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Emerging hybrid shigatoxigenic and enteropathogenic *Escherichia coli* serotype O80:H2 in humans and calves

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SUMMARY Attaching-effacing (AE) lesion- and Shiga toxin-producing *Escherichia* (*E.*) *coli* (AE-STEC), previously known as "enterohemorrhagic *E. coli*" (EHEC), are responsible for (hemorrhagic) enterocolitis (HC) and hemolytic uremic syndrome (HUS) in humans. The most frequent and pathogenic AE-STEC belong to a few O:H major serotypes that are responsible for the majority of cases and outbreaks worldwide. From time to time, one or another non-major O:H serotype can emerge, causing either local outbreaks or a progressive increase in clinical cases. One of these minor serotypes is O80:H2, which

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has been progressively emerging in Western Europe, especially in France, since 2010. AE-STEC O80:H2 are responsible for not only HC and HUS but also invasive infections with bacteremia and internal organ infection. In parallel to their emergence in humans, AE-STEC and enteropathogenic *E. coli* (EPEC) O80:H2 have also been emerging in young calves suffering diarrhea and enteritis and, more rarely septicemia, in Belgium since 2009. In this manuscript, an overview of AE-STEC and EPEC O80:H2 infections in humans and calves is presented, with particular focus on the clinical manifestations, the prevalence and incidence in Western Europe, and the identification of the potential reservoir(s). In addition, the results of a large-scale whole genome-based phylogenetic analysis of 417 published and unpublished genome sequences currently available in the literature and in the NCBI and EnteroBase databases are presented with hypotheses on the origin and evolution of this new hybrid AE-STEC and EPEC serotype.

KEYWORDS *Escherichia coli*, STEC, EPEC, O80:H2, humans, calves, hemorrhagic colitis, hemolytic uremic syndrome, bacteremia, septicemia, virulotyping, *eae* gene, *stx* genes, pS88 plasmid, pR444_A plasmid, phylogenetics, evolution, reservoir

INTRODUCTION

In 1980, the World Health Organization (WHO) published a report on diarrheagenic *Escherichia coli* (DEC) in humans and animals, describing “three [patho]types of *Escherichia* (*E.*) *coli*–enterotoxigenic (ETEC), enteropathogenic (EPEC), and enteroinvasive (EIEC)–that play important roles in the aetiology of acute diarrhoea” (1).

ETEC produce heat-stable and/or heat-labile enterotoxins and are important causes of diarrhea in infants, young children, and adults in developing countries, in travelers to these countries, in newborn farm animals (calves, piglets, and lambs), and in weaned piglets. EIEC have a pathological behavior similar to *Shigella* sp., invading the enterocytes and causing a dysentery-like syndrome in humans. EIEC have not been described in domestic animals. Both ETEC and EIEC belong to specific *E. coli* serotypes. Finally, the EPEC are associated with infantile diarrhea in many parts of the world and belong to specific serotypes, different from the serotypes of ETEC and EIEC. Nevertheless, EPEC were rather negatively defined: they neither produce any enterotoxins nor invade the enterocytes, although their actual diarrheagenic role had already been confirmed in 1978 by inoculating human volunteers (2). The WHO report ends with the description of research needs, more especially on EPEC to unveil their actual epidemiology, pathogenesis, and virulence properties.

During the following years, pathogenic mechanisms and virulence properties of EPEC were progressively unraveled, and different new pathotypes and acronyms of DEC were described not only in humans but also in animals: shigatoxigenic *E. coli* (STEC; synonymous with verotoxigenic *E. coli* or VTEC), attaching and effacing *E. coli* (AEEC), enterohemorrhagic *E. coli* (EHEC), enteroadherent *E. coli* (EAEC), diffusely-adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAggEC), necrotoxigenic *E. coli* (NTEC), etc (3–8). Several of these acronyms are redundant, whereas others are only rarely or even no longer used today. In addition, hybrid DEC have also been described combining different pathogenic mechanisms and virulence properties.

Reviews on the different pathotypes of DEC in humans and domestic animals have frequently been published since the years 1980s (9–17). After a general presentation of the shigatoxigenic and enteropathogenic *E. coli* in humans and cattle, the purpose of this manuscript was to review the current knowledge and understanding of the emerging STEC and EPEC serotype O80:H2 pathogens.

SHIGATOXIGENIC AND ENTEROPATHOGENIC *E. COLI* (STEC AND EPEC)

Definitions

Shigatoxigenic *E. coli* (STEC) are defined by the production of cytotoxins related to the Shiga toxin (Stx) of *Shigella dysenteriae* serotype 1 (3, 4, 10). *E. coli* Stx (synonymous with Verocytotoxins or VT) are AB_5 toxins and are divided into two families: Stx1 closely related to the Stx of *S. dysenteriae* with three variants (a,c,d) and Stx2 more distantly related to the Stx with 11 variants (a–k). They are produced in the intestinal tract and can cross the enterocytes by transcytosis. After reaching the bloodstream, they are transported by leucocytes to reach the endothelial cells of the small arteries and capillaries. After binding via the B subunits to the receptor on target cells, Stx are internalized, and the A subunit inhibits the protein synthesis by cleaving the 28S ribosomal RNA, causing cell death, microangiopathic damage, and small hemorrhages in the internal organs. *stx* genes are located on the genomes of temperate lambdoid bacteriophages (Stx phages) and can be transduced between different *E. coli* strains *in vivo* and *in vitro*. Conversely, Stx phages can be lost *in vitro* during storage or subcultures (18–23).

Attaching-effacing *E. coli* (AEEC) are defined by the production of the histological attaching and effacing (AE) lesion characterized by the effacement of the enterocyte microvilli and the intimate attachment of the bacterial cells to the nude enterocyte cytoplasmic membrane with the formation of a pedestal under the bacterial cells (5, 10). AEEC harbor a specific pathogenicity island (PAI), the locus of enterocyte effacement (LEE) that is responsible for the formation of the AE lesion. The LEE-located genes encode a type-3 secretion system (T3SS) and T3SS-secreted effectors that cause a cytoskeleton rearrangement in the enterocytes, leading to the effacement of the microvilli and the formation of the pedestal. The intimate attachment is mediated by one bacterial outer membrane protein adhesin, the intimin encoded by the LEE-located *eae* gene. More than 30 variants of the *eae* gene have been described that are designed by Greek letters (10, 24–28).

Enterohemorrhagic *E. coli* (EHEC) are a hybrid pathotype combining the pathogenic mechanisms and virulence properties of STEC and AEEC (10). Today, however, the EHEC acronym is considered obsolete, and the recommendation is to keep the STEC acronym for all strains producing Stx toxins (27). However, keeping only the STEC acronym can bring confusion since different hybrid STEC have been described. Therefore, use of combined acronyms based on the presence of another virulence-associated property was proposed (29): AE-STEC for strains also producing the AE lesion (that will be used throughout this manuscript), F18-STEC for strains causing the edema disease in weaned piglets, Agg-STEC for strains with an aggregative pattern of adherence on cells in culture like the STEC O104:H4, etc. STEC strains with no other property identified to keep the STEC acronym. Besides the production of the AE lesion and of Stx, another early described marker of several AE-STEC is the production of enterohemolysin (eHly) encoded by the *ehxA* gene that is located on the pO157 (or pEHEC) plasmid. Production of eHly cannot be detected by growing the strains on classical sheep blood agar plates, but only on Ehly agar plates containing washed red blood cells (11, 30).

AEEC, which do not produce any Stx, are still named enteropathogenic *E. coli* (EPEC) today. Their key virulence property is the production of the AE lesion. In earlier years, they were sometimes referred to as EPEC *sensu stricto* to differentiate them from the EPEC of the WHO report that were named EPEC *sensu lato*. For the sake of clarity, the acronym EPEC will be used in this manuscript to refer to those non-Stx-producing AEEC strains. Like several AE-STEC, EPEC can also produce eHly (24, 31).

AE-STEC in humans

In humans, AE-STEC are responsible for (hemorrhagic) enterocolitis (HC) with as sequelae the hemolytic uremic syndrome (HUS). After ingestion, AE-STEC colonize the intestinal tract producing the AE lesion and the Stx. The Stx are responsible for damage to the microvascular endothelium at the height of the intestinal cell wall and kidney glomeruli, causing HC and HUS, and in most severe cases in the brain and other organs (10, 18, 32).

Human AE-STEC belong to scores of O:H serotypes whose pathogenicity and epidemiological importance worldwide differ (15, 30). In 2003, a classification of AE-STEC and non-AE-STEC in five seropathotype groups (A–E) was proposed to define the risk assessment of the different AE-STEC based on these two features (33). Serotypes of groups A (O157:H7) and B (O26:H11, O103:H2, O111:H8, and O145:H28) are the most pathogenic AE-STEC with the highest epidemiologically incidence in humans. They are called the “major serotypes” and are more or less frequently responsible for outbreaks worldwide. The pathogenicity for humans and the epidemiological incidence of the serotypes of groups C and D that are either AE-STEC or non-AE-STEC differ between serotypes. Nevertheless, some of them can also cause dramatic, although usually short-lived and more local outbreaks. Finally, serotypes of group E, which are also either AE-STEC or non-AE-STEC, are considered nonpathogenic or very little pathogenic for humans, since they have never been isolated from humans up to date. Serotypes of groups C, D, and E are called the “minor serotypes.” These subdivisions are however not fixed, and some serotypes can move from one group to the other along the years, as for instance, serotypes O45:H2, O121:H19, and O165:H25 that are sometimes placed in group B (30, 34, 35). Moreover, the concept of seropathotype groups is challenged today because not all (AE-)STEC infections are fully characterized and coupled with reliable clinical information. Independent of the serotype, risk assessment of AE-STEC should today be based on the detection of the Stx subtypes (the Stx2a or Stx2d subtypes are considered the most pathogenic *in vivo*), *eae* gene, and other (virulence-associated) marker-encoding genes, like type III effector-encoding genes located in non-LEE genomic regions, identified by PCR and/or genome sequencing, whose full description is, however, beyond the scope of this manuscript (27, 30, 36–42).

Different ways of human infections by AE-STEC have been described, but the most frequent one is via foodstuffs (meat, dairy products, and vegetables) contaminated with fecal materials from healthy young and adult domestic and wild ruminants, most frequently cattle carrying (AE-)STEC in the intestinal tract (15, 30, 35, 43–45).

AE-STEC in calves

Besides their presence in healthy young and adult ruminants, a few AE-STEC serotypes are naturally and experimentally responsible for enteritis and diarrhea in <3-month-old young calves via the production of the AE lesions. Conversely, HC and HUS are not observed in calves. It has long been hypothesized that the absence of receptors on enterocytes was the cause of this lack of Stx toxicity in cattle, but Gb3 has been recently detected on bovine intestinal and renal cells. The actual reason is that Stx localize in the lysosomes of the enterocytes, leading to abrogation of transcytosis (46, 47). The most frequent serotypes are O26:H11 and O111:H-, two members of seropathotype group B, and O5:H-, a member of seropathotype group C. A few other serotypes are described from time to time, like O118:H16 last century and O103:H2, another member of seropathotype group B. Human and calf AE-STEC belonging to the same serotype are genetically related (11, 13, 14, 24, 48–51).

EPEC in humans and calves

EPEC are subdivided into typical (t) EPEC that harbor a specific “*E. coli* adherence factor” (EAF) plasmid carrying, among others, the genes coding for the bundle forming pili (BFP) and atypical (a) EPEC. tEPEC are almost exclusively present in humans and belong to host-specific serotypes and virulotypes, whereas aEPEC are isolated from humans and different animal species suffering non-bloody diarrhea, including young calves, and belong to several different O:H serotypes (10, 15, 52). The most frequent aEPEC isolated from diarrheic calves belong to serotype O26:H11. Although a few other serotypes have been identified, the majority of the calf aEPEC associated with diarrhea belong to still unidentified serotypes (31, 44, 51, 52).

