.M., Giddings, C.W., Ebert, J.O., Doetkott, C., Smith, M.H., Nolan, L.K., 2000. Complement resistance-related traits among *Escherichia coli* isolates from apparently healthy birds and birds with colibacillosis. *Avian Dis* 44, 23–33.

Piérard, D., De Greve, H., Haesebrouck, F., Mainil, J., 2012. O157:H7 and O104:H4 Vero/Shiga toxin-producing *Escherichia coli* outbreaks: respective role of cattle and humans. *Vet Res* 43, 13. https://doi.org/10.1186/1297-9716-43-13

Probert, W.S., McQuaid, C., Schrader, K., 2014. Isolation and identification of an *Enterobacter cloacae* strain producing a novel subtype of Shiga toxin type 1. *J Clin Microbiol 5*2, 2346–2351. https://doi.org/10.1128/JCM.00338-14

Projahn, M., Lamparter, M.C., Ganas, P., Goehler, A., Lorenz-Wright, S.C., Maede, D., Fruth, A., Lang, C., Schuh, E., 2021. Genetic diversity and pathogenic potential of Shiga toxin-producing *Escherichia coli* (STEC) derived from German flour. *Int J Food Microbiol* 347, 109197. https://doi.org/10.1016/j.ijfoodmicro.2021.109197

Pruimboom-Brees, I.M., Morgan, T.W., Ackermann, M.R., Nystrom, E.D., Samuel, J.E., Cornick, N.A., Moon, H.W., 2000. Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. *Proc Natl Acad Sci USA* 97, 10325–10329. https://doi.org/10.1073/pnas.190329997

Puño-Sarmiento, J., Medeiros, L., Chiconi, C., Martins, F., Pelayo, J., Rocha, S., Blanco, J., Blanco, M., Zanutto, M., Kobayashi, R., Nakazato, G., 2013. Detection of diarrheagenic *Escherichia coli* strains isolated from dogs and cats in Brazil. *Vet Microbiol* 166, 676–680. https://doi.org/10.1016/j.vetmic.2013.07.007

Quainoo, S., Coolen, J.P.M., van Hijum, S.A.F.T., Huynen, M.A., Melchers, W.J.G., van Schaik, W., Wertheim, H.F.L., 2017. Whole-Genome sequencing of bacterial pathogens:

the future of nosocomial outbreak analysis. *Clin Microbiol Rev* 30, 1015–1063. https://doi.org/10.1128/CMR.00016-17

Ramboarina, S., Fernandes, P.J., Daniell, S., Islam, S., Simpson, P., Frankel, G., Booy, F., Donnenberg, M.S., Matthews, S., 2005. Structure of the bundle-forming pilus from enteropathogenic *Escherichia coli*. *J Biol Chem* 280, 40252–40260. htt ps://doi.org/10.1074/jbc.M508099200

Ray, R., Singh, P., 2022. Prevalence and implications of Shiga toxin-producing *E. coli* in farm and wild ruminants. *Pathogens* 11, 1332. https://doi.org/10.3390/path ogens11111332

Renzhammer, R., Schwarz, L., Cabal Rosel, A., Ruppitsch, W., Fuchs, A., Simetz berger, E., Ladinig, A., Loncaric, I., 2024. Detection of *mcr*-1-1 positive enteropa thogenic *Escherichia coli* isolates associated with post-weaning diarrhoea in an or ganic piglet-producing farm in Austria. *Microorganisms* 12, 244. https://doi.org/10. 3390/microorganisms12020244

Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., Cohen, M.L., 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* 308, 681–685. https://doi.org/10.1056/NEJM198303243081203

Robaeys, G., Surmont, I., Lemmens, P., Coremans, G., Vantrappen, G., Vandepitte, J., 1987. Haemorrhagic colitis and verotoxin-producing *Escherichia coli* O157 in Belgium. *Lancet* 1, 1495–1496. https://doi.org/10.1016/s0140-6736(87)92250-1

Rodrigues, J., Scaletsky, I.C., Campos, L.C., Gomes, T.A., Whittam, T.S., Trabuls i, L.R., 1996. Clonal structure and virulence factors in strains of *Escherichia coli* of the classic serogroup O55. *Infect Immun* 64, 2680–2686. https://doi.org/10.1128 /iai.64.7.2680-2686.1996

Rodríguez-Rubio, L., Haarmann, N., Schwidder, M., Muniesa, M., Schmidt, H., 2021. Bacteriophages of Shiga toxin-producing *Escherichia coli* and their contribution to pathogenicity. *Pathogens* 10, 404. https://doi.org/10.3390/pathogens10040404

Rodwell, E.V., Chan, Y.-W., Sawyer, C., Carroll, A., McNamara, E., Allison, L., Browning, L., Holmes, A., Godbole, G., McCarthy, N., Jenkins, C., 2022. Shiga toxin-producing *Escherichia coli* clonal complex 32, including serotype O145:H28, in the UK and Ireland. *J Med Microbiol* 71. https://doi.org/10.1099/jmm.0.001579

Rodwell, E.V., Greig, D.R., Godbole, G., Jenkins, C., 2024. Clinical and public health implications of increasing notifications of LEE-negative Shiga toxin-producing *Escherichia coli* in England, 2014-2022. *J Med Microbiol* 73. https://doi.org/10.1099/jmm.0.001790

Rodwell, E.V., Vishram, B., Smith, R., Browning, L., Smith-Palmer, A., Allison, L., Holmes, A., Godbole, G., McCarthy, N., Dallman, T.J., Jenkins, C., 2021. Epidemiology and genomic analysis of Shiga toxin-producing *Escherichia coli* clonal complex 165 in the UK. *J Med Microbiol* 70. https://doi.org/10.1099/jmm.0.001471

Rounds, J.M., Rigdon, C.E., Muhl, L.J., Forstner, M., Danzeisen, G.T., Koziol, B. S., Taylor, C., Shaw, B.T., Short, G.L., Smith, K.E., 2012. Non-O157 Shiga toxin-producing *Escherichia coli* associated with venison. *Emerg Infect Dis* 18, 279–282. https://doi.org/10.3201/eid1802.110855

Ruano-Gallego, D., Álvarez, B., Fernández, L.Á., 2015. Engineering the controlled assembly of filamentous injectisomes in *E. coli* K-12 for protein translocation into mammalian cells. *ACS Synth. Biol*. 4, 1030–1041. https://doi.org/10.1021/acssynbio.5b00080

Russo, T.A., Johnson, J.R., 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. *J Infect Dis* 181, 1753–1754. https://doi.org/10.1086/315418

Sandoval, J.M., Arenas, F.A., García, J.A., Díaz-Vásquez, W.A., Valdivia-González, M., Sabotier, M., Vásquez, C.C., 2015. *Escherichia coli* 6-phosphogluconate dehydrogenase aids in tellurite resistance by reducing the toxicant in a NADPH-dependent manner. *Microbiol Res* 177, 22–27. https://doi.org/10.1016/j.micres.2015.05.002

Sandvig, K., van Deurs, B., 1996. Endocytosis, intracellular transport, and cytoto xic action of Shiga toxin and ricin. *Physiol Rev* 76, 949–966. https://doi.org/10.1 152/physrev.1996.76.4.949

Scaletsky, I.C., Silva, M.L., Trabulsi, L.R., 1984. Distinctive patterns of adherenc e of enteropathogenic *Escherichia coli* to HeLa cells. *Infect Immun* 45, 534–536. https://doi.org/10.1128/iai.45.2.534-536.1984

Scheutz, F., Teel, L.D., Beutin, L., Piérard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N.A., Melton-Celsa, A.R., Sanchez, M., Persson, S., O'Brien, A.D., 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J Clin Microbiol* 50, 2951–2963. https://doi.org/10.1128/JCM.00860-12

