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Spécificité d'hôte de souches entérohémorragiques et entéropathogéniques *d'Escherichia coli* : Etude comparative de souches isolées de bovins et d'humains.

Host specificity of enterohaemorragic and enteropathogenic *Escherichia coli* strains: a comprehensive comparison of strains isolated from bovines and from humans

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Résumé

L'espèce *Escherichia coli* est un bacille à Gram négatif appartenant au groupe des entérobactéries. Cette bactérie est commensale de la flore intestinale des mammifères. Cependant, il existe différentes souches pathogènes, notamment pour l'intestin. On les classe selon leurs propriétés de virulence : les entéropathogènes (EPEC), les entérotoxinogènes (ETEC), les entérohémorragiques (EHEC), les entéroinvasives (EIEC), les entéroaggrégatives (EAEC), les vérotoxinogènes (VTEC), les souches « diffuse adherent » (DAEC), les nécrotoxinogènes (NTEC), etc.

Les souches EPEC, EHEC et VTEC représentent actuellement un problème important en santé publique dans le monde entier. En effet, ces souches provoquent des diarrhées (infections à EHEC et EPEC) pouvant être hémorragiques dans le cas d'infection à EHEC et pouvant mener au syndrome hémolytique et urémique (HUS) et conduire à la mort dans le cas des souches EHEC et VTEC. De plus, il a été démontré que le réservoir majeur des souches EHEC et VTEC est le ruminant et que la majorité des contaminations de l'homme se réalisent via les denrées alimentaires animales et végétales souillées par des matières fécales. Le principal sérogroupe EHEC pathogène pour l'homme est O157, suivi des sérogroupes O26, O111 et O103.

Dans le domaine vétérinaire, quelques sérogroupes de souches EHEC (O26, O111, O118 par exemple) sont aussi directement associés à des troubles digestifs chez les veaux âgés de deux semaines à deux mois. Il en résulte des pertes économiques dues au retard de croissance et à la faiblesse de ces animaux.

La pathogénie de l'infection par des souches EHEC se déroule probablement en trois étapes : (1) l'adhérence initiale aux entérocytes via des adhésines particulières, (2) l'adhérence secondaire et la colonisation via la production de lésions d'attachement et d'effacement des entérocytes (A/E) et (3) la production de vérotoxines ou shigatoxines (Stx). Ces toxines agissent en provoquant l'arrêt de la synthèse protéique des cellules cibles (les cellules endothéliales *in vivo*), ce qui engendre des hémorragies locales et le dysfonctionnement des organes atteints. La lésion A/E consiste en l'induction de réarrangements à hauteur du cytosquelette des entérocytes provoquant la disparition (l'effacement) des microvillosités et en une adhérence très proche (intime) des bactéries à l'entérocyte par l'intermédiaire d'une protéine de la membrane externe. Les souches EPEC ne produisent pas de Stx (étape 3) et les souches VTEC ne

produisent pas de lésions d'attachement et d'effacement (étape 2). Actuellement, très peu d'adhésines intervenant dans l'adhérence initiale ont été décrites pour les souches EHEC et le mécanisme de l'adhérence initiale dans l'intestin par les souches EHEC reste encore grandement incompris à ce jour. Or, cette adhérence initiale représente une étape très importante dans la pathogénie. Sans elle, la bactérie ne peut poursuivre le processus d'infection de l'hôte. De plus, ces adhésines représentent la base de la spécificité d'hôte pour de nombreuses bactéries pathogènes. Il est d'autant plus important d'identifier ces structures que, ni les facteurs impliqués dans les lésions AE, ni les vérotoxines ne montrent une véritable spécificité d'hôte.

Dans ce travail, notre but est d'identifier des facteurs impliqués dans l'adhérence initiale et/ou dans la spécificité d'hôte (homme ou bovin) des souches EHEC appartenant au sérogroupe O26. La description de tels facteurs permettra d'accomplir un progrès important dans la connaissance de la pathogénicité des EHEC. De plus, elle permettra le développement d'outils supplémentaires de typage épidémiologique, ce qui facilitera, entre autre, l'analyse des risques encourus lors de l'isolement de souches EHEC dans les denrées alimentaires.

Le projet est basé sur quatre points : (1) la collecte de nouvelles souches EHEC, EPEC et VTEC et leur étude phylogénétique; (2) l'étude d'une collection de souches O26 d'origine bovine et humaine pour les facteurs de virulence et les adhésines déjà décrites dans la littérature (adhésines potentielles et polymorphisme des gènes *tir, eae* et *tccP2*); (3) la recherche de facteurs (éventuellement de virulence) spécifiques d'hôte ou impliqués dans l'adhérence initiale par la technique d'hybridation suppressive soustractive (SSH); (4) la comparaison des génomes de souches d'origine humaine et bovine par la technique de *Whole Genome PCR Scanning* (WGPS) et de typage basé sur la présence/absence de copie de l'IS621 par PCR.

a) Recherche de souches EHEC, EPEC et VTEC chez les veaux de boucherie et les cervidés

Une recherche de souches EHEC, EPEC et VTEC a été effectuée sur des matières fécales de veaux de boucherie en 2007 et en 2008 et sur des matières fécales de cervidés durant la saison de chasse de 2008 et de 2009. Douze pourcent des veaux de boucherie et 15% des cervidés sont porteurs d'un des trois pathotypes. De plus, 4% des veaux de boucherie sont porteurs de souches O26 ou O111 tandis qu'aucun cervidé n'est porteur de souche appartenant à un sérogroupe du « gang of five » (sérogroupes O157, O26, O111, O103, O145). De plus, nous avons également étudié les antibiorésistances de ces souches contre plusieurs antibiotiques.

Quatre-vingt trois pourcent et 22% des souches isolées respectivement chez les veaux et chez les cervidés sont résistantes à minimum deux antibiotiques.

b) Analyse de facteurs d'adhérence

- *Recherche de 27 adhésines déjà décrites*

La recherche de 27 adhésines connues dans la littérature pour les souches EHEC et EPEC a été effectuée sur notre collection de souches de sérogroupe O26. Quatre catégories d'adhésine ont été définies dans cette étude : les adhésines présentes dans toutes les souches O26, les adhésines présentes dans la plupart des souches O26, les adhésines présentes dans peu de souches O26 et les adhésines jamais présentes dans les souches O26. Concernant la spécificité d'hôte, aucune différence significative n'a été observée. Seule une adhésine (LdaE) est statistiquement associée aux souches EPEC/EHEC isolées de veaux diarrhéiques en comparaison aux veaux non-diarrhéiques.