Human and calf aEPEC are genetically related to AE-STEC belonging to the same O:H serotype (11, 13, 14, 24, 30, 31, 44, 50). For instance, aEPEC and AE-STEC O26:H11 share the presence of identical *eae* gene variants, the production eHly and IS*621* profiles. Therefore, several aEPEC may originate from AE-STEC after loss of the *stx* genes or Stx phages (aEPEC-like) or represent precursors of AE-STEC before acquisition of the *stx* genes by Stx phage infections. The different (virulence-associated) markers of AE-STEC have also been tested to increase the probability of identifying aEPEC-like from true aEPEC, especially within the major O:H serotypes with more or less discriminatory power (30, 40). Of these, the O genomic island (OI)–50- and OI-44-located type III effector-encoding *espK* and *espV* genes have recently received special attention (see "Genomic comparison and whole-genome sequence-based phylogenetics of human and calf AE-STEC and EPEC O80:H2" and "Reservoir(s) and detection of AE-STEC and EPEC O80:H2").

Here too, for the sake of clarity, the acronym EPEC will be used instead of aEPEC from now on.

ATTACHING-EFFACING SHIGATOXIGENIC AND ENTEROPATHOGENIC *E. COLI* (AE-STEC AND EPEC) O80:H2 IN DISEASES

Some years ago, an "unusual" AE-STEC serotype, O80:H2 was reported in France as an increasing cause of severe and fatal cases of HUS in infants, children, teenagers, but rarely in adults (53, 54). Until the year 2010, the serotype O80:H2 was indeed a member of the seropathotype groups C or D and had never been responsible for any large numbers of clinical cases. Today, the situation has changed and the specific objectives of the following sections are to review the knowledge on AE-STEC and EPEC O80:H2 with respect to (i) their association with diseases in humans and calves; (ii) the published microbiological data of the strains isolated from humans and calves; (iii) the phylogenetic analysis of published and unpublished genome sequences, currently available in the literature and in NCBI and EnteroBase databases; and (iv) the search for their reservoir(s).

E. coli O80

The somatic serogroup O80

The thermostable somatic "O" surface antigens of *E. coli* are the variable part of the polysaccharide moiety of the lipopolysaccharide, whose lipid A moiety is embedded in the bacterial outer membrane. During the years 1940s, 110 O serogroups, including the serogroup O80, were successively defined. The flagellar antigen H26 was early associated with the serogroup O80, but no capsular or capsular-like K antigen was described. Until the years 1980s, *E. coli* O80 was not associated with disease, either in humans or in animals in the literature. Therefore, the serogroup O80 was not included in any early serotyping scheme of disease-associated *E. coli* (55–57).

Association with diseases

To the authors' knowledge, the first associations of serogroup O80 with disease were reported in the years 1980s with the description of ETEC O80:H9 and O80:H_unknown (UK) isolated from patients with diarrhea (58, 59). Different non-EPEC non-STEC *E. coli* O80:H_UK and O80:non-H2 have also been regularly, although infrequently isolated since the years 1990s from diseased and healthy cattle, humans, piglets, and poultry (60–65). Next, EPEC O80 were identified in diarrheic lambs and kids (O80:H_UK) and in diseased or healthy poultry (O80:H_UK, O80:H19, O80:H26) (62, 66–68).

Before 2010, (AE-)STEC O80:H- or H_UK were isolated, although infrequently from diarrheic patients in Belgium (69), from a diarrheic calf in Germany (70) and from healthy or diarrheic cattle in Spain (71), whereas AE-STEC O80:H2 were reported only once along with AE-STEC O26:H11, during a small outbreak in France in 2005 that was linked to the consumption of raw milk camembert cheese (72, 73). Thereafter, retrospective studies identified AE-STEC O80:H2 isolated last century or in the early 2000 years from diseased or healthy humans and cattle in Belgium (74), France (54), Italy (75), Spain (54), and Switzerland (76). Nevertheless, between 2006 and 2010, the serogroup O80 represented only 1% of all AE-STEC identified at the National Reference Centre in France (77) and isolation of AE-STEC O80:H2 remained an exceptional random finding and not the result of any systematic survey.

AE-STEC and EPEC serotype O80:H2

Emergence of AE-STEC serotype O80:H2 in humans

The year 2010 marks the actual beginning of the emergence of the AE-STEC serotype O80:H2 in humans in France. Indeed, 57 AE-STEC O80:H2 were isolated from 54 patients between 2010 and 2014, in contrast to only 6 between 2005 and 2009 (54, 72). All 54 patients suffered from (bloody) diarrhea and 48 of them from HUS, of which 47 were children. The only adult patient with HUS was reported in 2013 (53). Quite unusual for AE-STEC, some of them also suffered from bacteremia and internal organ infections. After 2014, the yearly incidence of AE-STEC O80:H2 continued to increase until 2019, before stabilizing. Between 2017 and 2021, AE-STEC O80:H2 became one of the top three serotypes identified in France, along with O157:H7 and O26:H11, as much in cases of acute diarrhea, than of bloody diarrhea and of HUS, particularly in young children (<5 years) and in elderlies (>65 years). However, AE-STEC O80:H2 infections in humans remain sporadic, and no actual outbreak has been reported yet (39, 78, 79), with the exception of the small early outbreak in 2005 (72).

As a consequence, AE-STEC O80:H2 made their entry in 2014 in the yearly report of the European Food Safety Authority - European Center for Disease Prevention and Control, as a new member of the top 20 STEC serotypes (80). They were reported by three countries in 2014 up to nine countries in 2019 and represented 0.5% to 2.4% of all reported STEC (Table 1), although this may only reflect increased awareness and inclusion of this serotype in routine surveillance. They also represented 5%–13% of all STEC serotypes involved in HUS cases and ranked between the second and fifth places depending on the reporting year (Table 1) (80–89).

However, in contrast to France, the yearly incidence of AE-STEC O80:H2 remained low after 2010 in other European countries, including Belgium (Table 2) for some unknown reason (74–76, 90–95). Finally, only a very few AE-STEC O80:H2 isolated from clinical cases outside Europe are reported in the literature: in USA between 2013 and 2015, in Brazil in 2012 and in Japan in 2024 (63, 96, 97).

An unusual clinical property of human AE-STEC O80:H2, compared with other AE-STEC, is responsible for bacteremia and internal organ infection. This was first observed after isolation of the AE-STEC O80:H2 from the blood stream and internal organs of one adult patient in 2013 (53) and later confirmed or highly suspected in clinical cases reported in France and in The Netherlands (54, 62, 99). AE-STEC O80:H2 therefore represents a triple hybrid STEC and could be named extra-intestinal AE-STEC

TABLE 1 EFSA-ECDC reporting of AE-STEC O80 in humans, foods and animals since 2012 (80–89)

Year	No. of O80 human cases reported	No. of member states with O80 human cases reported	% STEC O80 among reported STEC isolates	% STEC O80 in HUS cases reported	STEC O80 ranking in HUS	STEC O80 in food and animals
<2012	No data	No data	No data	No data	No data	No data
2012[a]	4	1	0.1	No data	No data	0.1–1.0
2013[a]	8	3	0.2	No data	No data	0.1–1.0
2014[b]	18	3	0.4	No data	No data	0.1–1.0
2015	24	4	0.7	8.8	3rd	No data
2016	42	8	1.0	9.6	3rd	No data
2017	42	7	1.0	5.0	5th	No data
2018	64	8	1.3	6.7	4th	No data
2019	80	9	1.8	9.0	3rd	No data
2020	57	8	2.4	13.2	2nd	Not detected
2021[c]	No data	No data	0.1–1.0	11.0	3rd	Not detected
2022[c]	No data	No data	No data	6.1	3rd	No data
2023[c]	No data	No data	No data	5.3	4th	No data

[a] The results of years 2012 and 2013 are presented in the 2014 report.
[b] Entry of STEC O80 in the top 20 STEC serotypes identified.
[c] Serotypes are no more reported.

TABLE 2 Belgian STEC NRC reporting of AE-STEC O80:H2 isolated from humans between 2008 and 2023 (94) and AE-STEC and EPEC O80:H2 among eHly-producing *E. coli* isolated from calves at ARSIA between 2009 and 2023 (98) (Tables S1 and S2)

Year	Human strains		Calf strains		
	No. of STEC (no. of cases)	No. of AE-STEC O80:H2 (%)	No. of eHly + *E. coli* tested[a]	No. of EPEC O80:H2 (%)	No. of AE-STEC O80:H2 (%)
<2008	No data	No data	No data	No data	No data
2008	No data	1	15[b]	0	0
2009	No data	1	40	3 (7.5%)	0
2010	No data	0	48	8 (16.7%)	1 (2.1%)
2011	98 (99)	1 (1.0%)	34	5 (14.7%)	1 (2.9%)
2012	102 (102)	0	49	2 (4.1%)	0
2013	110 (110)	1 (1.0%)	41	6 (14.6%)	0
2014	88 (88)	2 (2.3%)	40	5 (12.5%)	0
2015	95 (95)	2 (2.1%)	26	8 (30.8%)	0
2016	106 (105)	2 (1.9%)	63	2 (3.2%)	3 (4.8%)
2017	114 (112)	0	74	5 (6.8%)	3 (4.1%)
2018	105 (104)	6 (5.7%)	68	3 (4.7%)	5 (7.8%)[c]
2019	123 (122)	4 (3.3%)	82	6 (7.3%)	8 (9.8%)
2020	81 (78)	2 (2.5%)	38	2 (5.3%)	1 (2.6%)
2021	119 (119)	2 (1.7%)	44	1 (2.3%)	3 (6.8%)
2022	179 (177)	3 (1.7%)	20	0	0
2023	332 (331)	9 (2.7%)	52	3 (5.8%)	2 (3.8%)
TOTAL	1653 (1641)	36 (2.1%)	734	59 (8.0%)	27 (3.7%)

[a] Number of *E. coli* producing hemolysis only on Ehly agar plates.

[b] Only eHly-positive *E. coli* isolated in November and December were studied.

[c] Three AE-STEC were isolated from the same calf suffering septicemia and internal organ colonization.

(Ex-AE-STEC) or septicemic AE-STEC (Se-AE-STEC), although we will keep the acronym AE-STEC in this manuscript for the sake of clarity.

This invasive property of AE-STEC O80:H2 presents clinicians with a dilemma since bacteremia and internal organ infection are life-threatening clinical conditions that need to be treated with antibiotics. However, the use of antibiotics in STEC infection is a matter of debate because some can increase the level of Stx released and therefore the risk for the patients of developing HUS. According to results obtained *in vitro*, the effect of these antibiotics is, nevertheless, dependent on the doses and on the type of antibiotic (100, 101). Sub-inhibitory levels of antibiotics that target DNA synthesis, including fluoroquinolones and trimethoprim-sulfamethoxazole, increase Stx production, whereas translation inhibitors, especially azithromycin do not induce Stx production (100). However, the use of macrolides is not appropriate to treat invasive infections with *E. coli*. Therefore, Cointe and collaborators suggest adding a macrolide such as azithromycin, in the treatment of invasive infections requiring systemic antibiotherapy. Since extended spectrum β lactamase-encoding genes of the $bla_{\text{CTX-M}}$ family have already been identified in some AE-STEC O80:H2, they suggest administering a combination of azithromycin and imipenem on the basis of *in vitro* results (102). Nevertheless, this antibiotic combination has not been assessed in clinical trials yet.

Since they have not been systematically searched, only a very few EPEC O80:H2 isolated from humans have also been reported in France or in other countries (Brazil, Spain and UK) (54, 62, 63, 103). Moreover, these EPEC may actually derive from AE-STEC after the loss of the Stx phages, like already observed or suspected (19, 62). Their actual identity will be discussed in the section "Origin and evolution of the serotype O80:H2".

AE-STEC and EPEC serotype O80:H2 in young calves

During a survey performed in 2014 in Belgium, 86 of the 206 eHly-producing *E. coli* isolated at the regional veterinary diagnostic laboratory in Wallonia ("Association régionale de Santé et d'Identification animale" <ARSIA>) between the end of 2008 and

2013 from <3-month-old calves with diarrhea and enteritis tested negative with PCR for the most frequent 10 O serogroups of human and calf AE-STEC (O5, O26, O103, O104, O111, O118, O121, O145, O157, and O165) (51). The O80 serogroup was later identified in six EPEC strains analyzed with the PCR O serogroup typing platform developed by Iguchi and collaborators (104). Subsequently, all eHly-producing *E. coli* isolated at ARSIA between 2008 and 2023 were tested with the PCR for the O80 serogroup and genome sequenced for further identification.