Schmidt, H., Beutin, L., Karch, H., 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect Immun* 63, 1055–1061. https://doi.org/10.1128/iai.63.3.1055-1061.1995

Schmidt, H., Bielaszewska, M., Karch, H., 1999. Transduction of enteric *Escherichia coli* isolates with a derivative of Shiga toxin 2-encoding bacteriophage phi3538 isolated from *Escherichia coli* O157:H7. Appl Environ Microbiol 65, 3855–3861. https://doi.org/10.1128/AEM.65.9.3855-3861.1999

Schmidt, H., Hensel, M., 2004. Pathogenicity islands in bacterial pathogenesis. *Clin Microbiol Rev* 17, 14–56. https://doi.org/10.1128/CMR.17.1.14-56.2004

Schmidt, H., Scheef, J., Morabito, S., Caprioli, A., Wieler, L.H., Karch, H., 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. *Appl Environ Microbiol* 66, 1205–1208. https://doi.org/10.1128/AEM.66.3.1205-1208.2000

Schnaitman, C.A., Klena, J.D., 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol Rev* 57, 655–682. https://doi.org/10.1128/mr.57.3.655-682.1993

Schumann, A., Gaballa, A., Wiedmann, M., 2024. The multifaceted roles of phos phoethanolamine-modified lipopolysaccharides: from stress response and virulence

to cationic antimicrobial resistance. *Microbiol Mol Biol Rev* 88, e0019323. https://doi.org/10.1128/mmbr.00193-23

Scotland, S.M., Smith, H.R., Willshaw, G.A., Rowe, B., 1983. Vero cytotoxin production in strain of *Escherichia coli* is determined by genes carried on bacteriophage. *Lancet* 2, 216. https://doi.org/10.1016/s0140-6736(83)90192-7

Seiler, C., Berendonk, T.U., 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Front Microbio* 3. https://doi.org/10.3389/fmicb.2012.00399

Sekiya, K., Ohishi, M., Ogino, T., Tamano, K., Sasakawa, C., Abe, A., 2001. Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. *Proc Natl Acad Sci USA* 98, 11638–11643. https://doi.org/10.1073/pnas.191378598

Shaikh, N., Tarr, P.I., 2003. *Escherichia coli* O157:H7 Shiga toxin-encoding bacteriophages: integrations, excisions, truncations, and evolutionary implications. *J Bacteriol* 185, 3596–3605. https://doi.org/10.1128/JB.185.12.3596-3605.2003

Sham, H.P., Shames, S.R., Croxen, M.A., Ma, C., Chan, J.M., Khan, M.A., Wickham, M.E., Deng, W., Finlay, B.B., Vallance, B.A., 2011. Attaching and effacing bacterial effector NleC suppresses epithelial inflammatory responses by inhibiting NF-κB and p38 mitogen-activated protein kinase activation. *Infect Immun* 79, 3552–3562. https://doi.org/10.1128/IAI.05033-11

Sharma, B., Modgil, V., Mahindroo, J., Kumar, A., Kaur, V., Narayan, C., Verma, R., Mohan, B., Taneja, N., 2023. Are non-lactose-fermenting *Escherichia coli* important diarrhoeal pathogens in children and adults? *Access Microbiol* 5, acmi000459.v3. https://doi.org/10.1099/acmi.0.000459.v3

Shepard, S.M., Danzeisen, J.L., Isaacson, R.E., Seemann, T., Achtman, M., Johns on, T.J., 2012. Genome sequences and phylogenetic analysis of K88- and F18-po sitive porcine enterotoxigenic *Escherichia coli*. *J Bacteriol* 194, 395–405. https://doi.org/10.1128/JB.06225-11

Shimizu, T., Sato, T., Kawakami, S., Ohta, T., Noda, M., Hamabata, T., 2007. Receptor affinity, stability and binding mode of Shiga toxins are determinants of toxicity. *Microb Pathog* 43, 88–95. https://doi.org/10.1016/j.micpath.2007.04.003

Silver, S., Phung, L.T., 1996. Bacterial heavy metal resistance: new surprises. *Annu Rev Microbiol* 50, 753–789. https://doi.org/10.1146/annurev.micro.50.1.753

Singh, P., Sha, Q., Lacher, D.W., Del Valle, J., Mosci, R.E., Moore, J.A., Scribner, K.T., Manning, S.D., 2015. Characterization of enteropathogenic and Shiga toxin-producing *Escherichia coli* in cattle and deer in a shared agroecosystem. *Front Cell Infect Microbiol* 5, 29. https://doi.org/10.3389/fcimb.2015.00029

Slinger, R., Lau, K., Slinger, M., Moldovan, I., Chan, F., 2017. Higher atypical enteropathogenic *Escherichia coli* (a-EPEC) bacterial loads in children with diarrhea are associated with PCR detection of the EHEC factor for adherence 1/lymphocyte inhibitory factor A (*efa1*/*lifa*) gene. *Ann Clin Microbiol Antimicrob* 16, 16. https://doi.org/10.1186/s12941-017-0188-y

Smith, D.L., Rooks, D.J., Fogg, P.C.M., Darby, A.C., Thomson, N.R., McCarthy, A.J., Allison, H.E., 2012. Comparative genomics of Shiga toxin encoding bacteriophages. *BMC Genomics* 13, 311. https://doi.org/10.1186/1471-2164-13-311

Smith, H.W., Halls, S., 1967. Studies on *Escherichia coli* enterotoxin. *J Pathol Bacteriol* 93, 531–543. https://doi.org/10.1002/path.1700930212

Smith, H.W., Linggood, M.A., 1971. The transmissible nature of enterotoxin production in a human enteropathogenic strain of *Escherichia coli*. J *Med Microbiol* 4, 301–305. https://doi.org/10.1099/00222615-4-3-301

Soleau, N., Ganet, S., Werlen, S., Collignon, L., Cointe, A., Bonacorsi, S., Serge ntet, D., 2024. First isolation of the heteropathotype Shiga toxin-producing and e xtra-intestinal pathogenic (STEC-ExPEC) *E. coli* O80:H2 in french healthy cattle: genomic characterization and phylogenetic position. *Int J Mol Sci* 25, 5428. http s://doi.org/10.3390/ijms25105428

Soysal, N., Mariani-Kurkdjian, P., Smail, Y., Liguori, S., Gouali, M., Loukiadis, E., Fach, P., Bruyand, M., Blanco, J., Bidet, P., Bonacorsi, S., 2016. Enterohemorrhagic *Escherichia coli* hybrid pathotype O80:H2 as a new therapeutic challenge. *Emerg Infect Dis* 22, 1604–1612. https://doi.org/10.3201/eid2209.160304

Steil, D., Bonse, R., Meisen, I., Pohlentz, G., Vallejo, G., Karch, H., Müthing, J., 2016. A topographical atlas of Shiga toxin 2e receptor distribution in the tissues of weaned piglets. *Toxins (Basel)* 8, 357. https://doi.org/10.3390/toxins8120357

Steimle, A., Autenrieth, I.B., Frick, J.-S., 2016. Structure and function: Lipid A modifications in commensals and pathogens. *Int J Med Microbiol* 306, 290–301. https://doi.org/10.1016/j.ijmm.2016.03.001

Steinberger, A.J., De Campos, J.L., Kates, A.E., Goldberg, T.L., Ruegg, P.L., Safdar, N., Sethi, A.K., Shutske, J.M., Suen, G., 2025. Assessing the Impacts of Dairy Farm Antimicrobial Use on the Bovine Fecal Microbiome. Animals 15, 1735. https://doi.org/10.3390/ani15121735