- *Polymorphisme des gènes* tir, eae *et* tccP2

Les gènes codant pour l'intimine (*eae*), pour Tir (*tir*) et pour TCCP2 (*tccP2*) de la collection de souches EHEC et EPEC de sérogroupe O26 ont été amplifiés par PCR dans leur entièreté et séquencés afin d'étudier leur polymorphisme. Un des objectifs de cette étude est de déterminer si certains polymorphismes sont associés à un hôte particulier, comme cela a été observé pour les souches du sérogroupe O157. Les résultats montrent que (1) les polymorphismes de *tir* et *eae* sont peu fréquents et aucun n'est associé à un hôte particulier (homme ou bovin) pour les souches étudiées et (2) que différent variants de *tccP2* ont été détectés et deux d'entre eux sont associés à un pathotype particulier.

c) Recherche de nouveaux facteurs spécifiques d'hôte par la technique d'hybridation suppressive soustractive

La méthode d'hybridation suppressive soustractive (SSH) est une technique qui permet d'identifier des gènes présents uniquement dans une souche (le *tester*) et non dans une autre (le *driver*). Dans notre étude, le génome d'une souche EHEC bovine O26:H11 a été comparé au génome d'une souche EHEC humaine O26:H11.

Plusieurs candidats potentiellement impliqués dans la spécificité d'hôte ont été mis en évidence : séquences homologues à des adhésines connues, fragments semblables à des gènes présents dans les phages P1 et P7, dans l'îlot de pathogénicité (PAI_{O113}) présent dans une souche VTEC de sérogroupe O113, dans l'espèce *Nitromonas europeae* et dans les genres *Shigella sp*. et *Salmonella sp.* (mais qui ne sont pas présents dans l'espèce *E. coli)*. Ces fragments ont été recherchés dans notre collection de souches EPEC et EHEC de sérogroupe O26 par hybridation sur colonie. L'îlot de pathogénicité provenant de la souche O113, PAI_{O113} (comprenant plusieurs gènes codant pour des adhésines) a été choisi pour une étude de répartition plus détaillée par PCR. Une grande partie de cet îlot est présent dans 8 souches parmi toutes celles étudiées. Aucune spécificité d'hôte n'a pu être observée. Cependant, ces résultats sont en contradiction avec ceux obtenus jusqu'à présent qui stipulaient que cet îlot est spécifique des souches VTEC (négatives pour le gène *eae*).

d) Comparaison génomique globale de souches bovines et humaines

- *WGPS*

La technique de *Whole Genome PCR Scanning* (WGPS) permet de comparer le génome de plusieurs souches et de déterminer les régions génomiques différentes qui existent entre ces souches. Le principe est basé sur l'amplification par PCR « *long range* » de tout le génome et sur la comparaison des amplicons obtenus. Dans notre étude, nous avons comparé dix souches O26 (5 souches EHEC humaines et 5 souches EHEC bovines).

Cette technique a permis de déterminer que les souches O26 ont, en moyenne, une diversité de 12% entre elles. Ces différences sont en grande majorité liées à la présence/absence d'IE (*Intregrative Elements*) ou de phages. Ensuite, 7 régions présentent des différences entre les souches bovines et humaines et pourraient comporter des gènes impliqués dans la spécificité d'hôte. Afin de confirmer leur caractère spécifique d'hôte, 55 souches EHEC et EPEC de sérogroupe O26 (humains et bovins) ont été testées par PCR pour ces régions. Cinq sur les sept régions présentent des différences en taille qui sont statistiquement spécifiques des souches EPEC isolées de veaux diarrhéiques.

- *Typage IS621*

Le WGPS a permis d'identifier 18 copies de l'IS621 (séquences d'insertion présentes en copies multiples) dans ces génomes. Des PCR de détection, basées sur des amorces situées en amont et en aval de chaque copie de l'IS621, permettent de déterminer leur présence ou leur

absence et une PCR multiplexe amplifiant 13 des 18 copies de l'IS621 a été mise au point. Septante-cinq souches EHEC et EPEC de sérogroupe O26 ont été étudiées à l'aide de cette PCR multiplexe et les profils ainsi obtenus ont été comparés entre eux selon l'origine des souches (hôtes, pays) et leur pathotype (EHEC ou EPEC). Cette nouvelle technique représente un outil de typage rapide et efficace qui pourrait être utilisé dans l'étude épidémiologique de souches EPEC et EHEC de sérogroupe O26.

En conclusion, ces différentes approches nous ont permis de comparer génomiquement des souches EHEC et EPEC de sérogroupe O26 isolées de bovins et d'humains afin de déterminer si une spécificité d'hôte basée sur une différence génomique existe. Aucune spécificité d'hôte n'a pu être mise en évidence. Seul un sous-groupe de souches EPEC isolées de veaux diarrhéiques semble apparaître par la présence de caractères génomiques communs. Ce sous-groupe de souches pourrait être adapté aux veaux et posséder les facteurs spécifiques et nécessaires pour provoquer des diarrhées chez ceux-ci, sans préjuger de leur potentiel zoonotique. Cependant, des analyses supplémentaires sur une plus grande population de souches devraient être effectuées avant de conclure à l'existence d'un tel sous-groupe. Pour les autres souches, une spécificité d'hôte potentielle pourrait être basée sur : (i) d'autres adhésines non détectées dans notre étude, (ii) des variations dans l'expression de gènes (adhésines par exemple) par rapport à son environnement, (iii) d'autres facteurs que des adhésines, comme des facteurs métaboliques. Une dernière possibilité à considérer est qu'il n'existe pas de spécificité d'hôte.

Summary

Enterohaemorrhagic *Escherichia coli* strains (EHEC) represent an important problem for public health in developed countries. Indeed, EHEC strains can infect the human beings by vegetal and animal foodstuffs soiled by ruminant faeces (ruminants are considered to be healthy carriers) and cause food poisoning with diarrhoeas. They are generally accompanied by haemorrhagic colitis (HC) with, in a few cases, occurrence of renal sequelae (HUS) that can lead to death. In the veterinary field, several serogroups of EHEC strains (O5, O26, O111, O118 for example) are directly associated with digestive disorders in two week- to two month-old calves. The consequences are economic losses due to delay of growth and weakness of calves.