From 2009 to 2015, only EPEC O80:H2 were identified with two exceptions in 2010 and 2011. From 2016, AE-STEC O80:H2 were also identified, at roughly the same yearly rate as EPEC. In summary, 27 AE-STEC and 59 EPEC O80:H2 were identified between 2008 and 2023, representing 11.7% of all eHly-producing *E. coli* studied (Table 2; Table S2) (68, 98). The actual identity of calf EPEC will also be discussed in the section “Origin and evolution of the serotype O80:H2” along with the human EPEC. Invasive infection with internal organ colonization was confirmed in 2018 with one AE-STEC, but not with any EPEC O80:H2.

Unfortunately, eHly-producing *E. coli* isolated from calves at ARSIA before November 2008 are no more available. However, different AE-STEC and EPEC tested in 1993 (105) at the Bacteriology laboratory of the Veterinary Faculty of the University of Liège (Belgium) were recovered. Five AE-STEC isolated in 1987 from the same calf tested positive for the O80:H2 serotype by PCR. Genome sequencing of two of them confirmed the PCR serotyping and identified them as AE-STEC (74, 104). To the authors’ knowledge, no survey on diseased calves has been performed in any other country.

AE-STEC and EPEC serotype O80:H2 from healthy cattle and other sources

Although very rarely, AE-STEC O80:H2 isolated from healthy cattle and dairy products before their emergence in France were reported in the literature (62, 63, 75). More recently, they were also isolated from healthy cows and calves on one single farm in France (106). In addition, a few EPEC O80:H2 have also been isolated from animals and from the environment: two from cattle in Spain and the USA, three from pigs or pig farm environments in Slovakia and Switzerland and one from water in Germany (62, 63, 107, 108).

GENOMIC COMPARISON AND WHOLE-GENOME SEQUENCE (WGS)-BASED PHYLOGENETICS OF HUMAN AND CALF AE-STEC AND EPEC O80:H2

Published WGS-based analyses

Since the first publication in 2016 (54), several authors analyzed and compared the virulotypes of AE-STEC and EPEC O80:H2 from humans and diarrheic calves by PCR or after genome sequencing in the frame of either serotype O80:H2-targeted or broader studies of (AE-)STEC. In addition, AE-STEC O80:H2 from humans were classified in WGS-based or “Clustered Regularly Interspaced Short Palindromic Repeats” (CRISPR)-based strain typing and phylogenetic analysis, with some publications also including AE-STEC and/or EPEC O80:H2 from diarrheic calves. The purpose of the following sections is to present the results of their genetic typing, population structure in WGS-based analyses, and lineage-associated genetic profiles.

Genetic typing of human and calf AE-STEC and EPEC O80:H2

Avoiding as much as possible duplicated strains in the different publications, 221 independent genome-sequenced human and calf AE-STEC and EPEC O80:H2 were found in the literature at the time of writing: 183 human AE-STEC, five human EPEC, 12 calf AE-STEC, and 21 calf EPEC. Conversely, the few AE-STEC and EPEC isolated from healthy cattle or other sources were not included (62, 63, 72, 75, 92, 93, 98, 103, 106–109).

All 221 AE-STEC and EPEC O80:H2 reported belong to the sequence type (ST) 301, a member of the clonal complex (CC) 165 along with, among others, ST165 (serotype

O80:H19) and ST189 (serotype O80:H26) that include human, porcine, and poultry *E. coli*. ST301 is, nevertheless not exclusive of the serotype O80:H2 and serotypes O45:H2, O55:H9, O119:H2, and O186:H2, for instance, also belong to ST301 (75, 93, 95, 103). Conversely, the *E. coli* O80:H6 and O80:H45, recently isolated from healthy cattle at slaughterhouses and in farms, are not members of the CC165 (65).

All 221 AE-STEC and EPEC O80:H2 harbor genes located on the LEE pathogenicity island, with, among others, a rare variant of the *eae* gene, *eae-xi* (*eaeξ*), being the only *eae* gene variant identified in all studies. The *eaeξ* gene was first described in three bovine AE-STEC O80:H- isolated in Spain (71) and Germany, under the name *eae-epsilon2* (*eae*ε_2) (70). The *eaeξ* gene is highly associated with ST301 but is not exclusive of the serotype O80:H2 (75, 93, 103). Of the 14 *stx* gene subtypes identified in the literature, the most frequent ones are *stx2d* (62.6%) and *stx2a* (32.8%). The *stx1a* and the *stx2f* subtypes are reported in only a few strains (4.6%) isolated as much from humans than from calves in Belgium, France, Italy, and the Netherlands (75, 98, 106). Moreover, the gene coding for eHly, *ehxA* (misnamed *hlyA* gene in some publications) is detected in the great majority (96.4%) of the 221 AE-STEC and EPEC O80:H2 studied, indicating the carriage of pO157-like plasmids. Other pO157 plasmid-located genes and different type III effector-encoding non-LEE-located genes were also detected (*espP* and *nleA/B/C*, respectively), or not (*katP* and *espI*, *espJ*, *cif*, respectively) in the majority of AE-STEC and EPEC O80:H2 from humans and calves in three independent studies (62, 93, 98). Furthermore, the OI-50- and OI-44-located *espK* and *espV* genes (40) were not searched in any of the published studies (see "EPEC in humans and calves").

As mentioned above, one unusual property of AE-STEC O80:H2 compared to other AE-STEC is to be responsible for bacteremia and internal organ infection. This was at first observed in one adult patient in 2013 (53) and later confirmed after isolation of some AE-STEC O80:H2 from blood and internal organs (54, 62), although not as frequently as could be expected from the presence of pS88-like plasmids carrying genes coding for invasive properties. The pS88 plasmid was first described in *E. coli* strains causing septicemia and infections of internal organs in poultry and neonatal meningitis in humans (110). Two plasmid replicons (FIB and FIIA) and up to nine virulence-associated genes or gene clusters (*cva*, *ets*, *hlyF*, *iro*, *iss*, *iuc/iutA, ompT*, and *sit*) can be located on pS88-like plasmids (62, 110, 111). The FIB replicon and the *hlyF* gene coding for an "avian hemolysin" can be considered specific markers of the pS88-like plasmids.

One pS88-like plasmid, pR444_A, was described in the human AE-STEC O80:H2 strain RDEx444 (62). In addition to the pS88-located replicons and genes, the pR444_A plasmid carries a composite resistance cassette, containing two copies of the two *rep* genes of the IncQ1 plasmid replicon (111, 112), the *mer* operon coding for the resistance to mercury, three copies of integron integrase-encoding genes, multiple copies of IS*26*, transposons (Tn*3* and Tn*1721*), and two or three copies of antibiotic resistance genes (*aph*, *dfrA*, *strAB*, *sul2*, *bla*, and *tetA* on Tn*1721*) (62). Most likely, the duplication/triplication of some genes was generated by the triplication of an IncQ1 plasmid integrated into the pS88-related plasmid. The *rep* genes of the IncQ1 replicon can be considered markers of this cassette and the pR444_A-like plasmids. The pR444_A plasmid also carries a set of *tra* genes for conjugal transfer, and its transmissibility to other *E. coli* strains, including an STEC O26:H11 strain, has been experimentally proven (62, 75). Moreover, strain RDEx444 contains two additional large plasmids: a pO157-like plasmid (pR444_C) carrying the *ehxA* gene coding for the eHly and the *espP* gene encoding a serine protease, and a cryptic phage-plasmid (pR444_B) (62).

Unfortunately, not all publications on genome-sequenced AE-STEC and EPEC O80:H2 identify the virulence- and resistance-associated genes located on the pS88 plasmid and on the cassette of the pR444_A plasmid. When searched (62, 63, 75, 92, 93, 98, 103, 106), the pS88-located genes are detected in a great majority of the genome-sequenced strains (95%–100%), with the exceptions of the *etsC* and *iucC* genes (60%). Remarkably, the presence/absence of both *etsC* and *iucC* genes are linked with only a very few exceptions. When searched (62, 75, 106), genes located on the resistance cassette of the pR444_A plasmid are also detected in the majority of human AE-STEC O80:H2 studied. Finally, the presence of the FIB, FIIA, and IncQ1 replicons has been reported in no published studies.

Besides the simultaneous presence/absence of the *etsC* and *iucC* genes in the 52 human and calf Belgian AE-STEC and EPEC studied, Habets and collaborators (98) observed that the *iha* gene coding for the "IrgA (iron regulatory gene A) homolog adhesin" and identified as the iha_{EDL933} gene subtype that is located on a chromosomal integrative element originally found in the AE-STEC O157:H7 strain Sakai ("Sakai prophage-like element 1-like" or SpLE1-like) (113, 114), was inversely present/absent in 46 (88.5%) of them, without any logical explanation at this time. Similar results were obtained on a more limited number of human Swiss AE-STEC O80:H2 (92), but the *iha* gene was not searched in the other studies.

Phylogenetic classification and lineage-associated gene profiles of human and calf AE-STEC and EPEC O80:H2

The population structure of AE-STEC and EPEC O80:H2 isolated from humans and diarrheic calves, and sometimes other sources are presented in a few WGS-based phylogenetic analyses. However, these studies include various sets of strains, as far as the source, the country, and the years of isolation are concerned. Cointe and collaborators (62) analyzed 32 strains with a majority of human AE-STEC from France (21 strains) or from Spain and Switzerland (five strains) and a few AE-STEC and EPEC from bovines, pig, or water isolated in Czechia, France, Germany, and Spain (six strains), whereas Rodwell and collaborators (93) analyzed human AE-STEC from UK (41 strains) and Habets and collaborators (98) analyzed human and calf AE-STEC and calf EPEC from Belgium (52 strains).

Cointe and collaborators divided the human AE-STEC O80:H2 from France into two clusters, C1 and C2, that can be differentiated by the presence/absence of the pR444_B cryptic plasmid of strain RDEx444 (62). C1 was further divided into two sub-clusters, SC1a and SC1b, that can be differentiated by the identity of the *stx* genes and the pS88 gene profiles. The strains in SC1a harbor the *stx2d* gene and their pS88-like plasmids carry the *iuc* and *ets* genes, whereas the strains of SC1b and of C2 harbor the *stx2a* gene and their pS88-like plasmids do not carry these genes. Several antibiotic resistance genes present on the cassette of the pR444_A plasmid are also detected in all pS88-like plasmid-positive strains. The animal and environmental AE-STEC and EPEC O80:H2 intermix with the human AE-STEC in SC1b and C2. A very similar AE-STEC O80:H2 population structure was described by Long and collaborators (63) in a CRISPR-based clustering analysis including 56 human, seven animal or environmental strains, and six strains of unknown sources, isolated in different European countries (France, Germany, Italy, Norway, Spain, and UK), Brazil, and USA.

Rodwell and collaborators also divided the 41 human AE-STEC O80:H2 from UK in two clusters, C1 and C2 (93), corresponding to the SC1a and SC1b of Cointe and collaborators and that can also be differentiated by the identity of the *stx* genes and by the pS88-like gene profiles. The strains in C1 harbor the *stx2d* gene and their pS88-like plasmid carry the *iuc* and *ets* genes, whereas the strains of C2 harbor the *stx2a* or *stx2d* gene and their pS88-like plasmid do not carry the *iuc* and *ets* genes. Antibiotic resistance genes present on the cassette of the pR444_A plasmid are also detected in pS88-like plasmid-positive strains, especially of C1. Moreover, AE-STEC of ST301 belonging to other serotypes intermix with the AE-STEC O80:H2 in C1, but not in C2.