Stevens, M.P., Frankel, G.M., 2014. The locus of enterocyte effacement and associated virulence factors of enterohemorrhagic *Escherichia coli*. *Microbiol Spectr* 2, EHEC-0007-2013. https://doi.org/10.1128/microbiolspec.EHEC-0007-2013

Stock, A.H., Shuman, M.E., 1956. Gastroenteritis in infants associated with specif ic serotypes of *Escherichia coli*. *Pediatrics* 17, 196–203. https://doi.org/10.1542/pe ds.17.2.196

Takeda, T., 1998. Strategy to prevent the progression of enterohemorrhagic *Escherichia coli* O157 infection to hemolytic uremic syndrome. Jpn J Med Sci Biol 51 Suppl, S124-128. https://doi.org/10.7883/yoken1952.51.supplement1_s124

Tarr, P.I., Bilge, S.S., Vary, J.C., Jelacic, S., Habeeb, R.L., Ward, T.R., Baylor, M.R., Besser, T.E., 2000. Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect Immun* 68, 1400–1407. https://doi.org/10.1128/IAI.68.3.1400-1407.2000

Taylor, D.E., 1999. Bacterial tellurite resistance. *Trends Microbiol* 7, 111–115. https://doi.org/10.1016/s0966-842x(99)01454-7

Taylor, D.E., Rooker, M., Keelan, M., Ng, L.-K., Martin, I., Perna, N.T., Burland, N.T.V., Blattner, F.R., 2002. Genomic variability of O islands encoding tellurite resistance in enterohemorrhagic *Escherichia coli* O157:H7 isolates. *J Bacteriol* 184, 4690–4698. https://doi.org/10.1128/JB.184.17.4690-4698.2002

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment. *Exp Suppl* 101, 133–164. https://doi.org/10.1007/978-3-7643-8340-4_6

Tesh, V.L., Burris, J.A., Owens, J.W., Gordon, V.M., Wadolkowski, E.A., O'Brien, A.D., Samuel, J.E., 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect Immun* 61, 3392–3402.

Thiry, D., De Rauw, K., Takaki, S., Duprez, J.-N., Iguchi, A., Piérard, D., Korsak, N., Mainil, J.G., 2018. Low prevalence of the "gang of seven" and absence of the O80:H2 serotypes among Shigatoxigenic and enteropathogenic *Escherichia coli* (STEC and EPEC) in intestinal contents of healthy cattle at two slaughterhouses in Belgium in 2014. *J Appl Microbiol* 124, 867–873. https://doi.org/10.1111/jam.13677

Thiry, D., Saulmont, M., Takaki, S., De Rauw, K., Duprez, J.-N., Iguchi, A., Pié rard, D., Mainil, J.G., 2017. Enteropathogenic *Escherichia coli* O80:H2 in young calves with diarrhea, Belgium. *Emerg Infect Dis* 23, 2093–2095. https://doi.org/10.3201/eid2312.170450

Tillman, G.E., Wasilenko, J.L., Simmons, M., Lauze, T.A., Minicozzi, J., Oakley, B.B., Narang, N., Fratamico, P., Cray, W.C., 2012. Isolation of Shiga toxin–producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 from ground beef using modified rainbow agar and post–immunomagnetic separation acid treatment. *Journal of Food Protection* 75, 1548–1554. https://doi.org/10.4315/0362-028x.jfp-12-110

Timoney, J.F., 1950. Oedema disease of swine. *Vet Rec* 62, 748–756. https://doi.org/10.1136/vr.62.49.748

Tobe, T., Beatson, S.A., Taniguchi, H., Abe, H., Bailey, C.M., Fivian, A., Younis, R., Matthews, S., Marches, O., Frankel, G., Hayashi, T., Pallen, M.J., 2006. An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdoid phages in their dissemination. *Proc Natl Acad Sci USA* 103, 14941–14946. https://doi.org/10.1073/pnas.0604891103

Tooke, C.L., Hinchliffe, P., Bragginton, E.C., Colenso, C.K., Hirvonen, V.H.A., Takebayashi, Y., Spencer, J., 2019. β-lactamases and β-lactamase inhibitors in the 21st century. *J Mol Biol* 431, 3472–3500. https://doi.org/10.1016/j.jmb.2019.04.002

Tóth, I., Schmidt, H., Dow, M., Malik, A., Oswald, E., Nagy, B., 2003. Transduction of porcine enteropathogenic *Escherichia coli* with a derivative of a Shiga toxin 2-encoding bacteriophage in a porcine ligated ileal loop system. *Appl Environ Microbiol* 69, 7242–7247. https://doi.org/10.1128/AEM.69.12.7242-7247.2003

Tóth, I., Schmidt, H., Kardos, G., Lancz, Z., Creuzburg, K., Damjanova, I., Pászti, J., Beutin, L., Nagy, B., 2009. Virulence genes and molecular typing of different groups of *Escherichia coli* O157 strains in cattle. *Appl Environ Microbiol* 75, 6282–6291. https://doi.org/10.1128/AEM.00873-09

Touchon, M., Perrin, A., De Sousa, J.A.M., Vangchhia, B., Burn, S., O'Brien, C.L., Denamur, E., Gordon, D., Rocha, E.P., 2020. Phylogenetic background and habitat drive the genetic diversification of *Escherichia coli*. *PLoS Genet* 16, e1008866. https://doi.org/10.1371/journal.pgen.1008866

Tozzoli, R., Scheutz, F. Diarrhoeagenic *Escherichia coli* infections in humans. In *Pathogenic* Escherichia coli: *Molecular and Cellular Microbiology*; Morabito, S., Ed.; Horizon Scientific Press and Caister Academic Press: Norwich, UK, 2014; pp. 1–18.

Trabulsi, L.R., Keller, R., Tardelli Gomes, T.A., 2002. Typical and atypical enter opathogenic *Escherichia coli*. *Emerg Infect Dis* 8, 508–513. https://doi.org/10.3201/eid0805.010385

Tsai, C.J.-Y., Loh, J.M.S., Proft, T., 2016. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 7, 214–229. https://doi.org/10.1080/21505594.2015.1135289

Turabelidze, G., Lawrence, S.J., Gao, H., Sodergren, E., Weinstock, G.M., Abubucker, S., Wylie, T., Mitreva, M., Shaikh, N., Gautom, R., Tarr, P.I., 2013. Precise dissection of an *Escherichia coli* O157:H7 outbreak by single nucleotide polymorphism analysis. *J Clin Microbiol* 51, 3950–3954. https://doi.org/10.1128/JCM.01930-13

Turner, R.J., Aharonowitz, Y., Weiner, J.H., Taylor, D.E., 2001. Glutathione is a target in tellurite toxicity and is protected by tellurite resistance determinants in *Escherichia coli*. *Can J Microbiol* 47, 33–40.