Pathogenicity of EHEC strains is probably divided in three stages: (1) colonisation of intestine by specific adhesins, (2) production by the bacteria of a signal that causes cytoskeleton's rearrangement in enterocytes and intimate adherence of bacteria to eukaryotes cells by specific proteins, the INTIMIN adhesins, (3) production of Shiga toxins (Stx) responsible for HC and HUS. During EPEC infections, strains do not produce Stx (step 3) and during VTEC infection, the step 2 is not accomplished. The initial adherence via specific adhesins (step 1) is actually still poorly understood for many strains whereas its importance is significant and resides in the following: 1) adherence is the first contact between bacteria and intestinal cells without which the other steps cannot occur, 2) adherence is the basis of host specificity for a lot of pathogens, 3) adhesins represent a good target for the development of a specific vaccinal prophylaxis.

The specific aim of this study on O26 EHEC and EPEC is double: (i) the identification of the factors implicated in initial attachment and/or in host specificity (human or cattle) of EHEC strains O26; and (ii) the whole genome comparison of EHEC strains isolated from human and cattle to identify host-specific sequences and/or clones. This work is therefore divided into four steps: (1) collection of new O26 EHEC, EPEC and VTEC strains and comparison by Pulsed Field Gel Electrophoresis (PFGE), (2) analysis of the distribution of already described adherence factors (putative adhesins and polymorphisms in *tir*, *eae* and *tccP2* genes), (3) search of new virulence factors implicated in the host specificity or in the initial adherence by Suppressive Subtractive Hybridisation (SSH), (4) comprehensive genomic comparison of bovine and human strains by Whole Genome PCR Scanning (WGPS) and multiple PCR-based IS fingerprint.

Firstly, we searched EPEC, EHEC and VTEC strains in veal calves and wild cervids with the aim to collect news strains. We showed in our study that the prevalence in Belgium of EPEC, EHEC and VTEC strains is about 12% in veal calves and about 15% in wild cervids. In addition, about 4% of the calves carried O26 or O111 serogroup strains, which are often associated to human infections. Moreover, we studied the resistance of the collected strains for several commonly used antibiotics. Results for the veal calves were significantly different from those obtained for the wild cervids: 83% of the strains isolated from the calves were resistant to two or more antibiotics in comparison to only 21.6% of the strains isolated from the wild animals.

Secondly, we decided to study the potential association of the pre-existing adhesins and their host. The first study that we performed is the search of 27 described adhesins in a collection of O26 EPEC and EHEC strains. Results showed that none of the adhesins was statistically associated to strains isolated from humans or bovines. Only the *ldaE* gene was more often found in EHEC and a-EPEC strains isolated from diarrhoeic bovine in comparison to healthy bovines. In the second work, we studied *tir, eae* and *tccP2* genes polymorphisms. Results showed that none of them were associated with bovine or human isolates. Nevertheless, two of the tccP2 variants were associated with a specific pathotype.

Thirdly, the SSH revealed the presence of DNA sequences with homology to genes or pathogenicity islands (PAIs) present in other specific *E. coli* pathotypes (e.g. PAI I_{CL3}) or other genders (e.g. *Klebsiella sp., Nitromonas sp.*), and not known to be present in EHEC and EPEC strains of serogroup O26, suggesting a horizontal transfer of genomic regions between bacteria. In addition, we showed that the Pathogenicity Island PAI_{O113} was present in EHEC and EPEC strains (and not specifically in VTEC strains as previously assumed). Nevertheless, no host specificity was observed.

Fourthly, the WGPS revealed a structural diversity in the genome of O26 EHEC strains of 12% in the average in comparison to the reference strain. Two-third of the variations were located in Integrative Elements (IE) or prophage regions. Besides, five regions were statistically associated with EPEC strains isolated from diarrhoeic bovines.

Fifthly, we developed a new and rapid (<24h) first-line surveillance typing assay of O26 EPEC and EHEC strains based on the results of the WGPS by the amplification of 13 IS*621* regions.

In conclusion, we did not obtain a specific factor associated with the isolate original host. Nevertheless, a sub-group of EPEC strains isolated from calves with diarrhoea appeared to

share specific genomic characteristics. This sub-group could possess some specific properties to produce diarrhoea in young calves not including their zoonotic potential at this stage. Specific analyses on a larger population of strains have to be performed before concluding that this kind of sub-group really exists. For the other strains, several hypotheses concerning a potential host specificity can be put forward: (i) other adhesins, not detected here, could be at the basis of the host-specific character, (ii) variations in the expression of some host-specific adhesins could be related to the growth environment, (iii) the host specificity could be based on properties other than adherence, such as metabolism. One other possibility is that there is no host specificity.

1. Introduction

1.1. Escherichia coli

1.1.1. History, taxonomy and characteristics

One hundred and twenty-seven years ago began the fabulous *Escherichia coli* history. In 1885, Theodor Escherich, a German medical doctor, isolated a common intestinal bacterium from infants stools and intestines (Escherich, 1885). He described the microorganism as a Gram-negative rod-shaped bacterium with curved extremities. This bacterium was originally called *Bacterium coli commune* by Dr. Escherich; then *Bacillus coli* and *Bacterium coli* by Migula in 1895 (Migula, 1895) to be finally renamed *Escherichia coli* in 1919 (Castellani and Chalmers).

The gender *Escherichia* belongs to the proteobacteria phylum, the gammaproteobacteria class, the enterobacteriales order and the enterobacteriaceae family (such as the genders *Salmonella, Shigella, Yersinia, Enterobacter, Escherichia, Klebsiella, Citrobacter*). After 88 years of loneliness, *Escherichia coli* finally got some companions and five other species have been described:

- *Escherichia blattae* (Burgess *et al.*, 1973): isolated from cockroach (*Blatta orientalis)* hind-gut without any known pathogenic properties;
- *Escherichia vulneris* (Brenner *et al.*, 1982): pathogenic species isolated from stool, wound and environment;
- *Escherichia hermanii* (Brenner *et al.*, 1982): pathogenic species isolated from stool, wound and environment;
- *Escherichia fergusonii* (Farmer *et al.*, 1985) pathogenic species isolated from stool, wound and environment;
- *Escherichia albertii* (Albert *et al.*, 1991, Huys *et al.*, 2003): diarrhoeagenic species isolated from stool.