Finally, Habets and collaborators identified two lineages, L1 and L2, among the 52 calf and human AE-STEC and EPEC O80:H2 from Belgium, with L1 being subdivided into four sub-lineages, SL1.1 to SL1.4, that here too can be differentiated by the identity of the *stx* genes and the pS88-like gene profiles (98). The strains in SL1.1 (23 strains) are mainly calf EPEC along with some calf and human AE-STEC harboring different *stx* genes (*stx1a*, *stx2a,* or *stx2d*), and their pS88-like plasmids do not carry the *ets* and *iuc* genes, except for one EPEC strain. The strains of SL1.2 (24 strains) and SL1.4 (two strains) are mainly human and calf AE-STEC, along with a few calf EPEC, harboring only the *stx2d* gene and the *etsC* and *iuc* genes. The strain of SL1.3 (single-member sub-lineage) harbors the *stx2a* gene, whereas the strains of L2 (two strains both isolated in 1987) harbor the *stx1a* gene and their pS88-like plasmid also carry the *iuc* and *ets* genes. Antibiotic resistance genes present on the cassette of the pR444_A plasmid were not searched.

Two studies compared human and calf AE-STEC O80:H2 along with a few bovine and/or food strains, but not calf EPEC, from different countries: 82 strains (including one from dairy food) isolated in Belgium, France, Italy, and the Netherlands (75) and 107 strains, (including a few from bovines and dairy food) isolated in Belgium, France, Switzerland, and UK (106). The results of these two WGS-based phylogenetic analyses confirmed the division of AE-STEC O80:H2 into two main evolutionary clusters/lineages, with one of them subdivided into at least two sub-clusters/sub-lineages, as already published (62, 93, 98). For instance, the 31 Belgian calf and human AE-STEC (98) are classified in the same SLs by Soleau and collaborators (106): the two Belgian calf *stx1a* AE-STEC of L2 isolated in 1987 grouped with three Swiss and French *stx2a* AE-STEC isolated between 2003 and 2008 in a particular lineage apart from all others strains; the 22 Belgian calf and human *stx2d* AE-STEC of SL1.2 and SL1.4 grouped along with *stx2d* British, French, and Swiss strains, and the remaining seven Belgian calf and human *stx1a*, *stx2a,* or *stx2d* AE-STEC of SL1.1 and SL1.3 grouped together with also British, French, and Swiss AE-STEC harboring different *stx* genes. A similar grouping was presented in the study by Gigliucci and collaborators (75).

In conclusion, the human and calf AE-STEC O80:H2 isolated since 1987 belong to two main clusters/lineages and two to four sub-clusters/sub-lineages, based on their phylogenetic relationship. They can be differentiated by their virulotypes, especially the (sub)types of *stx* genes and the profiles of genes of their pS88/pR444_A-like plasmids. The majority of calf EPEC O80:H2 belong to one SL along with human and calf AE-STEC (62, 75, 93, 98, 106).

Genotyping and global population structure inferred by the analysis of 417 O80:H2 genome sequences

As listed above, each published WGS-based phylogenetic analysis includes limited and various sets of strains, impairing a global analysis of the population structure of the human, calf, and other AE-STEC and EPEC O80:H2, whereas a total of more than 400 genome sequences are now available in public databases. Some of the additional genome sequences present in the databases are indirectly referred to in specific or more general publications about (AE-)STEC and EPEC (76, 91, 95, 103, 107, 109, 115, 116) or remain unpublished. Henceforth, to improve the current situation of genome sequencing, expand previous WGS-based phylogenetic analyses, and understand the population structure of AE-STEC and EPEC O80:H2 from a global point of view, we performed a core genome single nucleotide polymorphism (SNP)-based phylogenetic analysis of all genome sequences available in the NCBI and Enterobase databases (accessed on 31st August 2024). Additional unpublished genome sequences of Belgian human and calf AE-STEC and EPEC obtained from the sequencing platforms of the UZ Brussel STEC National Reference Center (Belgium), the Veterinary Faculty of the University of Liège (Belgium), and the Department of Bacteriology of the University of Kyushu (Japan) were included in the study and later uploaded to the NCBI database (Table S1).

After excluding low-quality genomes, duplicated genomes deposited in both databases, and those containing the same core genome SNP, 417 genome sequences of *E. coli* O80:H2 isolated between 1987 and 2024 were collected (Table 3; Table S1). Of these, 291 were from humans (70%) and 94 from calves (22%): 86 from diarrheic calves and eight from healthy calves. The remaining 32 genomes (8%) were from diverse or unknown sources. Of these 417 *E. coli* O80:H2, 61% were isolated in Belgium and France, and 90% in European countries.

TABLE 3 Country of origin and sources of the 417 *E. coli* O80:H2 whose genome sequences were obtained from the NCBI and EnteroBase databases (accessed on 31st August 2024) and from the sequencing platforms of the UZ Brussel STEC National Reference Center (Belgium), the Veterinary Faculty of the University of Liège (Belgium), and the Department of Bacteriology of the University of Kyushu (Japan) that were later uploaded to the NCBI database (Table S1)[a]

Countries	Sources					Total
	Humans	Calves[b]	Bovines[c]	Others	No data	
Belgium	39	86	0	0	0	125
France	128	0	2	0	0	130
Germany[d]	13	0	0	1	0	14
Switzerland	19	8	0	0	0	27
The Netherlands	8	0	0	0	0	8
United Kingdom[e]	59	0	0	1	7	67
Other Europe[f]	3	0	1	1	1	6
Asia[g]	3	0	0	0	0	3
North America[h]	14	0	2	0	0	16
South America[i]	2	0	0	0	0	2
No data	3	0	0	0	16	19
TOTAL	291	94	5	3	24	417

[a] Twenty-one genomes were excluded from the analysis because they were present in both databases (18 genomes from reference 62: the genomes present in the NCBI database were used) or because they were of low-quality (three genomes; <98.5% completeness or >2% contamination as estimated by the CheckM program [117]). Moreover, single nucleotide polymorphism (SNP) analysis revealed 33 groups of 86 duplicated genomes. Only one genome in each of the 33 groups was randomly chosen, further reducing the number of independent *E. coli* O80:H2 genomes to 417.

[b] The Belgian isolates were from diarrheic calves and the Swiss isolates from healthy calves.

[c] Other isolates from cattle.

[d] The other source was "water."

[e] The other source was "animal" with no further precision.

[f] From Spain (three human and one bovine isolates), Slovakia (one porcine isolate), and Poland (one "no data" isolate).

[g] From Bangladesh (two isolates) and India (one isolate).

[h] From USA (13 human and two bovine isolates) and Canada (one human isolate).

[i] From Brazil (one isolate) and Peru (one isolate).

Genetic typing

All but six of the 417 *E. coli* O80:H2 strains belonged to ST301, irrespective of their pathotypes and virulotypes, and the remaining six strains belonged to single locus variants (SLVs) of ST301: ST11915, ST12053, ST12702, ST13345, ST13689, and an untypeable ST (Table S2).

The overview of the major virulence-related genes and plasmid replicons detected in the 417 genomes is as follows (Table 4; Table S2): (i) 96.2% of the genomes were positive for the *eaeξ* gene and 1.2% for the *eaeρ* gene, whereas no *eae* gene was detected in 11 genomes (2.6%); (ii) the *stx2d* (55.2%) and *stx2a* (19.4%) genes were the most frequent, whereas the *stx1a* and *stx2f* genes were detected in a few genomes (3.3% and 1%, respectively) and 89 genomes (21.3%) were *stx*-negative (75.3% of them were isolated from calves); (iii) the *ehxA* gene was detected in the vast majority of the genomes (95.0%), indicating the carriage of pO157-like plasmids; (iv) the FIB replicon and various sets of pS88-located virulence genes were detected in 93.8% of the genomes indicating the carriage of pS88-like plasmids; (v) the IncQ1 replicon and various sets of resistance genes of the cassette of the pR444_A plasmid were detected in 90.5% of the genomes, indicating the carriage of pR444_A-like plasmids; (vi) of the *iha* gene subtypes (114), only the iha_{EDL933} gene was detected in 40.8% of the genomes, with those of human origin less frequently positive than the others (31.6% vs. 61.9%); and (vii) the search for type III effector-encoding *espK* and *espV* genes (see "EPEC in humans and calves") gave contrasting results: the *espK* gene was detected in all but two EPEC while the *espV* gene was detected in only two other EPEC and one *stx2d* AE-STEC.

TABLE 4 Overview of the gene profiles and sources of the 317 AE-STEC, 89 EPEC, and 11 STEC O80:H2 whose genome sequences were obtained from the NCBI and EnteroBase databases (accessed on 31st August 2024) and from the sequencing platforms of the UZ Brussel STEC National Reference Center (Belgium), the Veterinary Faculty of the University of Liège (Belgium) and the Department of Bacteriology of the University of Kyushu (Japan) that were later uploaded to the NCBI database (Table S1)[a]

Genes detected	Sources					Total
	Humans	Calves	Bovines	Others	No data	
eaeρ	4	0	0	0	1	5
eaeξ[b]	276	94	5	3	23	401
eae-	11	0	0	0	0	11
stx1a	5	8	0	0	1	14
stx2a	60	5	2	1	12	80
stx2d[c]	208	12	1	0	8	229
stx2f	1	2	0	0	1	4
stx2d/stx2f	1	0	0	0	0	1
stx-	16	67	2	2	2	89
ehxA+	275	93	5	2	22	396
ehxA-	17	1	0	1	2	21
FIB +*hlyF* + pS88 genes+	274	92	2	0	22	390
FIB +*hlyF*- pS88 genes+	1	0	0	0	0	1
FIB +*hlyF* + pS88 genes-	1	0	0	0	0	1
FIB- *hlyF*- pS88 genes-	15	2	3	3	2	25
pS88 +IncQ1+[d]	247	84	2	0	21	354
pS88 +IncQ1-	28	8	0	0	1	37
pS88- IncQ1+	2	0	0	1	0	3
pS88- IncQ1-	14	2	3	2	2	23
iha_{EDL933}+	92	56	5	2	15	170
iha_{EDL933}-	199	38	0	1	9	247
espK+	291	93	4	3	24	415
espK-	0	1	1	0	0	2
espV+	2	1	0	0	0	3
espV-	289	93	5	3	24	414

[a] The 417 genomes were searched for the presence of the marker genes of STEC and EPEC (*eae* coding for the intimin adhesin; *stx1* and *stx2* coding for the Stx1 and Stx2; *ehxA* coding for the eHly; *iha* gene coding for "IrgA <iron regulatory gene A > homolog adhesins"; *espK* and *espV* genes coding for T3SS effectors EspK and EspV), using BLASTN with the following identity and query coverage thresholds and reference sequences: *eae* gene and subtypes (>98% and >99%) (118); *stx* genes and subtypes (>99% and >99%) (20); *ehxA* gene (>95% and >90%; pR444_C plasmid [accession No. QBDM01000002.1: pos. 74,554–77,550]) (62); *iha* gene and subtypes (>98% and >60%) (114); *espK* and *espV* genes (>90% and >60%) (40). All 417 genomes of this study were also searched for the presence of a total of 41 genes associated with the pS88 and pR444_A plasmids with >98% identity and >60% query coverage thresholds and the gene sequences of the pR444_A plasmid (62) as references (Accession No. QBDM01000004.1) in an in-house database (Table S4) for repertoire analyses.

[b] Including the 317 AE-STEC.

[c] Including the 11 STEC.

[d] One human *stx2d* AE-STEC is positive for only one of the two IncQ1 replicon genes.

Global population structure and lineage-associated virulotypes

The phylogenetic analysis based on the SNPs in the core genome of the 417 O80:H2 genomes (n = 6,719) revealed three distinct lineages (L), referred to as L1, L2, and L3 in this manuscript (Table S3; Fig. 1 and 2). L3 can be further divided into three sub-lineages, SL3.1, SL3.2, and SL3.3. L1 and SL3.1 were identified and defined for the first time in the current analysis, whereas L2, SL3.2, and SL3.3 correspond to (sub)clusters, (sub)lineages, and (sub)clades defined in previous studies (Fig. 1) (62, 75, 93, 98, 106). The numbers of genomes belonging to each (sub)lineage were as follows (Table S3): five (1.2%) in L1; 18 (4.3%) in L2; 11 (2.6%) in SL3.1; 141 (33.8%) in SL3.2; 241 (57.8%) in SL3.3; and one not assigned to any lineage.