Tzschoppe, M., Martin, A., Beutin, L., 2012. A rapid procedure for the detection and isolation of enterohaemorrhagic *Escherichia coli* (EHEC) serogroup O26, O103, O111, O118, O121, O145 and O157 strains and the aggregative EHEC O104:H4 strain from ready-to-eat vegetables. *Int J Food Microbiol* 152, 19–30. https://doi.org/10.1016/j.ijfoodmicro.2011.10.009

Valleau, D., Little, D.J., Borek, D., Skarina, T., Quaile, A.T., Di Leo, R., Houliston, S., Lemak, A., Arrowsmith, C.H., Coombes, B.K., Savchenko, A., 2018. Functional diversification of the NleG effector family in enterohemorrhagic *Escherichia coli*. *Proc Natl Acad Sci USA* 115, 10004–10009. https://doi.org/10.1073/pnas.1718350115

van Diemen, P.M., Dziva, F., Stevens, M.P., Wallis, T.S., 2005. Identification of enterohemorrhagic *Escherichia coli* O26:H- genes required for intestinal colonization in calves. *Infect Immun* 73, 1735–1743. https://doi.org/10.1128/IAI.73.3.1735-1743.2005

Van Duyne, G.D., Landy, A., 2024. Bacteriophage lambda site-specific recombination. *Mol Microbiol* 121, 895–911. https://doi.org/10.1111/mmi.15241

van Hoek, A.H.A.M., van Veldhuizen, J.N.J., Friesema, I., Coipan, C., Rossen, J.W.A., Bergval, I.L., Franz, E., 2019. Comparative genomics reveals a lack of evidence for pigeons as a main source of *stx2f*-carrying *Escherichia coli* causing disease in humans and the common existence of hybrid Shiga toxin-producing and enteropathogenic *E. coli* pathotypes. *BMC Genomics* 20, 271. https://doi.org/10.1186/s12864-019-5635-z

Vasoo, S., Barreto, J.N., Tosh, P.K., 2015. Emerging issues in gram-negative bacterial resistance: an update for the practicing clinician. *Mayo Clin Proc* 90, 395–403. https://doi.org/10.1016/j.mayocp.2014.12.002

Vávrová, S., Grones, J., Šoltys, K., Celec, P., Turňa, J., 2024. The tellurite resistance gene cluster of pathogenic bacteria and its effect on oxidative stress response. *Folia Microbiol (Praha)* 69, 433–444. https://doi.org/10.1007/s12223-024-01133-8

Verhaegen, B., De Reu, K., Heyndrickx, M., De Zutter, L., 2015. Comparison of Six chromogenic agar media for the isolation of a broad variety of Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) serogroups. *Int J Environ Res Public Health* 12, 6965–6978. https://doi.org/10.3390/ijerph120606965

Vieira, M.A., Andrade, J.R., Trabulsi, L.R., Rosa, A.C., Dias, A.M., Ramos, S.R., Frankel, G., Gomes, T.A., 2001. Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry EAE and lack the EPEC adherence factor and Shiga toxin DNA probe sequences. *J Infect Dis* 183, 762–772. https://doi.org/10.1086/318821

Vu-Khac, H., Cornick, N.A., 2008. Prevalence and genetic profiles of Shiga toxin-producing *Escherichia coli* strains isolated from buffaloes, cattle, and goats in central Vietnam. *Vet Microbiol* 126, 356–363. https://doi.org/10.1016/j.vetmic.2007.07.023

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J.E., Dahbi, G., Mora, A., López, C., González, E.A., Blanco, J., 2007. Serotypes, virulence genes, intimin types and PFGE profiles of *Escherichia coli* isolated from piglets with diarrhoea in Slovakia. *Vet J* 174, 176–187. https://doi.org/10.1016/j.tvjl.2006.05.019

Wagner, Patrick L., Acheson, D.W.K., Waldor, M.K., 2001. Human neutrophils and their products induce Shiga toxin production by enterohemorrhagic *Escherichia coli*. *Infect Immun* 69, 1934–1937. https://doi.org/10.1128/IAI.69.3.1934-1937.2001

Wagner, P. L., Neely, M.N., Zhang, X., Acheson, D.W., Waldor, M.K., Friedman, D.I., 2001. Role for a phage promoter in Shiga toxin 2 expression from a patho genic *Escherichia coli* strain. *J Bacteriol* 183, 2081–2085. https://doi.org/10.1128/JB.183.6.2081-2085.2001

Wagner, P.L., Waldor, M.K., 2002. Bacteriophage control of bacterial virulence. *Infect Immun* 70, 3985–3993. https://doi.org/10.1128/IAI.70.8.3985-3993.2002

Wang, L., Bai, X., Ylinen, E., Zhang, J., Saxén, H., Matussek, A., 2023. Genetic characterization of intimin gene (*eae*) in clinical Shiga toxin-producing *Escherichi a coli* strains from pediatric patients in Finland. *Toxins (Basel)* 15, 669. https://d oi.org/10.3390/toxins15120669

Wang, L., Rothemund, D., Curd, H., Reeves, P.R., 2003. Species-wide variation i n the *Escherichia coli* flagellin (H-antigen) gene. *J Bacteriol* 185, 2936–2943. htt ps://doi.org/10.1128/JB.185.9.2936-2943.2003

Wani, S.A., Bhat, M.A., Samanta, I., Nishikawa, Y., Buchh, A.S., 2005. *Escherichia coli* O4:NM associated with an outbreak of calf diarrhoea. *Vet J* 169, 300–302. https://doi.org/10.1016/j.tvjl.2004.01.001

Watson, V.E., Jacob, M.E., Flowers, J.R., Strong, S.J., DebRoy, C., Gookin, J.L., 2017. Association of atypical enteropathogenic *Escherichia coli* with diarrhea and related mortality in kittens. *J Clin Microbiol* 55, 2719–2735. https://doi.org/10.112 8/JCM.00403-17

Weinstein, D.L., Jackson, M.P., Samuel, J.E., Holmes, R.K., O'Brien, A.D., 1988. Cloning and sequencing of a Shiga-like toxin type II variant from *Escherichia coli* strain responsible for edema disease of swine. *J Bacteriol* 170, 4223–4230. https://doi.org/10.1128/jb.170.9.4223-4230.1988

Whelan, K.F., Colleran, E., Taylor, D.E., 1995. Phage inhibition, colicin resistance, and tellurite resistance are encoded by a single cluster of genes on the IncHI2 plasmid R478. *J Bacteriol* 177, 5016–5027. https://doi.org/10.1128/jb.177.17.5016-5027.1995

White, A.P., Sibley, K.A., Sibley, C.D., Wasmuth, J.D., Schaefer, R., Surette, M.G., Edge, T.A., Neumann, N.F., 2011. Intergenic sequence comparison of *Escherichia coli* isolates reveals lifestyle adaptations but not host specificity. *Appl Environ Microbiol* 77, 7620–7632. https://doi.org/10.1128/AEM.05909-11

WHO Scientific Working Group, 1980. *Escherichia coli* diarrhoea. Bull World Health Organ 58, 23–36.

Wieczorek, K., Osek, J., 2020. Identification and molecular characteristics of vero toxin-producing *Escherichia coli* (VTEC) from bovine and pig carcasses isolated in Poland during 2014-2018. *Food Microbiol* 92, 103587. https://doi.org/10.1016/j.fm.2020.103587

Wijnsma, K.L., Schijvens, A.M., Rossen, J.W.A., Kooistra-Smid, A.M.D., Schreuder, M.F., van de Kar, N.C.A.J., 2017. Unusual severe case of hemolytic uremic syndrome due to Shiga toxin 2d-producing *E. coli* O80:H2. *Pediatr Nephrol* 32, 1263–1268. https://doi.org/10.1007/s00467-017-3642-3

Wilczyński, J., Stępień-Pyśniak, D., Wystalska, D., Wernicki, A., 2022. Molecular and serological characteristics of avian pathogenic *Escherichia coli* isolated from various clinical cases of poultry colibacillosis in Poland. *Animals (Basel)* 12, 1090. https://doi.org/10.3390/ani12091090

Willetts, N.S., Paranchych, W., 1974. Inhibition of Flac transfer by the fin+ I-like plasmid R62. *J Bacteriol* 120, 101–105. https://doi.org/10.1128/jb.120.1.101-105.1974

Willis, L.M., Whitfield, C., 2013. KpsC and KpsS are retaining 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) transferases involved in synthesis of bacterial capsules. *Proc Natl Acad Sci U S A* 110, 20753–20758. https://doi.org/10.1073/pnas.1312637110