Most *Escherichia coli* (*E. coli*) strains are harmless commensal bacteria acquired during the few hours after birth and form a part of the normal microbiota of warm-blooded organisms gut (Tenaillon *et al.*, 2010). Its proportion in the intestinal flora depends on several factors (e.g. country of birth, feeding practice, delivery method), is different from person to person and evolves during life (Adlerberth and Wold, 2009). They are to the advantage of their hosts by assisting vitamin K absorption by the host (Meganathan, 2001, Ramotar *et al.*, 1984) and by inhibiting pathogenic bacteria growth within the intestine (Kruis *et al.*, 2004).

This rod-shaped bacterium (cell length average: 2 microns; cell diameter average: 0.8 micron) is Gram-negative and does not sporulate. Some strains, but not all, possess peritricheous flagella and are motile (Bergey, 1984).

E. coli (Figure 1) is a facultative anaerobic bacteria and consequently can grow with or without oxygen. Under anaerobic conditions, *E. coli* can grow by means of fermentation (producing gases and acids; e.g. lactic acid, succinic acid, acetic acid, formic acid, carbon dioxide) or anaerobic respiration (using NO_2, SO_3, fumarate as final electron acceptors). Therefore, it may live on a large variety of substrates. Some *E. coli* biochemical characteristics are used to identify the species: glucose and several other carbohydrate fermentation; methyl red positive and Voges-Proskauer negative; no growth on citrate; production of catalase, tryptophanase and lipase, but not of peroxidase or H_2S (from cystein); and negative for urease production. Moreover, 95% of *E. coli* has the ability to ferment lactose by β-galactosidase enzyme action, which hydrolyses lactose into glucose and galactose (Bergey, 1984).

The optimal *E. coli* growth is at 37°C, but most strains can grow at a temperature range from 7.5°C up to 49°C (Neidhardt, 1987). Furthermore, this bacterium can survive at a temperature close to 0°C for several days. Additionally, *E. coli* can grow at pH from 4 to 9 and is able to survive at lower pH using acid resistance mechanisms (Foster, 2004). Consequently, *E. coli* is capable to survive and to remain during a long period (several weeks to several months) in the environment or in food.

Finally, *E. coli* strains (Figure 1) can be identified by the characterisation of three antigens (Orskov and Orskov, 1992, Stenutz *et al.*, 2006, Whitfield, 2006):

- O-antigen (derived from the German expression "Ohne Kapsel", meaning "without capsule"): antigenicity of the distal carbohydrate moiety of the lipopolysaccharide (LPS) attached to the cell outer membrane. At least 180 O-antigens have been described so far;
- H-antigen (derived from the German word "Hauch"): flagellar protein antigenicity of motile strains. at least 70 H-antigens have been described; strains without flagella are noted as H- or NM (non motile);
- K-antigen (derived from the German family name "Kauffmann"): antigenicity of acidic capsular polysaccharide. At least 80 K-antigens have been described.

E. coli strains are first identified using O-antigen, then H- and K-antigens are specified (e.g. O104:H4, O157:H7, O26:H11). Using this division, hundreds of serotypes have been described. But only a few are commonly found in pathogenic strains.

1.1.2. Pathogenic strains

Besides commensal strains belonging to the normal microbiota, some *E. coli* strains harbour virulence factors and properties (such as adhesins, toxins, siderophores, serum resistance, invasins, etc) that enable them to cause diseases in humans or animals (e.g. enteric diseases, urinary tract infections, septicaemia, …) (Kaper *et al.*, 2004, Nataro and Kaper, 1998). Rapidly after its discovery, *E. coli* was associated with peritonitis in humans and animals by the Belgian doctor Laruelle (Laruelle, 1889). And, in 1893, the Danish veterinarian Carl Oluf Jensen showed that *E. coli* was the cause of white scour in calves and suggested the species heterogeneity formed from "pathogenic" and "non-pathogenic" strains (Jensen, 1893).

The strains serotyping (O-, H- and K-typing) was the first method to distinguish pathogenic from non-pathogenic strains (Hechemy *et al.*, 1974, Orskov *et al.*, 1984, Terlecki and Sojka, 1965). Indeed, some serotypes were frequently associated with diseases and others were seldom, if ever found in pathogenic *E. coli.*

Another way of categorisation is based on the colonisation place (intestine, urinary tract or other). Two main categories are defined: the diarrhoeagenic *E. coli* strains (DEC), colonising the intestines, and the extra-intestinal *E. coli* strains (ExPEC), subdivided into uropathogenic strains (UPEC) and invasive/septicaemic (SePEC), including the strains causing Neonatal Meningitis in infants (NMEC) and Avian Pathogenic *E. coli* (APEC) strains causing septicaemia and systemic infections in birds.

Today pathogenic *E. coli* strains are classified according to the virulence factors/properties of the bacteria causing specific pathologies (Cooke, 1985, Pohl *et al.*, 1993). Each unique combination is defined as one pathotype and each pathotype can be classified inside one of the "clinical" categories defined above (Table 1). The following eight pathotypes are the most frequent today (Kaper *et al.*, 2004, Nataro and Kaper, 1998) (Table 1):

- Enterotoxigenic *E. coli* (ETEC): causing traveller's and infantile diarrhoea in humans and watery diarrhoea in newborn animals via heat-stable (ST) and heat-labile (LT) enterotoxins production;
- Enteropathogenic *E. coli* (EPEC): causing diarrhoea in humans and animals via the attaching and effacing lesions (A/E lesions) production and inflammation in the gut;
- Verotoxigenic *E. coli* (VTEC): causing enterotoxaemia (oedema disease) in weaned piglets and diarrhoea and Haemolytic Uraemic Syndrome (HUS) in humans via the production of verotoxins (VT), also called Shiga toxins (Stx);

- Enterohaemorrhagic *E. coli* (EHEC): causing watery or bloody diarrhoea and HUS in humans and diarrhoea in animals via the A/E lesions and the Stx toxins production;
- Enteroinvasive *E. coli* (EIEC): causing diarrhoea in humans and capable to proliferate intracellularly;
- Enteroaggregative *E. coli* (EAEC): causing diarrhoea in humans and capable to aggregately adhere to epithelial cells;
- Diffuse adherent *E. coli* (DAEC): causing diarrhoea and urinary tract infections in humans and capable to diffusely adhere to epithelial cells;
- Necrotoxigenic *E. coli* (NTEC): causing diarrhoea, urinary tract infection and septicaemia in humans and animals via Cytotoxic Necrotic Factors (CNF) production.