L1 comprises five *eaeρ* EPEC isolated between 2009 and 2013 (three strains) or after 2014 (two strains) from humans in Asia (Bangladesh and India) and South America (Peru) (Table S3; Fig. 2). These five EPEC are the first described AE-STEC or EPEC O80:H2 not

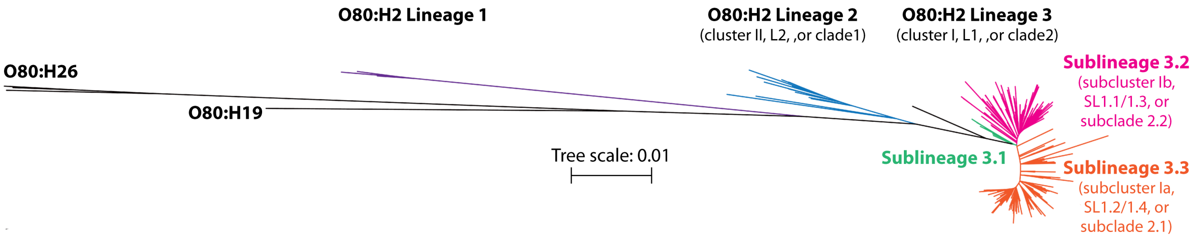

FIG 1 Phylogenetic relationship in a radiation scheme of the genomes of the 317 AE-STEC, 89 EPEC, and 11 STEC O80:H2 and of four close relatives O80:non-H2 whose sequences were obtained from the NCBI and EnteroBase databases (accessed on 31st August 2024) and from the sequencing platforms of the UZ Brussel STEC National Reference Center (Belgium), the Veterinary Faculty of the University of Liège (Belgium) and the Department of Bacteriology of the University of Kyushu (Japan) that were later uploaded to the NCBI database (Table S1).

harboring the *eaeξ* gene, although the *eaeρ* gene has already been identified in AE-STEC or EPEC belonging to other serotypes (103, 118, 123). Of the 41 pR444_A plasmid-located replicons and genes, only the FIB replicon and a few resistance genes, such as *strAB*, *sul2,* and/or *bla*$_{\text{TEM-1B}}$ were detected in one and three strains respectively, suggesting that neither pS88-like nor pR444_A-like plasmids are distributed in this lineage and that these resistance genes are located on different non-pR444_A genetic elements. The *ehxA* and *iha* genes were not detected either.

L2 comprises 14 AE-STEC and four EPEC isolated from humans, diarrheic calf, cattle, and pigs in different European and North American countries and in Brazil and corresponds to the L2 defined by Habets and collaborators (98) and to the C1 defined by Soleau and collaborators (106). Seven strains were isolated between 1987 and 2014, eight strains after 2014, and the year was not reported for three strains (Table S3; Fig. 2). The *eae* gene belong to the *ξ* subtype, whereas the *stx* genes belong to the *stx1a* (three strains), *stx2a* (eight strains), or *stx2d* (three strains) subtypes. The eight *stx2a* AE-STEC isolated from humans and cattle in France and Switzerland contain 27–32 of the 41 plasmid-located genes searched, including the FIB and IncQ1 replicons, but not the *ets* and *iuc/iutA* genes (Table 5), suggesting the presence of pR444_A-like plasmids lacking the *ets* and *iuc/iutA* operons, which were called “the incomplete form of pR444_A-like plasmids” (103). In addition, the FIB operon, most pS88-located virulence-associated genes and some antibiotic resistance genes, but neither the *ets* and *iuc/iutA* operons nor the IncQ1 replicon was detected in one calf *stx1a* AE-STEC isolated in Belgium and two human *stx2d* AE-STEC isolated in the USA, suggesting the presence of “the incomplete form of pS88-like plasmids” lacking the resistance cassette. The *ehxA* and *iha* genes were also detected in these 11 plasmid-positive AE-STEC (Table 5). The remaining seven strains were negative for all or most of the pS88/pR444_A-located replicons and genes, indicating that they contain neither pS88-like, nor pR444_A-like plasmids. In particular, the Brazilian human and the Slovakian porcine EPEC, which early separated from the other strains (Fig. 2), were negative for not only all 41 genes but also the *ehxA* gene, suggesting that pO157-like plasmids are not present. The predominant pathotype of the L2 strains is therefore *stx2a* AE-STEC containing pR444_A-like plasmids lacking the *ets* and *iuc/iutA* operons (eight strains, 44%).

L3 comprises all remaining AE-STEC and EPEC as well as the 11 STEC strains lacking the *eae* gene. As these *eae*-negative strains are sporadically distributed in L3, the LEE or part of it may be deleted in these strains. The *eae* genes of the other strains belonged to the *eaeξ* variant and nearly all strains contained the *ehxA* gene, suggesting the presence of pO157-like plasmids. Although pS88/pR444_A-like plasmid-encoded replicons and virulence and resistance genes were also detected in nearly all strains, there are notable variations in their repertoires.

Of the three sub-lineages of L3, SL3.1, which first separated from the other L3 strains comprises 10 AE-STEC and one EPEC isolated between 2017 and 2022 from only humans in three European countries and in USA (one strain) (Table S3; Fig. 2). The *stx* genes belong to the *stx2a* (nine strains) or *stx2d* (one strain) subtypes. All 11 strains contain 27

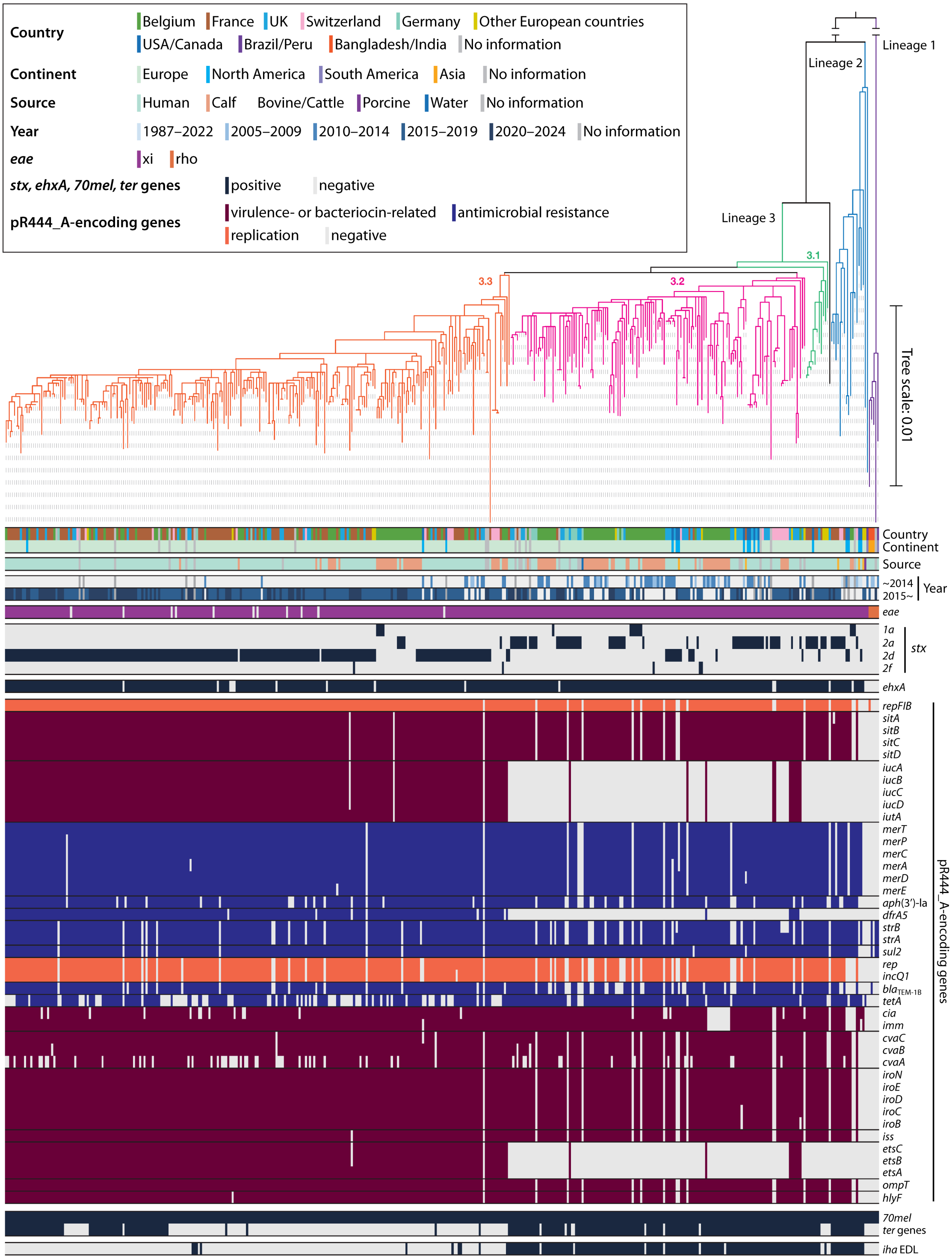

FIG 2 Phylogenetic relationship in a single nucleotide polymorphism (SNP)-based tree of the 317 AE-STEC, 89 EPEC, and 11 STEC O80:H2 whose genome sequences were obtained from the NCBI and EnteroBase databases (accessed on 31st August 2024) and from the sequencing platforms of the UZ Brussel STEC National Reference Center (Belgium), the Veterinary Faculty of the University of Liège (Belgium), and the Department of Bacteriology of the University of Kyushu (Japan) that were later uploaded to the NCBI database (Table S1). The SNP detection and the phylogenetic analysis were performed as previously described (98) with a slight modification. Based on the 6,719 SNP sites which were identified on the prophage (PP)- and integrative element (IE)-free and recombination-free chromosome backbone sequences conserved in all 417 analyzed genomes (2,972,816 bp) using MUMmer (119) and Gubbins (120), a maximum likelihood (ML) tree was constructed based on these SNPs using RAxML (121). The tree was rooted by the four genomes of three *E. coli* O80:H26 and one *E. coli* O80:H19 (Table S1) from the NCBI database (62), used as outgroups, and displayed using iTOL (122).

TABLE 5 Correlation between the (sub)lineages, pS88 plasmid marker replicon and gene (FIB and *hlyF*), the pR444_A resistance cassette marker replicon (IncQ1), the *ets* and *iuc/iutA* genes, the *iha*$_{EDL933}$ gene, and the *eae* and *stx* virulotypes of the 304 AE-STEC, 76 EPEC, and 11 STEC O80:H2 harboring one pS88 plasmid[a]

(Sub)lineages (total no. of genomes)	pS88 plasmid markers[b]	pR444_A cassette markers	pS88 plasmid genes		*iha*$_{EDL933}$ gene	No. of genomes	*eae* and *stx* virulotypes (no. of genomes)
	FIB/*hlyF*	IncQ1	*etsA/B/C*	*iuc/iutA*			
L2 (18)	+	+	–	–	+	8	*eaeξ / stx2a* (8)
	+	–	–	–	+	3	*eaeξ / stx1a* (1), *eaeξ / stx2d* (2)
SL3.1 (11)	+	+	–	–	+	8	*eaeξ / stx2a* (7), *eaeξ / stx2d* (1)
	+	+	–	–	–	3	*eaeξ* (1), *eaeξ / stx2a* (2)
SL3.2 (141)	+	+	–	–	+	99	*eaeξ* (42),*eaeξ / stx1a* (5), *eaeξ / stx2a* (41), *eaeξ / stx2d* (9), *eaeξ / stx2f* (2)
	+	–	–	–	+	17	*eaeξ* (7),*eaeξ / stx2a* (8), *eaeξ / stx2d* (2)
	+	+	–	–	–	5	*eaeξ* (3),*eaeξ / stx2a* (1), *eaeξ / stx2f* (1)
	+	+	+	+	+	7	*eaeξ* (4),*eaeξ / stx2a* (3)
	+	–	+	+	+	1	*eaeξ* (1)
SL3.3 (241)	+[c]	+[d]	+	+	–	210	*eaeξ* (16), *eaeξ / stx1a* (4), *eaeξ / stx2a* (4), *eaeξ / stx2d* (174)[a,b], *eaeξ / stx2f* (1), *eaeξ / stx2d / stx2f* (1), *stx2d* (10)
	+	–	+	+	–	16	*eaeξ / stx2a* (1), *eaeξ / stx2d* (14), *stx2d* (1)
	+	+	+	–[e]	–	2	*eaeξ* (1), *eaeξ / stx2d* (1)[d]
	+	+	+/–/–	+	–	1	*eaeξ / stx2d* (1)
	+	+	+	+	+	10	*eaeξ* (1), *eaeξ / stx2d* (9)
	+	+	–	–	+	1	*eaeξ / stx2d* (1)

[a] The identity and query coverage thresholds and the reference sequences are described in Table 4.
[b] The repFIIA replicon was not looked for.
[c] One isolate is negative for the *hlyF* gene.
[d] One isolate is positive for only one of the two *repIncQ1* genes.
[e] This isolate is positive for the *iutA* gene only.