Yang, X., Liu, Q., Sun, H., Xiong, Y., Matussek, A., Bai, X., 2022. Genomic Characterization of *Escherichia coli* O8 strains producing Shiga toxin 2l subtype. *Microorganisms* 10, 1245. https://doi.org/10.3390/microorganisms10061245

Yang, X., Ma, Y., Chu, F., Wang, H., Sui, X., Liu, Q., Zhang, P., Bai, X., Duan, B., Xiong, Y., 2024. Characterization of *Escherichia coli* strains producing Shiga Toxin 2f subtype from domestic Pigeon. *Sci Rep* 14, 24481. https://doi.org/10.1038/s41598-024-76523-6

Yang, X., Sun, H., Fan, R., Fu, S., Zhang, J., Matussek, A., Xiong, Y., Bai, X., 2020. Genetic diversity of the intimin gene (*eae*) in non-O157 Shiga toxin-producing *Escherichia coli* strains in China. *Sci Rep* 10, 3275. https://doi.org/10.1038/s41598-020-60225-w

Yin, X., Wheatcroft, R., Chambers, J.R., Liu, B., Zhu, J., Gyles, C.L., 2009. Contributions of O island 48 to adherence of enterohemorrhagic *Escherichia coli* O157:H7 to epithelial cells in vitro and in ligated pig ileal loops. *Appl Environ Microbiol* 75, 5779–5786. https://doi.org/10.1128/AEM.00507-09

Yoshida, S., Tanaka, E., Kiuchi, Z., Nunokawa, S., Kawahara, A., Iyoda, S., Narita, M., 2024. O80:H2-associated hemolytic uremic syndrome without hemorrhagic colitis: a case report. *Case Rep Nephrol Dial* 14, 97–103. https://doi.org/10.1159/000539403

Zaheri, H., Ghanbarpour, R., Jajarmi, M., Bagheri, M., Ghanadian, A., Askari Badouei, M., 2020. Public health aspects of Shiga toxin-producing *Escherichia coli* (STEC) strains in sheep and goats of Bakhtiari pastoral tribe, Iran. *Trop Anim Health Prod* 52, 2721–2724. https://doi.org/10.1007/s11250-020-02245-2

Zalewska, M., Błażejewska, A., Gawor, J., Adamska, D., Goryca, K., Szeląg, M., Kalinowski, P., Popowska, M., 2024. A newly identified IncY plasmid from multi-drug-resistant *Escherichia coli* isolated from dairy cattle feces in Poland. *Microbiol Spectr* 12, e0087724. https://doi.org/10.1128/spectrum.00877-24

Zhang, W.L., Bielaszewska, M., Liesegang, A., Tschäpe, H., Schmidt, H., Bitzan, M., Karch, H., 2000. Molecular characteristics and epidemiological significance of Shiga toxin-producing *Escherichia coli* O26 strains. *J Clin Microbiol* 38, 2134–2140. https://doi.org/10.1128/JCM.38.6.2134-2140.2000

Zhang, W.L., Köhler, B., Oswald, E., Beutin, L., Karch, H., Morabito, S., Caprioli, A., Suerbaum, S., Schmidt, H., 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *J Clin Microbiol* 40, 4486–4492. https://doi.org/10.1128/JCM.40.12.4486-4492.2002

Zhu, C., Agin, T.S., Elliott, S.J., Johnson, L.A., Thate, T.E., Kaper, J.B., Boedeker, E.C., 2001. Complete nucleotide sequence and analysis of the locus of enterocyte effacement from rabbit diarrheagenic *Escherichia coli* RDEC-1. *Infect Immun* 69, 2107–2115. https://doi.org/10.1128/IAI.69.4.2107-2115.2001

STUDY 3 ANNEXES

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Table 2. Virulotype, metadata, Biosample numbers, and reference of calf and human AE-STEC and EPEC O80:H2, O80:nonH2 strains.Virulence-associated genes highlighted in yellow indicate virulence factors considered important for serotype O80:H2.