1.2. Enteropathogenic, enterohaemorrhagic and verotoxigenic *E. coli* strains

1.2.1. Definition

The main virulence property that defines the EPEC strains is the specific "attaching and effacing (A/E) lesion" production characterised by microvilli loss and intimate attachment (via the INTIMIN adhesin, an outer membrane protein) of the bacteria to the host enterocytes (see section 1.3.1). Moon and collaborators first reported this histopathologic A/E lesion in 1983 (Moon *et al.*, 1983). Even, if EPEC were known since a long time as diarrhoeagenic *E. coli* (Ewing, 1956, Quilligan and Shadomy, 1958, Shuman and Stock, 1956), it was only at that time that those strains were associated with the lesion and defined as a specific pathotype (Moon *et al.*, 1983). Additionally, EPEC strains are subdivided into two groups: "typical EPEC" (t-EPEC) and "atypical EPEC" (a-EPEC) strains, depending on the EPEC Adherence Factor (EAF) plasmid presence, responsible of the "localised adherence" (LA) phenotype, and coding for the Bundle-forming Pili (BFP), a type4 fimbrial adhesin (Kaper *et al.*, 2004).

The main VTEC strains virulence property is the production of Shiga toxins (Stx) that are lethal for eukaryotic cells (see section 1.3.2). VTEC were first reported by Konowalchuk *et al.* in 1977 who observed a cytotoxic effect of the toxins produced by these strains on the Vero cells (Konowalchuk *et al.*, 1977).

As for EHEC strains, their main virulence properties are the A/E lesion and Stx production. They were recognised as a distinct class of pathogenic *E. coli* in 1983 after two

outbreaks in the United States caused by contaminated hamburgers (Wells *et al.*, 1983). They were called enterohaemorrhagic *E. coli* because of the produced diarrhoea bloody character.

The figure 2 shows the relationship existing between the three pathotypes and their virulence properties.

1.2.2. Pathology in humans

EPEC strains can colonise the small (the duodenum, the jejunum and the ileum) and, to a lesser extent, the large intestine and may cause profuse watery diarrhoea in humans. They were responsible for frequent infant diarrhoea outbreaks in the United States and the United Kingdom in the 1940s and 1950s (Robins-Browne, 1987). Nowadays, EPEC are still a major cause of child diarrhoea in developing countries, especially in infants younger than 2 years. After a decrease in the number of t-EPEC infections in developed countries, a-EPEC strains became more and more frequent in post-industrial countries (Campos *et al.*, 2004). The death rate in developed countries is low today, but it can reach up to 30% in developing countries (Nataro and Kaper, 1998, Senerwa *et al.*, 1989). Transmission occurs via oral-faecal contact with contaminated hands or foods. According to epidemiological surveys, asymptomatic carriage by humans could also be at the root of contamination (Nataro and Kaper, 1998). Serotypes usually associated with EPEC strains are O55:H6, O86:H34, O111ab:H2, O119:H6, O127:H6, 0127:H40, O142:H6, O142:H34 for t-EPEC and O26:[H11], O55:[H7], O111ac:H9, O119;H2, O125ac:H6, O128ab:H2 for a-EPEC according to Campos et collaborators (Campos *et al.*, 2004).

Infections by EHEC strains in humans are characterised by (bloody) diarrhoea production as a consequence of (haemorrhagic) colitis (HC) development, and followed by, in a few percent of the cases, renal lesions that can lead to renal failure and to death (= Haemolytic Uremic Syndrome or HUS). Today, they represent an important problem for public health in developed countries all over the world. The most common EHEC serotype is O157:H7, but other serogroups such as O26, O111, O145 and O103 are also very important in some countries. In the United States, O157:H7 have been estimated to cause 73 000 illnesses annually and non-O157 EHEC serotypes, at least 37 000 illnesses (Brooks *et al.*, 2005). In Europe and Japan, infection by EHEC has also a real importance as a foodborne disease (Caprioli *et al.*, 2005). In 2010, 4 037 cases were reported in the European Union (including 84 cases in Belgium) (EFSA, 2012). Cattle, other domestic ruminants and wild ruminants are the most important animal species in terms of reservoir for human infection and are mainly responsible for food contamination

(Hornitzky *et al.*, 2005). Indeed, in many cases, EHEC strains infect man via vegetal and animal food soiled by ruminant faeces. Other well-recognised sources of contamination are secondary transmissions from infected persons (Rangel *et al.*, 2005, Rowe *et al.*, 1993).

In humans, VTEC infections are not frequent but, when they occur, they are frequently associated with HUS syndrome. Nevertheless, they have also been associated with bloody diarrhoea (Beutin *et al.*, 2004). The main serotypes of VTEC strains in humans are O91:H21 and O113:H21 (Bettelheim, 2007, Bielaszewska *et al.*, 2009, Mellmann *et al.*, 2009). Recently, a VTEC strain of an unusual serogroup, O104:H4, was responsible of an outbreak in Europe (mainly in Germany) with more than 4000 cases and a progression to HUS in more than 800 patients. It appears that this strain shares 93% of one EAEC strain genome and is probably born from the acquisition of a Stx-encoding phage by a pre-existing EAEC pathogen (Denamur, 2011, Rasko *et al.*, 2011, Ruggenenti and Remuzzi, 2011).

1.2.3. Pathology in animals

Infection with EPEC strains is associated with diarrhoea in young calves (one week to eight weeks old) (Doughty *et al.*, 2002). There is no mortality in calves, but diarrhoea can become chronic, causing economic cost. The main EPEC serogroup infecting calves is O26. In addition to human and bovine infections, EPEC strains are also found in dogs, cats, rabbits, pigs, goats, sheep and wild ruminants (Krause *et al.*, 2005). In rabbits, EPEC strains infect neonates and freshly weaned animals causing mild to severe diarrhoea, with important economic losses due to high mortality rate that can reach 100% (Blanco *et al.*, 1996). In dogs, cats and piglets, colonisation by EPEC strains can occasionally lead to diarrhoea. Finally, in goats, sheep and wild ruminants, EPEC strains can be found but the association with diarrhoea has not yet been demonstrated (Mainil, 1999).

In the veterinary field, several EHEC strains serogroups are directly associated with diarrhoea in two week- to two month-old calves (Hornitzky *et al.*, 2005, Moxley and Francis, 1986). The major serotypes in calves are O5, O26, O111, and O118. The consequences are economic losses due to a delay in growth and weakness of calves. EHEC strains can also be found in the faecal flora of a wide variety of animals (cattle, sheep, goats, pigs, cats, dogs, chickens, and gulls) (Mainil and Daube, 2005).