(the EPEC from Spain) or 32 (all AE-STEC) of the 41 pR444_A-like plasmid-located replicons and genes searched, but not the *ets* and *iuc/iutA* operons, indicating the presence of "the incomplete form of pR444_A-like plasmids" (Table 5). Moreover, the resistance genes located on pR444_A-like plasmids are well conserved, except for the *dfrA5* gene. Of note, the *iha*$_{EDL933}$ gene was detected in only eight of them: seven of the *stx2a* AE-STEC and the *stx2d* AE-STEC. The predominant pathotype of the SL3.1 strains is therefore *stx2a* AE-STEC containing pR444_A-like plasmids lacking the *ets* and *iuc/iutA* operons (nine strains, 82%).

SL3.2 comprises 81 AE-STEC and 60 EPEC isolated, when reported between 2009 and 2014 (39 strains) or after 2014 (97 strains), from essentially humans (67 strains) and diarrheic calves (52 strains), in several European countries (126 strains) and in USA (eight strains) (Table S3; Fig. 2). SL3.2 corresponds to the SL1.1/SL1.3 defined by Habets and collaborators (98) and to the SC2.2 defined by Soleau and collaborators (106). The *stx* genes belong to the *stx1a* (seven strains), *stx2a* (58 strains), *stx2d* (13 strains), or *stx2f* (three strains) subtypes. One *stx2a* AE-STEC and one EPEC belong to SLVs of ST301. One hundred and four strains (73.8%) contain pR444_A-like plasmids with a gene set similar to that of L2 and SL3.1 (Table 5), also lacking the *ets* and *iuc/iutA* operons. Seventeen other strains (12.1%) contain plasmids lacking not only the *ets* and *iuc/iutA* operons but also all or several resistance genes of the pR444_A plasmid suggesting the absence of the resistance cassette of the pR444_A plasmid. These 121 strains contain "incomplete forms of the pS88/pR444_A-like plasmids." Conversely, eight strains (5.7%) contain pS88/pR444_A-like plasmids carrying the *ets* and *iuc/iutA* operons ("complete forms of the pS88/pR444_A-like plasmids"). In nine strains (6.4%), the FIB and IncQ1 replicons and several of the pR444_A plasmid-located genes as well were not detected, suggesting the absence of pS88/pR444_A-like plasmids. Interestingly, the remaining three strains (2.1%) do not contain most pS88-located virulence genes but contain the incQ1 replicon and most of the pR444_A-located resistance genes, suggesting the absence of pR444_A-like plasmids and the translocation of the resistance cassette to other genomic location(s). The *iha*$_{EDL933}$ gene was detected in all but six strains, of which five harbor incomplete pS88/pR444_A plasmids. Hence, two predominant pathotypes characterize the SL3.2 strains: *stx2a* AE-STEC (50 strains, 35.5%) and EPEC (52 strains, 36.9%) containing pS88/pR444_A-like plasmids lacking the *ets* and *iuc/iutA* operons. A majority of the 86 Belgian strains (60.5%) isolated from diarrheic calves belong to SL3.2 (seven AE-STEC and 45 EPEC), compared with L2 (two AE-STEC) and SL3.3 (18 AE-STEC and 14 EPEC).

SL3.3 comprises 212 AE-STEC, 18 EPEC, and 11 STEC isolated essentially from humans (82%) and healthy or diarrheic calves (15%) in several European countries (229 strains; 95%) after 2014 (226 strains; 94%), when reported (Table S3; Fig. 2). SL3.3 corresponds to the SL1.2/SL1.4 defined by Habets and collaborators (98) and to the SC2.1 defined by Soleau and collaborators (106). The *stx* genes belong to the *stx1a* (four strains), *stx2a* (five strains), *stx2d* (212 strains, including the 11 STEC), or *stx2f* (one strain) subtypes, and both *stx2d* and *stx2f* genes were detected in one strain. Four *stx2d* AE-STEC belong to SLVs of ST301. Noteworthy, strain RDEx444 belong to SL3.3 (62). The most striking contrast of the SL3.3 strains is their plasmid gene profiles, when compared with L2, SL3.1, and SL3.2 strains. For instance, 213 of the SL3.3 strains (88.5%) contain a full or nearly full set of the 41 pR444_A plasmid-located genes, and 16 other strains (6.7%), a full or nearly full set of the nine pS88-located genes, including the *ets* and *iuc/iutA* operons, indicating the presence of "the complete form of pS88/pR444_A-like plasmids. Conversely, the chromosomal *iha*$_{EDL933}$ gene was not detected in any of them (Table 5). Of the 12 remaining strains, 10 harbor the complete form and one harbors the incomplete form of the pR444_A-like plasmid, in addition to the *iha*$_{EDL933}$ gene. Neither the pS88/pR444_A-like plasmid nor the *iha*$_{EDL933}$ gene was detected in the last strain. In sharp contrast to L2, SL3.1, and SL3.2, the predominant pathotype of SL3.3 strains is therefore *stx2d* AE-STEC containing pS88/pR444_A-like plasmids carrying the *ets* and *iuc/iutA* operons (199 strains, 82.5%). The great majority of the 128 French human AE-STEC (89.1%) belong to SL3.3, compared with L2 (four strains), SL3.1 (six strains), and SL3.2 (four strains).

Finally, the phylogenetically unassigned strain is an *eaeξ* EPEC isolated in 2023 from a human in UK and negative for the *ehxA*, *iha,* and all virulence and resistance genes searched for, suggesting the absence of pO157- and pS88/pR444_A-like plasmids.

Conclusions

The WGS-based phylogenetic analysis of the 417 (AE-)STEC and EPEC O80:H2 genomes not only confirms but also extends the data already published on human, calf, bovine, and other AE-STEC and EPEC O80:H2 isolated in different countries between 1987 and 2024 (62, 63, 75, 92, 93, 98, 103, 106, 109).

In summary:

1. two new (sub)lineages are defined (L1 and SL3.1);
2. a second *eae* gene variant (*eaeρ*) is present in the five EPEC of L1 isolated in Asian and South American countries;
3. calf and bovine AE-STEC and EPEC O80:H2 are intermixed with and can be closely related to human strains in SL3.2 and SL3.3, suggesting possible cross-transfer (see section "Reservoir(s) and detection of AE-STEC and EPEC O80:H2");
4. the majority (90.5%) of the 391 pS88-like plasmid-positive strains harbor the resistance cassette of the pR444_A-like plasmid;
5. the resistance cassette of the pR444_A plasmid is similarly distributed in the (AE-)STEC and EPEC harboring the complete form (93%) or the incomplete form (86%) of the pS88/pR444_A-like plasmids;
6. the *ets* and *iuc/iutA* operons and the *iha*$_{EDL933}$ gene are inversely present/absent in 92.6% of the 391 (AE-)STEC and EPEC O80:H2 harboring pS88/pR444_A-like plasmids;

7. the distribution of the type III effector-encoding *espK* and *espV* genes is not different among AE-STEC and EPEC O80:H2, in contrast to the classical major serotypes (40);
8. a striking contrast is observed between the strains in SL3.3 and the strains in L2, SL3.1, and SL3.2, regarding their virulotypes and plasmid gene profiles.

Origin and evolution of the serotype O80:H2

The results of the WGS-based phylogenetic analyses of the 417 (AE-)STEC and EPEC O80:H2 genomes help speculate about the origin of the serotype O80:H2, the evolution of AE-STEC and EPEC O80:H2 in different (sub)lineages and the timing of acquisition of the different LEE, *eae* genes, Stx phages, and pS88/pR444_A-like plasmids.

Two studies (62, 63) suggested that the serotypes O80:H19 (ST165) or O80:H26 (ST189), which are also members of the CC165, could be at the origin of the serotype O80:H2. This hypothesis is tempting since poultry EPEC O80:H26 have been described (62, 63, 67, 68). However, the *eae* gene present in the poultry EPEC O80:H26 is the *eaeβ* variant (Table S2), which is closely related neither to the *eaeξ* nor to the *eaeρ* variants present in AE-STEC and EPEC O80:H2 (71, 118). Moreover, this hypothesis does not take into account the non-O80 serotypes also belonging to ST 301 and/or CC165.

More recently, Cointe and collaborators (103) proposed a model of the evolution of ST301 serotypes from a common ancestor with tentative timing of the acquisition of the different virulence genes, based on the results of core genome SNP-based phylogenetic analysis of 23 representative O80:H2 strains, 57 non-O80:H2 ST301 strains, and 13 CC165 O80 strains belonging to ST165 or ST189. In this model, a common ancestor O_UK:H2 would have acquired the *eaeξ* LEE and successively different Stx phages, pO157-like plasmids and the two "complete" and "incomplete" pS88-like plasmids, whereas the O antigen would have evolved to O80. From the serotype O80:H2, other serotypes in ST301, such as O45:H2, O55:H9, O119:H2, and O186:H2 might have emerged by O and H antigen switches while acquiring the other additional sets of virulence genes. Whatever the actual origin of the ST301 serotypes, the acquisition of the LEE most probably occurred before the acquisition of Stx phage(s) and the acquisition of the pO157-like and pS88-like plasmids. However, from the results of the analysis of the 417 (AE-)STEC and EPEC O80:H2 genomes, it can be hypothesized that the history of *E. coli* O80:H2 is certainly more complex.

This alternative model indeed does not take into account (i) the presence of the *eaeρ* LEE in the EPEC of L1 (Fig. 1 and 2); (ii) the possibility of the acquisition of Stx phages encoding the same *stx* subtypes by multiple events, like in other AE-STEC serotypes (124–126); (iii) the within-lineage diversification and evolution of the pS88/pR444_A-like plasmids and of the resistance cassette identified on the pR444_A plasmid (Table 5; Tables S2 and S3; Fig. 2); and (iv) the existence of at least two groups of EPEC that are intermixed with AE-STEC, especially in SL3.2 and SL3.3 (Table S3), as already suggested by Habets and collaborators (98).

The presence of the *eaeρ* gene in the five Asian and South American EPEC of L1 but of the *eaeξ* gene in all other AE-STEC and EPEC of L2 and L3 (Table 4; Table S3) indicates a change in the *eae* gene variant in either L1 or L2 or the existence of two independent acquisition events of the LEE after their separation (Fig. 1 and 2). Nevertheless, such a difference in the *eae* gene variant and the acquisitions of the pO157- and pS88/pR444_A-like plasmids by the L2 and L3 strains suggest that a prototype of the hybrid pathotype Ex-AE-STEC O80:H2 emerged after the separation of L2/L3 from L1, which was long before O80:H2 was recognized as an important pathogen in Europe.