Host / Pathotype	ID ML-tree	Isolate collection codes[†]	Serotype	Isolation year	Clinical sign / Lesion	Age	Sex	Province / Country	NGS technology	*eae* and *stx* gene subtypes			LEE-located Type 3 Secretion System- and Type 3-secreted effector-encoding genes				Non-LEE-located Type 3 Secretion System- and Type 3-secreted effector-encoding genes							pS88 plasmid-located genes									pO157 plasmid	Genes coding for other virulence-associated factors											GenBank BioSample accession numbers	References
										eae	*stx1*	*stx2*	*espA*	*espB*	*espF*	*tir*	*nleA*	*nleB*	*nleC*	*cif*	*espI*	*espJ*	*cia*	*cvaA*	*hlyF*	*iroN*	*iss*	*ompT*	*sitA*	*etsC*	*iucC*	*espP*	*efa1*	*ehxA*	*gad*	*mchB*	*mchC*	*mchF*	*astA*	*cba*	*ireA*	*cma*	*iha*		
Calf_EPEC	Bc001	EH2850 / SES0022	O80:H2	2009	Diarrhea	UNK	UNK	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN13876229	De Rauw *et al.* 2019; Habets et al., 2021
	Bc002	EH2851 / SES0023	O80:H2	2009	Diarrhea	UNK	UNK	NAMUR	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN13876233	
	Bc003	SES0055	O80:H2	2009					MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927717	Mainil et al.,2025
	Bc004	SES0056	O80:H2	2010					MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927718	
	Bc005	SES0061	O80:H2	2010					MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927719	
	Bc006	SES0071	O80:H2	2010					MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927720	
	Bc007	SES0074	O80:H2	2010					MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927721	
	Bc008	EH2846 / SES0094	O80:H2	2010	Diarrhea	1 M	M	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	SAMN13876234	De Rauw *et al.* 2019; Habets et al., 2021
	Bc009	SES0076	O80:H2	2010					MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927722	Mainil et al.,2025
	Bc010	SES0103	O80:H2	2010					MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927723	
	Bc011	SES0087	O80:H2	2010					MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927724	
	Bc012	SES0120	O80:H2	2011	Diarrhea	UNK	UNK	HAINAUT	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927725	
	Bc013	SES0119	O80:H2	2011	Diarrhea	1 W	UNK	BRABANT WALLON	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927726	
	Bc014	SES0128	O80:H2	2011	Diarrhea	UNK	UNK	HAINAUT	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	+	-	-	-	+	+	SAMN46927727	
	Bc015	SES0130	O80:H2	2011	Diarrhea	1W	UNK	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927728	
	Bc016	SES0136	O80:H2	2011	Diarrhea	UNK	UNK	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927729	
	Bc017	EH2845 / SES0150	O80:H2	2012	Diarrhea	6.5 M	M	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN13876235	De Rauw *et al.* 2019; Habets et al., 2021
	Bc018	SES0145	O80:H2	2012	Diarrhea	UNK	UNK	NAMUR	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927730	Mainil et al.,2025
	Bc019	EH2849 / SES2812	O80:H2	2014	Diarrhea	16 D	M	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14087589	De Rauw *et al.* 2019; Habets et al., 2021
	Bc020	SES0194	O80:H2	2013	Enteritis	UNK	M	NAMUR	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927731	Mainil et al.,2025
	Bc021	SES0205	O80:H2	2013	Diarrhea	UNK	UNK	HAINAUT	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927732	
	Bc022	SES0213	O80:H2	2013	Diarrhea	UNK	UNK	HAINAUT	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927733	
	Bc023	SES0214	O80:H2	2013	Enteritis	1 M	UNK	HAINAUT	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927734	
	Bc024	EH2847 / SES0212	O80:H2	2013						ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927735	
	Bc025	SES0215	O80:H2	2013	Diarrhea	UNK	UNK	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927736	
	Bc026	SES2779	O80:H2	2014	Diarrhea	UNK	UNK	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927737	
	Bc027	SES2775	O80:H2	2014	Septicemia	3 W	M	HAINAUT	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927738	
	Bc028	SES2807	O80:H2	2014	Diarrhea	UNK	UNK	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	-	-	SAMN46927739	
	Bc029	SES2876	O80:H2	2014	Enteritis	1 M	F	BRABANT WALLON	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927740	
	Bc030	EH2848 / SES2858	O80:H2	2015	Diarrhea	17 D	M	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN13876249	De Rauw *et al.* 2019; Habets et al., 2021
	Bc031	EH3299 / SES2789	O80:H2	2015	Enteritis, pneumonia	3 M	F	HAINAUT	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14086249	Mainil et al.,2025
	Bc032	EH3300 / SES2790	O80:H2	2015	Enteritis	UNK	UNK	LUXEMBOURG	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14086250	
	Bc033	EH3301 / SES2860	O80:H2	2015	Enteritis	UNK	UNK	HAINAUT	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14086251	
	Bc034	EH3303 / SES2853	O80:H2	2015	Septicemia, salmonellosis	2 W	M	LUXEMBOURG	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	-	-	+	-	SAMN14086255	
	Bc035	EH3304 / SES2851	O80:H2	2015	Septicemia, salmonellosis	10 D	F	LUXEMBOURG	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14086265	
	Bc036	EH3305 / SES2818	O80:H2	2015	Enteritis	2 W	F	LUXEMBOURG	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14086266	
	Bc037	EH3306 / SES2953	O80:H2	2015	Diarrhea	UNK	UNK	LUXEMBOURG	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14086268	
	Bc038	EH3308 / SES2973	O80:H2	2016	Diarrhea	UNK	UNK	LUXEMBOURG	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14086269	
	Bc039	EH3309 / SES2987	O80:H2	2016	Hemorrhagic colitis	1 M	F	LUXEMBOURG	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14086270	
	Bc040	EH3312 / SES3019	O80:H2	2017	Diarrhea	UNK	UNK	BRABANT WALLON	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14086995	
	Bc041	EH3313 / SES3022	O80:H2	2017	Hemorrhagic colitis	1 W	M	HAINAUT	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14087009	
	Bc042	EH3314 / SES3024	O80:H2	2017	Enteritis	UNK	F	GD Luxembourg	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14087010	
	Bc043	EH3318 / SES3069	O80:H2	2017	Septicemia	1 W	M	HAINAUT	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087011	
	Bc044	EH3319 / SES3073	O80:H2	2017	Diarrhea	UNK	UNK	LUXEMBOURG	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	SAMN14087012	
	Bc045	EH3322 / SES3122	O80:H2	2018	Pleuropneumonia	1 W	M	NAMUR	NovaSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087013	
	Bc046	SES5423	O80:H2	2018	Diarrhea	1 W	UNK	HAINAUT	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927741	
	Bc047	SES5422	O80:H2	2018	Diarrhea	2 M	UNK	HAINAUT	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN46927742	
	Bc048	SES5291	O80:H2	2019	Diarrhea	UNK	UNK	NAMUR	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN46927743	
	Bc049	SES5292	O80:H2	2019	Diarrhea	UNK	UNK	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927744	
	Bc050	SES5294	O80:H2	2019	Diarrhea	1 W	UNK	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927745	
	Bc051	SES5295	O80:H2	2019	Diarrhea	1 W	UNK	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927746	
	Bc052	SES5318	O80:H2	2019	Diarrhea	UNK	UNK	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927747	
	Bc053	SES5362	O80:H2	2019	Diarrhea	1 D	UNK	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927748	
	Bc054	SES5378	O80:H2	2020	Enteritis	1 D	M	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927749	
	Bc055	SES5385	O80:H2	2020	Enteritis, pneumonia	1 M	F	LUXEMBOURG	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927750	
	Bc056	SES6199	O80:H2	2021	Diarrhea	UNK	UNK	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	SAMN46927751	
	Bc057	SES6256	O80:H2	2023	Enteritis	18 D	M	LIEGE	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN46927752	
	Bc058	SES6258	O80:H2	2023	Diarrhea	1 W	UNK	NAMUR	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927753	
	Bc059	SES6268	O80:H2	2023	Diarrhea	UNK	UNK	NAMUR	MiSeq	ξ	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	SAMN46927754	
Calf_AE-STEC	Bc060	EH2882 / FMV36819_3[‡]	O80:H2	1987	Diarrhea	2.5 M	UNK	BRABANT WALLON	MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	SAMN13876256	De Rauw et al. 2019; Habets et al., 2021
	Bc061	EH2883 / FMV36819_5[‡]	O80:H2	1987					MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	-	-	+	SAMN13876258	
	Bc062	SES0057	O80:H2	2010					MiSeq	ξ	-	f	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SAMN46927755	Mainil et al.,2025
	Bc063	SES0108	O80:H2	2011	Diarrhea	UNK	UNK	NAMUR	MiSeq	ξ	-	f	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	-	-	SAMN46927756	
	Bc064	EH3307 / SES2959	O80:H2	2016	Diarrhea	UNK	UNK	HAINAUT	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14087032	De Rauw et al. 2019; Habets et al., 2021
	Bc065	EH3310 / SES3008	O80:H2	2016	Diarrhea	UNK	UNK	LUXEMBOURG	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087033	Mainil et al.,2025
	Bc066	EH3311 / SES3009	O80:H2	2016	Diarrhea	UNK	UNK	HAINAUT	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-	-	+	SAMN14087039	
	Bc067	EH3315 / SES3038	O80:H2	2017	Diarrhea	UNK	UNK	HAINAUT	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-	-	+	SAMN14087040	
	Bc068	EH3317 / SES3064	O80:H2	2017	Enteritis	1 W	F	LUXEMBOURG	NovaSeq	ξ	-	?[£]	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087041	
	Bc069	EH3320 / SES3090	O80:H2	2017	Diarrhea	UNK	UNK	HAINAUT	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087146	
	Bc070	EH3154 / RU_BLA947	O80:H2	2018	Enteritis, pneumonia	4 W	F	NAMUR	HiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	SAMN14087147	
	Bc071	EH3155 / RU_BLA1040[§]	O80:H2	2018	Enteritis	1 W	F	NAMUR	HiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087149	
	Bc072	EH3160 / RU_BLA1042[§]	O80:H2	2018					HiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087151	
	Bc073	EH3338 / RU_BLA1043[§]	O80:H2	2018	Septicemia				NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087161	
	Bc074	SES5287	O80:H2	2018	Diarrhea	15 D	UNK	LUXEMBOURG	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927757	
	Bc075	SES5298	O80:H2	2019	Enteritis	2 W	M	LUXEMBOURG	MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927758	
	Bc076	SES5314	O80:H2	2019	Septicemia	1 W	M	LUXEMBOURG	MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927759	
	Bc077	SES5320	O80:H2	2019	Diarrhea	1 W	UNK	LUXEMBOURG	MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	+	SMAN35130764	Ikeda et al., 2023b
	Bc078	SES5336	O80:H2	2019	Septicemia	UNK	F	HAINAUT	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927760	Mainil et al.,2025
	Bc079	SES5341	O80:H2	2019	Diarrhea	2 W	UNK	NAMUR	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927761	
	Bc080	SES5348	O80:H2	2019	Diarrhea	UNK	UNK	LUXEMBOURG	MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927762	
	Bc081	SES5363	O80:H2	2019	Enteritis	1 W	M	LUXEMBOURG	MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SMAN35130765	Ikeda et al., 2023b
	Bc082	SES5369	O80:H2	2019	Diarrhea	UNK	UNK	LUXEMBOURG	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN46927763	Mainil et al.,2025
	Bc083	SES5388	O80:H2	2020	Enteritis	2 W	M	LIEGE	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	SAMN46927764	
	Bc084	SES6209	O80:H2	2021	Diarrhea	3 M	UNK	LUXEMBOURG	MiSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN46927765	
	Bc085	SES6211	O80:H2	2021	Diarrhea	2 W	F	HAINAUT	MiSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927766	
	Bc086	SES6226	O80:H2	2021	Diarrhea	UNK	UNK	LIEGE	MiSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927767	
	Bc087	SES6283	O80:H2	2023	Enteritis	10 D	M	LUXEMBOURG	MiSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN46927768	
	Bc088	SES6292	O80:H2	2023	Diarrhea	UNK	UNK	LIEGE	MiSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN46927769	
Human_AE-STEC	Bh001	EH1752	O80:H2	2008	Diarrhea	1 Y	F	BRABANT WALLON	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	SAMN14087242	De Rauw et al. 2019; Habets et al., 2021
	Bh002	EH1764	O80:H2	2008	Diarrhea	72 Y	F	UNK	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	SAMN14087243	