In animals, VTEC strains are found in many different species but their pathogenic role has only been confirmed in swine. Indeed, in piglets, VTEC strains are responsible for oedema disease up to two weeks after weaning (Moxley, 2000). VTEC strains colonise the intestine thanks to the production of a fimbrial adhesin (F18) and produce the Stx2e toxin, which is responsible for clinical signs.

1.2.4. Healthy carriage and host specificity

Among EPEC, EHEC and VTEC strains, only a few are or seem to be specific to their host. It is the case of EPEC strains that infect specifically humans (Trabulsi *et al.*, 2002) or rabbits (Marches *et al.*, 2000) and of porcine VTEC strains that infect specifically piglets (Mainil, 1999). The host specificity is here based on host-specific fimbrial adhesins (BFP in human t–EPEC and F18 in porcine VTEC), INTIMIN adhesin (α variant in t-EPEC) and/or Stx variants (Stx2e variant in porcine VTEC) production. For the other strains (EHEC and most EPEC and VTEC), the host specificity remains unclear and three situations are reported for these strains: healthy carriage in cattle, disease in humans and disease in calves.

First, the detection of EPEC, EHEC and VTEC strains from healthy animals has been described worldwide in a large number of reports, with a positive animals proportion varying significantly from study to study (Blanco *et al.*, 1996, Burnens *et al.*, 1995, Caprioli *et al.*, 2005, China *et al.*, 1998, Goffaux *et al.*, 2000, Hernandes *et al.*, 2009, Jeon *et al.*, 2006, Karch *et al.*, 2009, Kaufmann *et al.*, 2006, Krause *et al.*, 2005, Leotta *et al.*, 2006, Mainil, 1999). Cattle are considered to be the major reservoir of EHEC and VTEC strains implicated in human diseases and direct (by contact with the animal) or indirect (via contaminated foodstuffs consumption) transmissions (Figure 3) have been widely documented (Buvens *et al.*, 2011, Caprioli *et al.*, 2005, Mainil, 1999, Morgan *et al.*, 1993, Pao *et al.*, 2002, Tutenel *et al.*, 2003). These strains colonised the large intestine and the cattle rectum (Pearson *et al.*, 1999, Stevens *et al.*, 2002). Concerning EPEC strains, even though no evidence of transmission exists, many strains isolated from animals belong to serogroups highly virulent to humans (Chen and Frankel, 2005, Goffaux *et al.*, 2000, Krause *et al.*, 2005, Leomil *et al.*, 2005, Trabulsi *et al.*, 2002, Yuste *et al.*, 2006). Other healthy ruminants (e.g. sheeps, goats, water buffalos, roe and red deers) can also be considered as a potential reservoir of such pathogenic strains (Ahn *et al.*, 2009, Chapman and Ackroyd, 1997, Dunn *et al.*, 2004, Horcajo *et al.*, 2010, Keene *et al.*, 1997, La Ragione *et al.*, 2009, Lorusso *et al.*, 2009, Seker *et al.*, 2010). EPEC, EHEC and VTEC strains have also sporadically been isolated from animals other than ruminants (e.g. from dogs, cats, horses, birds) (Beutin *et al.*,

1995, Goffaux *et al.*, 2000, Kataoka *et al.*, 2010, Lengacher *et al.*, 2010, van Duijkeren *et al.*, 2000).

Then, two groups can be distinguished regarding the pathogenicity in animals. In one hand, several serogroups of EHEC strains (such as O157, O145, O103) and VTEC strains severely infect humans but not ruminants, which can be healthy carrier of these strains. In the other hand, several serogroups of EHEC and EPEC strains (such as O26, O111, O118) can infect both humans and calves, but are also carried by healthy ruminants. To date, no one can say whether these strains are homogenous inside the same serogroup or if some strains are specific to human or calves. The actual situation regarding the host specificity remains unknown especially concerning EHEC and non-classical EPEC strains.

1.3. Major virulence factors

1.3.1. The locus of enterocyte effacement (LEE)

The ability to produce the attaching and effacing lesion (A/E lesion) is shared by EHEC and EPEC. Strains that produce the A/E lesion form the "attaching and effacing" *E. coli* group (AEEC). Staley *et al.* first described this lesion in 1969, but the name "attaching and effacing lesion" and its association with EPEC strains were only given by Harley W. Moon in 1983 (Moon *et al.*, 1983, Staley *et al.*, 1969).

The microvilli disappearing (=effacing) and the intimate attachment of the bacteria to the epithelial cell (=attaching) characterise the A/E lesion (Figure 4). Beyond, a pedestal is created accompanied with the cytoskeleton rearrangement and the actin polymerisation.

The locus of enterocyte effacement (LEE) is the pathogenicity island responsible of the A/E lesion. This genomic region is 35 to 45 kb long depending on the strain/pathotype (EPEC or EHEC) and is divided in five polycistronic operons (LEE1 to LEE5). Genes present on the LEE may be functionally divided in three categories:

- Type III secretion systems (TTSS) proteins that allow the bacteria to translocate virulence factors into the host cells;
- Proteins translocated by the TTSS (e.g. effectors) into the host cells;
- The INTIMIN adhesin and the Translocated Intimin Receptor (Tir) that enable the intimate attachment to the host cell.

After initial adherence to the host cell, the bacteria translocate the effectors into the enterocyte cytoplasm via the TTSS that act as a molecular syringe. The translocated effectors induce the cytoskeleton rearrangement with the effacement of enterocytes microvilli as a consequence (Figure 5). Then, the bacteria intimately adhere to the cell via the intimin (present at the bacterial surface) and its specific receptor Tir (Rosenshine *et al.*, 1992). This one was previously translocated to the host cell via the TTSS and phosphorylated on Y474 residue in the enterocytes cytoplasm prior to be integrated into the host cell membrane in a hair loop topology (Kenny and Finlay, 1997). Following the phosphorylation of residue Y474, Tir furthermore interacts with the cytoskeleton by utilising the host adaptor protein Nck to trigger actin polymerisation and induce pedestal production under the adherent bacteria through the accumulation of polymerised actin (Figure 5). This first scheme was established for t-EPEC strain. Since, other pathways to trigger actin polymerisation, which leads to the formation of the pedestal, have been described (Frankel and Phillips, 2008). For O157:H7 and non-O157 EHEC strains e.g., two non-LEE-encoded (Nle) bacterial TTSS proteins, the Tir-cytoskeleton-coupling Protein (TccP) and the Tir-cytoskeleton-coupling Protein 2 (TccP2), are used as an adaptor in place of Nck to trigger actin polymerisation. The actin-signalling pathway depends on the type of the bacteria (e.g. t-EPEC, a-EPEC, O157 EHEC, non-O157 EHEC, Sorbitol-fermenting O157 EHEC) (Figure 6).