From the published and the current (Fig. 2) phylogenetic analyses, multiple independent acquisition events of Stx phages can be extrapolated even for phages carrying the same *stx* gene. The majority of strains of L2 and L3 (80%) are indeed (AE-)STEC, and several different subtypes of the *stx* genes (*stx1a*, *stx2a, stx2d*, or *stx2f*) were identified in all (sub)lineages (Tables 4 and 5; Table S3; Fig. 2). Moreover, as mentioned above, the major *stx* subtypes of L2, SL3.1, and SL3.2 is *stx2a* (44%), whereas that of SL3.3 is *stx2d* (88%). These observations suggest that deletion and exchange of Stx phages occurred frequently amongst strains of L2 and L3, although Stx2d phage appears to be rather stably maintained among strains in SL3.3. Interestingly enough, *in vitro* introduction of Stx2d phage was also successfully performed after isolation from one AE-STEC O80:H2, into not only K-12 laboratory strains but also an *E. coli* O80:H26 strain (127), emphasizing the possibility of horizontal transfer between O80 strains of different H serotypes *in vivo*. Moreover, one Belgian calf AE-STEC strain lost its *stx2* gene during storage between the identification by PCR and the genome sequencing (98). Finally, phylogenetic analyses from previous studies and from our current analysis (Fig. 2) and the analysis of chromosomal scars of Stx prophages strongly suggest that at least some EPEC O80:H2 have derived from AE-STEC by loss of Stx phages (62, 98, 103). However, the possibility that other EPEC represent the precursors of AE-STEC O80:H2 cannot be formally excluded at this stage. To understand the mechanisms underlying the acquisition/loss of Stx phages and the variations in *stx* genes among (AE-)STEC O80:H2, more detailed analyses of the Stx phages identified in the different *E. coli* serotypes belonging to ST301 and CC165 are required.

Various scenarios can also be proposed regarding the acquisition and evolution of the pS88-like plasmids and the pR444_A resistance cassette. Noteworthy, the original pS88 plasmid identified in a neonatal meningitis-associated *E. coli* carries the *ets* and *iuc/iutA* operons, but not the resistance cassette (110). The first scenario is the acquisition of the original "complete" pS88-like plasmid by the common SL2/SL3 ancestor of AE-STEC and EPEC O80:H2 before their evolution in different (sub)lineages, followed by the integration of the pR444_A resistance cassette at one stage, most probably through the integration of an IncQ1 plasmid. The "incomplete" pS88-like plasmids would have been generated by deletion of the *ets* and *iuc/iutA* operons in most L2/SL3.1/SL3.2 strains, whereas they were stably maintained in SL3.3 strains. Such a scenario would explain that a few strains in SL3.2 still contain the "complete" pS88-like plasmids and that the "complete" and "incomplete" pS88-like plasmids carry the resistance cassette at roughly the same rates, 93% vs. 86%. Deletion of other genes also frequently occurs in pS88/pR444_A-like plasmids, generating a notable variation in their repertoire of genes (Table S3; Fig. 2). In a second scenario, the presence of the two operons in almost all strains in SL3.3 and their absence in the majority of strains in L2/SL3.1/SL3.2 (Table 5; Table S3; Fig. 2) suggests that the "complete" and "incomplete" pS88-like plasmids existed prior to their independent acquisition by the different (sub)lineages of AE-STEC and EPEC O80:H2, followed by independent integration events of the pR444_A resistance cassette. This second scenario is consistent with the presence of the "incomplete" pS88-like plasmids in the other AE-STEC serotypes in ST301 and with the hypothesis that these new heteropathotypes might have derived from AE-STEC O80:H2 strains carrying the "incomplete" pS88-like plasmid (103). Whichever the right scenario is, some recently isolated strains would also have been cured of their pS88-like plasmids either *in vivo* or *in vitro* generating pS88-like plasmid-free strains (Tables S2 and S3). More detailed comparative genomic analysis of the pS88/pR444_A-like plasmids, such as analyses using complete plasmid sequences, in each (sub)lineage is required to understand the complexity of their evolution and diversification in AE-STEC and EPEC O80:H2, as well as in their close relatives in ST301 and CC165.

In conclusion, the evolution of the AE-STEC and EPEC O80:H2 is not as linear and straightforward as early studies suggested, but most probably follows multiple events in multiple pathways. Such events involved not only the LEE, the Stx phages, and the pS88/pR444_A-like plasmids but also other genetic elements, such as the pR444_B cryptic plasmid, the pO157-like plasmids, the SpLE1-like integrative elements, and other genomic islands. Clearly, more detailed WGS-based phylogenetic studies, including CRISPR analysis (30, 63), of AE-STEC and EPEC O80:H2 isolated from humans, calves, adult cattle, other animal species, and the environment, especially before their emergence ca. 2010 in humans, and of their close relatives in ST301 and CC165, are needed to bring answers to the different questions and hypotheses about serotype O80:H2 origin and evolution.

RESERVOIR(S) AND DETECTION OF AE-STEC AND EPEC O80:H2

Besides the microbiological questions about the evolutionary origin of the AE-STEC and EPEC O80:H2, several epidemiological questions also remain unanswered with regard to their reservoir(s) and the way of infection of humans by AE-STEC O80:H2.

As mentioned earlier, the source of the majority of infections of humans by AE-STEC is the consumption of foods (meat, dairy products, and vegetables) contaminated by the feces of ruminants (especially cattle), which can be healthy carriers in different segments of their gastrointestinal tracts (15, 30, 43). Therefore, ruminants and foodstuffs were suspected to be at the origin of the infection of humans by AE-STEC O80:H2. Indeed, the first two *eaeξ stx1* AE-STEC O80:H- reported (71) were isolated from cattle feces, and the small outbreak of 2005 in France was linked to the consumption of raw milk camembert cheese (72, 73). The few *stx2a* or *stx2d* AE-STEC and EPEC O80:H2 isolated from healthy cattle and dairy products in different countries, which were included in later studies, also seem to support this hypothesis (54, 62, 63, 75, 107). Moreover, no obvious bias in the isolation source was observed in the distribution of the genomes of AE-STEC and EPEC O80:H2 isolated from humans, diarrheic calves, and cattle in the different (sub)lineages of phylogenetic analyses, with some strains being even closely related (Fig. 2) (98, 106), suggesting the circulation of O80:H2 between humans and cattle.

However, 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 raw dairy products, and confirm that cattle is at the origin of human contamination (39, 78, 128). Moreover, the results of the recently published surveys in healthy cattle are not all successful. Although AE-STEC and EPEC O80:H2 were isolated from healthy calves and adult cattle in one farm in France (106) and from young healthy calves bought in markets and different farms in Switzerland (115), other surveys failed to isolate AE-STEC or EPEC O80:H2 from healthy dairy calves and healthy cattle at slaughterhouse or in farms, in which they had been isolated from diarrheic calves, in Belgium (44, 65, 129).

At least, three reasons can explain this failure to more frequently isolate AE-STEC and EPEC O80:H2 from healthy cattle at slaughterhouses and in farms: (i) their presence in (very) low numbers in healthy cattle feces because their ecological niche would not be the posterior segments of the intestines, unlike the recto-anal junction for AE-STEC O157:H7 (130, 131); (ii) the possibility of biases in the results based on the country and/or population sampled: for instance, France and Switzerland with positive results vs. Belgium with negative results; and (iii) the absence of more specific and selective isolation procedures of the serotype O80:H2.

Most of the published surveys, with positive or negative results, indeed applied classical procedures using different selective media for the major AE-STEC serotypes (44, 65, 106, 115, 129), whereas the procedure at ARSIA is based on the production of eHly on Ehly agar plates, followed by PCR and/or genome sequencing of isolated colonies. These non-specific procedures can be successful on fecal samples of diseased humans and of diarrheic calves excreting high concentrations of *E. coli* O80:H2, but with much greater difficulty on fecal samples of healthy cattle carriers, excreting low bacterial concentrations ($<10^2$ colony forming units [CFU] of AE-STEC O157:H7 per gram of feces), with the exceptions of the few super-shedders (sometimes $>10^7$ CFU/g of feces) (132).

Recently, however, a selective methodology based on non-melibiose fermentation and resistance to the piperacillin antibiotic was successfully applied to isolate AE-STEC O80:H2 from diseased human patients (133) but has not been assessed on fecal samples from healthy cattle to the authors' knowledge. The non-melibiose fermentation is the consequence of the replacement of part of the *mel* operon by a 70 bp-long DNA fragment (*70mel*) (133). The piperacillin was added at a concentration of 6 mg/L to inhibit most of the other non-melibiose fermenting bacterial species while AE-STEC

O80:H2 are resistant due to the presence of the *bla*$_{\text{TEM-IB}}$ gene on the resistance cassette of the pR444_A-like plasmid present in human AE-STEC O80:H2. Nevertheless, ureidopenicillins are not permitted in veterinary medicine in European Union ([Implementing regulation - 2022/1255 - EN - EUR-Lex [europa.eu]]) and the antibiotic resistance profiles of fecal *E. coli* from healthy cattle, including the AE-STEC O80:H2, are difficult to predict and relatively low (106, 134), compared with human and calf clinical isolates (62, 95, 102, 109, 135). Also noteworthy, the *bla*$_{\text{TEM-1B}}$ gene was not detected in 5.1% of the 354 pR444_A-like plasmid-positive AE-STEC and EPEC O80:H2 of different origins in our current phylogenetic analysis. Similarly, only 12 (19%) of the 63 AE-STEC and EPEC O80:H2 not harboring the pR444_A-like plasmid are positive for the *bla*$_{\text{TEM-1B}}$ gene (Tables S2 and S3). Another selective procedure was therefore assessed, based on non-melibiose fermentation and tellurite resistance (136), since tellurite resistance is common amongst AE-STEC and EPEC (137).

The presence of the *70mel* DNA sequence inactivating the *mel* operon in the 52 calf and human AE-STEC and EPEC isolated in Belgium (98) was confirmed (136). Unfortunately, although the majority of calf *stx1a* AE-STEC and EPEC O80:H2 of SL1.1 are resistant to high concentrations of tellurite and harbor the *ter* operon, the majority of *stx2d* AE-STEC of SL1.2 were not. Moreover, no AE-STEC or EPEC O80:H2 could be isolated from fecal samples at one slaughterhouse following this procedure (136). The analysis of the 417 genomes of (AE)STEC and EPEC O80:H2 also confirms the presence of the *70mel* sequence in all AE-STEC and EPEC O80:H2, including in the five *eaeρ* EPEC, and its absence in the four *E. coli* O80:non-H2 (Tables S2 and S3; Fig. 2), as previously observed (133, 136). This result strongly suggests that a *70mel*-involved inactivation of the *mel* operon occurred in the common ancestor of the *E. coli* O80:H2 strains and that all are unable to ferment melibiose whichever their virulotype and (sub)lineage. Conversely, the *ter* operon was not detected in 45.5% of the 417 genomes of (AE-)STEC and EPEC O80:H2 (Tables S2 and S3; Fig. 2). In particular, most strains in SL3.3 do not contain the *ter* operon, confirming that tellurite is not any efficient selective agent of AE-STEC and EPEC O80:H2.

Similar results have been reported in the food industry. For instance, no AE-STEC or EPEC O80:H2 could be isolated from raw milk and raw milk cheese in France, even if qPCR targeting the *stx*, *eaeξ,* and *wzx*$_{O80}$ genes were positive (138). This result can be explained not only by the presence of low numbers of AE-STEC or EPEC O80:H2 but also by the presence of the *eaeξ* gene in other serotypes, for instance, O45:H2, O55:H9, O119:H2, and O186:H2 (70, 75, 95, 103) and of non-CC165 O80 serotypes, like O80:H6 and O80:H45 (65). Following previous studies on the usefulness of the type III effector-encoding *espK* and *espV* genes to more specifically detect and identify highly pathogenic AE-STEC serotypes in beef and dairy samples (138–140), the USDA-FSIS MLG5C reference method was updated (MLG5C.04) (141). Applying the MLG5C.04 method, Tran and collaborators recently reported that non-AE-STEC O80 *E. coli* are probably much more prevalent on ground beef and carcasses than AE-STEC O80:H2, if any (142), similar to the results obtained on cattle fecal materials (65, 136). However, they did not attempt to isolate the *E. coli* O80 from the positive samples, to the understanding of the authors. Nevertheless, the *espK* and *espV* genes would not be able to differentiate between AE-STEC and EPEC O80:H2 according to the results on 417 genomes (Table 4; Table S2), in contrast to the classical major AE-STEC serotypes (40).