	Bh003	EH2069	O80:H2	2011	Diarrhea	1 Y	F	WEST VLAANDEREN	NovaSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+		unpublished
	EB241	EH2262	O80:H2	2013	Diarrhea	49 Y	F	VLAAMSE BRABANT	MiSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	SAMN14087244	De Rauw et al. 2019; Habets et al., 2021
	Bh004	EH2400	O80:H2	2014	Diarrhea	61 Y	F	WEST VLAANDEREN	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	SAMN14087245	
	Bh005	EH2436	O80:H2	2014	UNK	78 Y	F	BRABANT WALLON	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+	-	-	-	-	-	SAMN14087253	
	Bh006	EH2549	O80:H2	2015	Bloody diarrhea	60 Y	F	ANTWERPEN	MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	-	+	+	+	+	+	+	+	-	+	-	-	+	SAMN14087304	
	Bh007	EH2644	O80:H2	2015	Diarrhea/HUS	11 M	F	BRABANT WALLON	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14087454	
	Bh008	EH2786	O80:H2	2016	UNK	2 Y	F	BRUSSELS CAPITAL	MiSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14087455	
	Bh009	EH2808	O80:H2	2016	HUS	61 Y	F	UNK	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	SAMN14087456	
	EB101	EH3045	O80:H2	2018	Diarrhea	68 Y	M	LIEGE	HiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	SAMN14090730	
	EB087	EH3138	O80:H2	2018	Bloody diarrhea	63 Y	F	OOST VLAANDEREN	MiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	SAMN14090731	
	EB085	EH3168	O80:H2	2018	Bloody diarrhea	72 Y	F	ANTWERPEN	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14090732	
	EB086	EH3172	O80:H2	2018	Diarrhea	66 Y	F	LIEGE	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14090733	
	EB083	EH3173[¶]	O80:H2	2018	UNK	1 Y	M	HAINAUT	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14090734	
	EB084	EH3180[¶]	O80:H2	2018	UNK	4 Y	M	HAINAUT	MiSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14090735	
	EB088	EH3205	O80:H2	2019	Diarrhea	33 Y	M	OOST VLAANDEREN	HiSeq	ξ	a	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	SAMN14090736	
	EB089	EH3212	O80:H2	2019	Diarrhea/HUS	1 Y	F	BRUSSELS CAPITAL	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14090737	
	EB102	EH3257	O80:H2	2019	Bloody diarrhea/HUS/Death[$]	1 Y	M	NAMUR	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14090738	
	EB103	EH3278	O80:H2	2019	Diarrhea/HUS	11 M	F	HAINAUT	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN14090739	
	EB148	EH3467	O80:H2	2020	UNK	62 Y	F	OOST VLAANDEREN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA112721049	Nouws et al., 2023
	EB192	EH3505	O80:H2	2020	Bloody diarrhea	17 Y	F	ANTWERPEN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA112729147	Nouws et al., 2023
	EB223	EH3539	O80:H2	2021	Diarrhea	65 Y	F	LIEGE	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	ESC_XA0926AA	unpublished
	EB222	EH3551	O80:H2	2021	UNK	26 Y	F	LIEGE	NovaSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	ESC_XA0914AA	
	EB245	EH3661	O80:H2	2022	Diarrhea	70 Y	F	ANTWERPEN	NovaSeq	ξ	a	-	+	+	+	+	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	+	ESC_AB4971AA	
	EB270	EH3821	O80:H2	2022	Diarrhea	25 Y	F	OOST VLAANDEREN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	ESC_CB4954AA	
	EB275	EH3837	O80:H2	2022	UNK/HUS/Death	59 Y	M	VLAAMSE BRABANT	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	ESC_DB7710AA	
	EB276	EH3876	O80:H2	2023	Diarrhea	92 Y	F	OOST VLAANDEREN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA114295715	
	EB313	EH4030	O80:H2	2023	Bloody diarrhea	1 Y	F	OOST VLAANDEREN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115368896	
	EB329	EH4163	O80:H2	2023	Diarrhea	18 Y	F	LIEGE	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115376150	
	EB330	EH4167	O80:H2	2023	UNK	1 Y	F	ANTWERPEN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115376157	
	EB331	EH4173	O80:H2	2023	Diarrhea	12 Y	M	LIEGE	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115376164	
	EB332	EH4177	O80:H2	2023	Diarrhea	75 Y	F	ANTWERPEN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115376168	
	EB333	EH4193	O80:H2	2023	Bloody diarrhea	79 Y	M	ANTWERPEN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115376179	
	EB335	EH4228	O80:H2	2023	Diarrhea	84 Y	M	OOST VLAANDEREN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	SAMEA115376210	
	EB336	EH4233	O80:H2	2023	Bloody diarrhea	66 Y	M	ANTWERPEN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115376218	
	Bh010	EH4296	O80:H2	2024	Diarrhea	81 Y	M	OOST VLAANDEREN	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115975597	
	EB353	EH4319	O80:H2	2024	UNK	1 Y	F	LIEGE	NovaSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115784826	
	Bh011	EH4328	O80:H2	2024	Diarrhea	71 Y	F	LIEGE	NovaSeq	-	-	d	-	-	-	-	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	+	-	-	-	-	-	SAMEA115975637	
	Bh012	EH4329	O80:H2	2024	UNK	70 Y	M	VLAAMSE BRABANT	NovaSeq	ξ	-	d	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115975583	
	Bh013	EH4367	O80:H2	2024	UNK	1 Y	M	LUXEMBOURG	NovaSeq	ξ	-	a	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMEA115975592	
	C033	RDEx444	O80:H2	2016	HUS	---	---	FRANCE		ξ	-	d	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	SAMN08915508	Cointe et al., 2018
	ref	2013C-4991	O80:H2	2013	---	---	---	USA	PacBio	ξ	-	d	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	+	+	+	+	-	-	-	-	+	SAMN08579568	Patel *et al.* 2018
O80:nonH2																																													
Serotype collection	H26_1	EH3161	O80:H26	+/-1960	?		?	?	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN35130763	Ikeda et al., 2023b (Séquencée par Klara)
AO 3426-2 (poultry)	H26_2	EH3162 / AV431	O80:H26	1999	?		?	FRANCE	MiSeq	β	-	-	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	SAMN08579568	Schouler et al., 2012 (Séquencée par Klara)
ES 351 (poultry)	H26_3	EH3164 / AV1112	O80:H26	1991	?		?	SPAIN	MiSeq	β	-	-	+	+	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-		
ES 465 (poultry)	H26_4	EH3165 / AV1241	O80:H26	1992	?		?	SPAIN	MiSeq	-	-	-	-	-	-	-	+	+	-	+	-		-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-		
AP 1353 (turkey)	H19_1	EH3166 / AV1390	O80:H19	2000	?		?	FRANCE	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-		
Serotype collection	---	---	O78:H4	+/-1960	?		?	?		-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	SAMN35130762	Ikeda et al., 2023b (Séquencée par Keiji)
O80:nonH2 from heathy adult cattle																																													
b_Abattoir O80:nonH2	SES5589		O80:H45		2019			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092019	Ikeda et al., 2023a
	SES5725		O80:H45		2019			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092020	
	SES5726		O80:H45		2019			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092021	
	SES5845		O80:H45		2019			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092022	
	SES5849		O80:H45		2019			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092023	
a_duck strain	CanClin81		O80:H45		2009/10			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	SAMN32092025	
b_Farms O80:nonH2	SES6039		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092024	Ikeda et al., 2023a
	SES6040		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092025	
	SES6041		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092026	
	SES6042		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092027	
	SES6043		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092028	
	SES6044		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092029	
	SES6045		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092030	
	SES6046		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092031	
	SES6047		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092032	
	SES6048		O80:H6		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092033	
	SES6156		O80:H45		2021/2022			BELGIUM	MiSeq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	SAMN32092034	