Several *eae* (coding for the INTIMIN adhesin) and *tir* (coding for Tir) genes subtypes have been identified. Currently, up to 28 *eae* variants and 20 *tir* variants have been described (designated according to the Greek alphabet). Some variants are preferentially associated with some serogroup (e.g. β subtype with O26 a-EPEC and EHEC strains).

The A/E lesion is responsible of the apparition of diarrhoea via the local modifications that it induces (Nataro and Kaper, 1998). Several mechanisms could be involved: the active secretion of Chlorine, a malabsorption due to the microvilli loss and a local inflammatory response.

1.3.2. The Shiga toxins

The ability to produce Shiga toxins (Stx) is shared by EHEC and VTEC strains. These toxins were first described in 1977 by Konowalchuk and collaborators (Konowalchuk *et al.*, 1977). They isolated *E. coli* strains, from diarrhoeic patients, producing cytotoxins that were lethal for Vero cells. Therefore, these toxins were called Verotoxins (VT). Thereafter, they were

renamed Shiga-like toxins (SLT) (or more simply Shiga toxins, Stx) owing to their similarity with toxins from *Shigella dysenteriae* (O'Brien *et al.*, 1982).

The toxins are encoded by genes located on bacteriophages that have a chromosomal localisation in the bacteria. Hence, it was suggested that the toxin genes could easily move from strain by phage excision (Bielaszewska *et al.*, 2007, Joris *et al.*, 2011). Two main types of Stx exist: Stx1 and Stx2. They share 55% amino acid similarities (Kaper *et al.*, 2004). Each family is as well divided in sub-variants:

- Stx1, Stx1c and Stx1d for Stx1 variants and;
- Stx2, Stx2c, Stx2c2, Stx2d, Stx2d$_{act}$, Stx2e, Stx2f and Stx2g for Stx2 variants.

Some variants (e.g. Stx2, Stx2c, Stx2d$_{act}$) appear to be related with more severe diseases such as HC and HUS (Friedrich *et al.*, 2002, Persson *et al.*, 2007).

Structurally, Stx toxins (Figure 7) are composed of two subunits (A-B structure type). Each toxin comprises one A-subunit (32 kDa) and five B-subunits (7.7 kDa each). The enzymatic active part is the A-subunit, while the pentameric B-subunit is involved in the binding of the toxin to the receptor (Sandvig, 2001).

After the intestine colonisation by the bacteria, the toxins are excreted outside the bacteria via a type II secretion system (Sandvig, 2001). Then, Stx toxins cross the bowels by transcytosis to enter the bloodstream. Stx will bind specific receptors (the globotriaosylceramide, Gb3) present at the surface of a variety of epithelial and endothelial cells. One exception is the Stx2e variant that preferentially binds to the globotetraosylceramide (Gb4) (Muthing *et al.*, 2009, Samuel *et al.*, 1990). After the internalisation of the toxins by endocytosis, Stx toxins are translocated into the endoplasmic reticulum via the Golgi apparatus (Figure 7). From there, the A-subunit is translocated into the cytoplasm where it is cleaved into two parts (A1 and A2). The 28 kDa A1 fragment possesses N-glycosidase activity, which enables the enzyme to cleave a specific residue of the eukaryotic cell 60S ribosome. Thereof, the protein elongation step is inhibited and it induces the targeted cells death. HC, HUS or oedema disease lesions are several examples of consequences of this cell destruction.

By binding to Gb3 present on microvascular endothelial cells of the kidney, the intestine and the brain, the Shiga toxins are respectively responsible of the Haemolytic Uremic Syndrome (HUS), the Haemolytic Colitis (HC) and the Thrombotic Thrombocytopenic Purpura (TTP) in humans (Muthing *et al.*, 2009, Schuller, 2011). On the other hand, in contrast to human infections, HUS is never observed in calves. The usual hypothesis suggested to explain this fact is that endothelial bovine cells lack Stx receptors, which toxins are responsible of this

syndrome. Nevertheless, Hoey *et al.* identified Gb3 in bovine intestinal and renal cells but without having any cytotoxic activity against them. A different intracellular trafficking in ruminants and a Gb3 isoform configuration were advanced to explain these differences with humans (Hoey *et al.*, 2002, Hoey *et al.*, 2003).

1.3.3. Other virulence factors

Two widely known virulence factors other than Stx and LEE are the enterohaemolysin (Ehly) and the ferric uptake system. Both will be briefly described.

The enterohaemolysin differ from other classical haemolysins (α and β produced by other pathogenic *E. coli*) by its haemolytic activity on Sheep Blood Agar (SBA) containing washed erythrocytes. Different types of Ehly have been identified but only EHEC-hly or Ehx is specific to EHEC strains. Schmidt and collaborators have first described this haemolysin on a 60 MDa plasmid (so called pEHEC or pO157) isolated from EHEC O157:H7 strain EDL933 (Schmidt *et al.*, 1995). Four genes encoded the Ehx: *ehxA*, *ehxB*, *ehxC* and *ehxD* (Schmidt *et al.*, 1996). *ehxA* gene code for the structural protein and present 60% homology with *hlyA* gene coding for the α-Hly. The three other proteins are involved in the EhxA secretion. This monomeric pore-forming toxin, belonging to the "Repeats in ToXin" family (RTX), is inserted into eukaryotic cell cytoplasmic membrane and creates pores causing targeted cells osmotic lyses. The Ehx role in pathology is still confusing. Several laboratories hypothesise the potential involvement of Ehx in iron release from haemoglobin in the environment (Law and Kelly, 1995, Mills and Payne, 1995, Torres and Payne, 1997). Moreover, in 2007, Aldick and collaborators suggested a conceivable Ehx toxicity to microvascular endothelial cells and thus a contribution to HUS pathogenesis (Aldick *et al.*, 2007).