As conclusion, two important questions remain unanswered today (39): (i) Are the ruminants, especially cattle, healthy carriers of AE-STEC and EPEC O80:H2? and (ii) Are they at the origin, directly or indirectly, of human infections? Clearly, more bacteriological and epidemiological studies are needed to identify the reservoir(s) and decipher the way of transmission of AE-STEC and EPEC O80:H2 in order to more efficiently prevent and handle human infections. Regarding the epidemiological studies, the absence of any other outbreak than a small one in France in 2005 (72) hampers the understanding of the way of transmission. As far as the bacteriological studies are concerned, the identification of the resistance cassette of the pR444_A plasmid in 84.9% of the 417 AE-STEC and EPEC O80:H2 analyzed may help design and assess procedures based on new selective media to isolate AE-STEC and EPECO80:H2 from healthy cattle. However, since 15.1% of these 417 AE-STEC and EPEC O80:H2 analyzed are pR444_A resistance cassette-negative and/or pS88/pR444_A-like plasmid-negative (Tables S2 and S3), a combination of selective procedures should be used in future surveys. Moreover, other potential reservoirs than ruminants should not be forgotten during such surveys, like other domestic animals, wild animals, environment, or even humans. For instance, EPEC O80:H2 have already been isolated from pigs, pig farms, and water in different European countries (54, 62, 108).

GENERAL CONCLUSIONS AND PERSPECTIVES

The triple hybrid Ex-AE-STEC O80:H2 illustrate once more the high flexibility of and the potential of accumulating virulence and resistance genes in the genomes of *E. coli* in general and of STEC in particular, as dramatically illustrated by the outbreak of the aggregative STEC (Agg-STEC) O104:H4 in 2011 in Germany (29, 143). Today, the AE-STEC O80:H2 can certainly move to the STEC seropathotypes B group, as defined by Karmali and collaborators (33). The actual question is today: which serotype will be the next one?

When comparing the stories of the STEC O80:H2 and O104:H4, epidemiological and microbiological differences can be listed:

1. in contrast to Agg-STEC O104:H4, AE-STEC O80:H2 have not caused any dramatic outbreak yet but have progressively emerged during the 2010 s;
2. AE-STEC O80:H2 are still emerging, whereas the 2011 outbreak by Agg-STEC O104:H4 was short-lived;
3. AE-STEC O80:H2 are highly associated with HUS cases in children and elderly more like AE-STEC O157:H7, whereas Agg-STEC O104:H4 caused HC and HUS primarily in adults;
4. AE-STEC O80:H2 are also present and cause diseases in animals, especially young calves in contrast to Agg-STEC O104:H4 that were restricted to humans;
5. AE-STEC O80:H2 can also be invasive owing to the presence of the pS88/pR444_A-like plasmids, in contrast to Agg-STEC O104:H4 (and to other AE-STEC, like serotype O157:H7 for instance);
6. human and calf EPEC O80:H2 are phylogenetically related to human and calf AE-STEC O80:H2, whereas Agg-EPEC O104:H4 have not been described.

The final questions are: why did these AE-STEC and EPEC O80:H2 emerge around the year 2010? In other words, which event(s) prompted this serotype to emerge at that particular time and not before? Why are they still persisting today? Compared with the Agg-STEC O104:H4 outbreaks, there is no answer yet, since no common source of contamination, especially not any food consumption, could be identified despite different epidemiological studies (39, 78, 128).

According to Darwinism, acquired properties persist only if they give an advantage to the organism. We can therefore wonder about the advantage given to the serotype O80:H2 by acquiring the *eaeξ* LEE, the different *stx* genes and, more especially the pS88/pR444_A-like plasmids, which seem to be stably maintained. Although there exist no mammalian experimental models for STEC, lepidoptera, like the *Galleria mellonella* moth larvae (144, 145), can be used as a first-line model to assess the role of the different properties of AE-STEC and EPEC O80:H2 in their virulence, as recently performed (135): (i) AE-STEC and EPEC O80:H2 are lethal for the larvae with some difference in the lethal concentrations according to the strain; (ii) the Stx2d and, to a lesser extent, the Stx1a and the pS88-like plasmids are responsible for the lethality of the larvae by the *E. coli* O80:H2; (iii) the EPEC O80:H2 harboring the pR444_A-like plasmid containing the *ets* and *iuc/iutA* operons is statistically almost twice as lethal than the EPEC strain harboring one pS88-like plasmid not carrying these two operons. More experiments are now needed to explore the respective role of each property encoded by the pS88-like plasmids in *G. mellonella* larvae.

As a general conclusion of this review manuscript, to better understand and prevent future contaminations and maybe outbreaks not only by AE-STEC O80:H2 but also by other serotypes belonging to ST301 and CC165 (for instance, O45:H2, O119:H2, O186:H2, and O55:H9), intensive surveillance plans, and more studies are necessary to identify (i) the actual reservoir(s) and way(s) of contamination; (ii) the origin, acquisition times, evolution, and actual role in pathogenicity of each virulence property; (iii) the relationship between AE-STEC and EPEC from humans and animals belonging to the same serotype and/or lineage; and (iv) the antibiotic resistance patterns beyond the resistance genes present on the pR444_A cassette.

For instance, among the non-O80:H2 serotypes in ST301, AE-STEC O55:H9 are of particular clinical importance in humans. In addition to the *eaeξ*-positive LEE, different *stx* genes and "incomplete" pS88-like plasmids, they sometimes acquired the *fyuA* gene coding for the yersiniabactin extra-intestinal virulence factor and one of the three sub-lineages also acquired genes coding for extended-spectrum β lactamases (ESBLs). Up to date, AE-STEC O55:H9 have been identified in France, Germany, and UK (93, 103).

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (CMR00011-25-s0001.xlsx). List of the 417 AE-STEC, EPEC, and STEC O80:H2 and four *E. coli* O80:non-H2 strains analyzed in this study.

Table S2 (CMR00011-25-s0002.xlsx). List of the 417 AE-STEC, EPEC, and STEC O80:H2 and four *E. coli* O80:non-H2 strains according to their virulotypes.

Table S3 (CMR00011-25-s0003.xlsx). List of the 417 AE-STEC, EPEC, and STEC O80:H2 strains according to their place in (sub)lineages in the core gene SNP-based phylogenetic tree.

Table S4 (CMR00011-25-s0004.xlsx). The pR444_A (Accession No. QBDM01000004.1)-encoding genes in an in-house database for repertoire analyses.

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AUTHOR BIOS

Jacques G. Mainil, DVM, PhD, Doctorate, is an emeritus professor of the ULiège, Faculty of Veterinary Medicine, Department of Infectious Diseases, Laboratory of Bacteriology. After graduating as a DVM from ULiège in 1981, he began his career as an assistant professor and received his PhD in 1988 and his Doctorate in 2003. He was confirmed associate professor in 1994, promoted to professor in 2007 and retired in 2021. He spent 40 years studying the virulence properties and the molecular epidemiology of bacterial species causing enteritis, enterotoxaemia and/or septicemia, focusing on pathogenic *Escherichia coli* in cattle. He began to work on STEC and EPEC in the mid-1980s during a sabbatical year at the NADC, Ames, IA, under the supervision of Prof. Harley W. Moon. The work on the Belgian STEC and EPEC O80:H2 in humans and calves began in 2016 in collaboration with the three institutes listed.

Keiji Nakamura, DVM, PhD, obtained a Doctor of Veterinary Medicine degree in 2008 from Osaka Prefecture University, Japan, and his PhD in 2012 at the Graduate School of Life and Environmental Sciences, Osaka Prefecture University. Subsequently, he worked as a postdoctoral fellow at Osaka Prefecture University (1 year) and Osaka University (3 years). In 2016, he joined the Department of Bacteriology at the Graduate School of Medical Sciences, Kyushu University, and progressed to Lecturer in 2023. His research interest is consistently bacterial pathogens of public health concern, including *Clostridium botulinum*, *Bordetella pertussis*, and enterohemorrhagic *Escherichia coli* (EHEC). He is currently analyzing the pathogenic evolution of EHEC, the diversification of Shiga toxin-transducing phages (Stx phages), and the prophage-prophage interactions by utilizing whole genome sequencing and bioinformatics.

Rie Ikeda, DVM, graduated as a Doctor of Veterinary Medicine from Osaka Prefecture University, Japan, in 2020. Her interest in infection diseases and veterinary bacteriology already developed during a summer internship in 2018 at the ULiège, Faculty of Veterinary Medicine, Department of Infectious Diseases, Bacteriology, Belgium. In November 2020, she started her PhD at the ULiège. Her PhD research focuses on *Escherichia coli* serotype O80:H2, with particular emphasis on the identification of their reservoir and on the comparison of strains from calves and humans. In the future, she aims to keep contributing to public and global health through her research.

Florence Crombé, Sc, PhD, is a molecular biologist at the "Universitair Ziekenhuis Brussel", Department Clinical Biology, Laboratory of Microbiology and Infection Control. She graduated in Biomedical Sciences at the "Katholieke Universiteit Leuven", Belgium, in 2007 and received her PhD at the Faculty of Veterinary Medicine of the "Universiteit Gent" in 2012. She is co-responsible for the National Reference Center for Shiga toxin-producing *Escherichia coli* since 2019, where she participates to the surveillance of STEC infections in humans through molecular testing.

Jacob Diderich, DVM, graduated from the Faculty of Veterinary Medicine, ULiège, Belgium, in 2021. During his student thesis, he worked on the transduction of Shiga toxin-encoding genes from Shiga toxin-producing *Escherichia coli* (STEC) O80:H2 to non-STEC strains. After graduation, he collaborated with the surveillance network of microbial and parasitic infections in wildlife and participated to in vivo preclinical trials of SARS-CoV-2 treatments at the Veterinary Faculty of ULiège. More recently, he analyzed genome sequences of calf *E. coli* O80:H2. Today, his research work focuses on the use of bacteriophages to control *E. coli* mastitis in dairy cattle, as main purpose of his PhD in Veterinary Bacteriology.

Marc Saulmont, DVM, graduated from the Faculty of Veterinary Medicine, ULiège, Belgium, in 1998 and worked as a field practitioner in France for 6 years. In 2004, he began to work at ARSIA asbl ("Association régionale de Santé et d'Identification animale"), Ciney, Belgium. Today, he is in charge of the laboratory of bacteriology, parasitology and pathology. His main activities are the bacteriological diagnosis on samples received from farms or from the post-mortem room (hundreds of enterobacteria are isolated and typed each year) and the monitoring of antibiotic resistance in animals (since 2013).

Denis Piérard, MD, PhD, is an emeritus professor at "Vrije Universiteit Brussel", Belgium. After graduate education at "Université Libre de Bruxelles", he specialized in Medical Microbiology. He promoted in 1998 with a thesis entitled "Epidemiology, clinical impact and virulence factors of verocytotoxin-producing *Escherichia coli* in Belgium". He was member of several scientific societies, in particular the Belgian Society of Infectiology and Clinical Microbiology. He retired in 2021, but is still active as consultant. During his career, he developed reference activities for Shiga toxin-producing *Escherichia coli*, *diphtheria*, *pertussis*, *Legionella*, *Burkholderia* and AIDS in the frame of Belgian National Reference Centers. He published 298 indexed articles on these subjects and on other microbiological themes, such as antibiotic susceptibility of anaerobic bacteria, mass-spectrometry identification, and automation in the clinical microbiology laboratory. He (co-)supervised 10 PhD students and is still supervising one PhD student who works on the topic of *diphtheria* and *pertussis*.

Damien Thiry, DVM, PhD, is a Professor of Bacteriology at the Department of Infectious Diseases of the Faculty of Veterinary Medicine, ULiège, Belgium. He graduated as a DVM in 2009 and obtained his PhD in 2015, whose subject was the interactions between hepatitis E virus and pigs, as part of a collaboration between the Veterinary Virology laboratory (ULiège) and the Scientific Institute of Public Health (Sciensano, Brussels). In 2014, he was hired as an assistant professor by the Bacteriology laboratory (ULiège). He performed post-doctoral stays at the Pasteur Institute (Paris) and at the "Katholieke Universiteit Leuven", where he developed a *Galleria mellonella* model of phage therapy against *Klebsiella pneumoniae*. He was promoted associate professor in 2021, following retirement of Prof. Mainil, and professor in 2024. Today, his main research topics are related to the use of bacteriophages as alternative treatment against antibiotic resistant bacterial infections.