Table 3. Description of each similar sequence searched by Web BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Subject	Strain Description	Scientific Name	Serotype	isolation source	Query Coverage (%)	Per. ident (%)	Accession No.	Reference
STX2f phage	2015C-4136CT1 chromosome	*Escherichia coli*	O145:H34	Human:	100%	99.49	CP027550.1	Patel et al., 2018
	377323_2f chromosome	*Escherichia coli*	O63:H6	Human	100%	99.44	CP039404.1	Greig, unpublish 2019
	2011C-4180 chromosome,	*Escherichia albertii*	-	Human	99%	99.43	CP126912.1	Carter et al., 2023
STX2f phage (Region including phage morphogenesis genes and the terminal part of SES0057)	2013C-4991 chromosome,	*Escherichia coli*	O80:H2	Human	98%	98.86	CP027355.1	Patel et al., 2018
	97-3250 chromosome,	*Escherichia coli*	O26:H11	Human	99%	98.46	CP027599.1	Patel et al., 2018
	2003-3014 chromosome,	*Escherichia coli*	O26:H11	Human	96%	98.36	CP101292.1	Kalalah and Eppinger, unpublish 2022
	99-3311 chromosome,	*Escherichia coli*	O145	Human	92%	98.26	CP101310.1	Kalalah and Eppinger,unpublish 2022
	O145:H28 112648 DNA,	*Escherichia coli*	O145:H28	Human	92%	98.22	AP019706.1	Nakamura et al., 2020
	O145 strain RM12275-C1 chromosome,	*Escherichia coli*	O145:H28	Unknown	92%	98.22	CP031341.1	Carter and Pham, unpublish 2020
	Stx1a-converting phage Stx1_1380 DNA,	*Stx1a-converting phage Stx1_1380*	O145:H28	Unknown	82%	97.38	LC645433.1	Nakamura and Hayashi, unpublish 2020
***nleG* (SES0057)**	O157:H7 strain MB41-1 chromosome,	*Escherichia coli*	O157:H7	Cattle	100%	100	CP039834.1	Bono, unpublish, 2020
	2013C-4991 chromosome,	*Escherichia coli*	O80:H2	Human	100%	100	CP027355.1	Patel et al., 2018
	O26:H11 strain FWSEC0001 chromosome,	*Escherichia coli*	O26:H11	Human	100%	99.9	CP031922.1	Tyson et al., unpublish, 2019
***cma* (SES0057)**	APEC E19025 plasmid pEND_Eco 19025-1,	*Escherichia coli*	O78:H51	Chicken	100%	100	CP126932.1	Ha, unpublish 2023
	APEC E18005 plasmid pEND_Eco 18005,	*Escherichia coli*	O78:H51	Chicken	100%	100	CP126947.1	Ha, unpublish 2023
	APEC E12049 plasmid pEND_Eco 12049-1,	*Escherichia coli*	O78:H4	Chicken	100%	100	CP126953.1	Ha, unpublish 2023
	ExPEC_A186 plasmid pA186_p1,	*Escherichia coli*	Unknown	Swine	100%	100	CP142551.1	Li et al., unpubloish 2024
	N921 plasmid pN921_1,	*Escherichia coli*	Unknown	Human	100%	100	CP176482.1	Dona et al., unpublish 2024
	EC12 plasmid pEC12_2,	*Escherichia coli*	Unknown	Avian	100%	100	CP095832.1	Dona et al., unpublish 2024
	EC31 plasmid pEC31_1,	*Escherichia coli*	Unknown	Avian	100%	100	CP095837.1	Dona et al., unpublish 2024
	FY10 plasmid pFY10_1,	*Escherichia coli*	Unknown	Avian	100%	100	CP095817.1	Dona et al., unpublish 2024
SpLE1-like element (SES0057)	2013C-4991 chromosome,	*Escherichia coli*	O80:H2	Human	100%	99.68	CP027355.1	Patel et al., 2018
	CE0007a chromosome,	*Escherichia coli*	O155:K:H	Cattle (calf)	100%	99.28	CP119011.1	Burgess et al., unpublish 2023
	O104:H4 strain FWSEC0009 chromosome,	*Escherichia coli*	O104:H4	Human	95%	99.71	CP031902.1	Tyson et al., unpublish, 2019
	1070/13 chromosome,	*Escherichia coli*	O104:H4	Human	95%	99.72	CP171474.1	Whelan et al., unpublish 2024
	Escherichia coli O104:H4 strain LB226692 chromosome	*Escherichia coli*	O104:H4	Human	95%	99.71	CP171472.1	Whelan et al., unpublish 2024
	Escherichia coli O104:H4 strain FDAARGOS_349 chromosome,	*Escherichia coli*	O104:H4	Human (HUS)	95%	99.72	CP024992.1	Saile et al., 2018
	Escherichia coli O104:H4 strain FDAARGOS_348 chromosome,	*Escherichia coli*	O104:H4	Human (HUS)	95%	99.72	CP027394.1	Minogue et al., unpublish 2019
***cdt* (SES0108)**	Escherichia coli strain STECUZB005 chromosome, complete genome	*Escherichia coli*	O63:H6	Human	100%	100	CP171880.1	Crombé et al., 2024
	Escherichia coli strain STECUZB006 chromosome, complete genome	*Escherichia coli*	O157:H7	Human	100%	100	CP171880.3	Crombé et al., 2024
	Escherichia coli strain 377323_2f chromosome,	*Escherichia coli*	O63:H6	Human	100%	100	CP039404.1	Greig, unpublish, 2019
	Escherichia coli strain 2015C-4136CT1 chromosome,	*Escherichia coli*	O145:H34	Human	100%	100	CP027550.1	Patel et al., 2018
	Escherichia albertii strain 2011C-4180 chromosome,	*Escherichia albertii*	-	Human	100%	99.93	CP126912.1	Carter et al., 2023
	O1:K1:H7 strain APEC E18006 chromosome,	*Escherichia coli*	O1:K1:H7	Chicken	100%	99.28	CP126942.1	Ha, unpublish 2023
	O1:K1:H7 strain APEC E16002 chromosome,	*Escherichia coli*	O1:K1:H7	Chicken	100%	99.28	CP126944.1	Ha, unpublish 2023
	O78:H4 strain APEC E12049 chromosome,	*Escherichia coli*	O78:H4	Chicken	100%	99.28	CP126952.1	Ha, unpublish 2023
	N441 plasmid pN441 DNA,	*Escherichia coli*	Unknown	Human	97%	99.9	LC567071.1	Suzuki et al., unpublish 2024

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