Ferric iron is a substantive nutrient for most microorganisms. Under iron starvation conditions, many bacteria (including *E. coli*) synthesise iron-complexing molecules (called siderophores), and ferric siderophore complex transport systems to acquire iron from the surrounding environment (Figure 8). Thus, siderophores are secreted in the environment and bind to ferric iron (Fe^{+3}). These complexes are retrieved in the bacteria through specific receptors to release iron in the bacterial cytosol. If the intracellular iron concentration is sufficient, Fur (Ferric Uptake Regulation) protein acts as a transcriptional repressor by inhibiting siderophores and transport proteins transcription. Various kinds of siderophores have been described (e.g. aerobactin, enterobactin, colibactin, yersiniabactin, salmochelin) and

the common hemin uptake system found in *E. coli* is represented by an outer membrane protein encoded by *chuA* (*E. coli* heme utilisation) gene. Nevertheless, in 2007, Kresse *et al.* have studied the iron uptake system in O157 and non-O157 EHEC strains and concluded that the strategies for obtaining iron probably differ between O157 and non-O157 strains because: 1) *chuA* gene is rare among non-O157 EHEC and prevalent in O157 strains; 2) none of the O157 and O26 strains produced aerobactin or colibactin (in contrary to other non-O157 strains); 3) all O157 and O26 strains utilised heme as iron source (in contrary to other non-O157 strains) (Kresse *et al.*, 2007).

1.4. Article 1: "Initial adherence of EPEC, EHEC and VTEC to host cells"

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Review article

Initial adherence of EPEC, EHEC and VTEC to host cells

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Abstract – Initial adherence to host cells is the first step of the infection of enteropathogenic *Escherichia coli* (EPEC), enterohaemorrhagic *Escherichia coli* (EHEC) and verotoxigenic *Escherichia coli* (VTEC) strains. The importance of this step in the infection resides in the fact that (1) adherence is the first contact between bacteria and intestinal cells without which the other steps cannot occur and (2) adherence is the basis of host specificity for a lot of pathogens. This review describes the initial adhesins of the EPEC, EHEC and VTEC strains. During the last few years, several new adhesins and putative colonisation factors have been described, especially in EHEC strains. Only a few adhesins (BfpA, AF/R1, AF/R2, Ral, F18 adhesins) appear to be host and pathotype specific. The others are found in more than one species and/or pathotype (EPEC, EHEC, VTEC). Initial adherence of EPEC, EHEC and VTEC strains to host cells is probably mediated by multiple mechanisms.

adherence / adhesin / enteropathogenic *E. coli* / enterohaemorrhagic *E. coli* / verotoxigenic *E. coli*

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1. INTRODUCTION

1.1. Enteropathogenic, enterohaemorrhagic and verotoxigenic *Escherichia coli*

The *Escherichia coli* species is a Gram-negative bacterium that belongs to the *Enterobacteriaceae* family. It is the predominant facultative anaerobe of the human colonic flora. However, some strains have developed the ability to cause disease of the gastrointestinal, urinary, or central nervous system. Pathogenic gastrointestinal strains of *E. coli* are classified according to the properties of their virulence: enteropathogenic *E. coli* (EPEC), enterotoxinogenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), verotoxinogenic *E. coli* (VTEC), "diffusely adherent" *E. coli* (DAEC), necrotoxinogenic *E. coli* (NTEC) [77].

The main virulence property of EPEC strains is the production of a specific lesion called "attaching and effacing (A/E) lesion" characterised by loss of microvilli and intimate attachment of the bacteria to the host [71], whereas the main virulence property of VTEC is the production of verotoxins (or shiga-toxins) that are lethal for eukaryotic cells [56]. As for EHEC strains, their main virulence properties are the production of A/E lesions and verotoxins [23].

EPEC strains colonise the small intestine and cause profuse watery diarrhoea in humans. They were responsible for frequent outbreaks of infant diarrhoea in the USA and the United Kingdom in the 1940s and 1950s [92]. In contrast to the limited importance of EPEC in developed countries nowadays, EPEC are still a major cause of child diarrhoea in developing countries, especially in infants younger than 2 years. The death rate in developed countries is low today but it can reach up to 30% in developing countries [77, 96]. Transmission occurs via oral-faecal contact with contaminated hands or foods. According to epidemiological surveys, asymptomatic carriage by humans could also be at the root of contamination [77]. In bovines, infection with EPEC strains is associated with diarrhoea in young calves (1 week to 8 weeks old) [32]. There is no mortality in calves, but diarrhoea can become chronic, causing economic loss as a consequence. In addition to human and bovine infections, EPEC strains are also found in dogs, cats, rabbits, pigs, goats, and sheep [57]. In rabbits, EPEC strains infect neonates and recently weaned animals causing mild to severe diarrhoea, with important economic loss [17]. In dogs, cats and piglets, colonisation by EPEC strains can occasionally lead to diarrhoea. Finally, in goats and sheep, EPEC strains can be found but the association with diarrhoea has not yet been demonstrated [68].

Infections by EHEC strains in humans are characterised by the production of diarrhoea generally accompanied by haemorrhagic colitis (HC) with, in a few percent of cases, renal sequelae (haemolytic uremic syndrome, HUS), which can lead to death. EHEC strains were recognised as a distinct class of pathogenic *E. coli* in 1983 after two outbreaks in the USA [124]. Today, they represent an important problem for public health in developed countries all over the world. The most common EHEC serotype is O157:H7, but other serogroups such as O26, O111, O145 and O103 are also very important in some countries. In the USA, O157:H7 have been estimated to cause 73 000 illnesses annually and non-O157 EHEC serotypes, at least 37 000 illnesses [19]. In Europe and Japan, infection by EHEC also has a real importance as a foodborne disease [23]. In the veterinary field, several serogroups of the EHEC strain (O26, O111, O118 for example) are directly associated with diarrhoea in 2 week to 2 month old calves [46, 73]. The consequences are economic losses due to a delay in growth and weakness of

calves. EHEC strains can also be found in the faecal flora of a wide variety of animals (cattle, sheep, goats, pigs, cats, dogs, chickens, and gulls) [70]. Cattle, other domestic ruminants and wild ruminants are the most important animal species in terms of a reservoir for human infection and are mainly responsible for food contamination [46]. Indeed, in many cases, EHEC strains infect man via vegetal and animal food soiled by ruminant faeces. Other well-recognised sources of contamination are secondary transmissions from infected persons [88, 93].

VTEC strains cause pathology mainly in humans and piglets. But they can also be found in a large spectrum of domestic and wild animals [126]. In humans, VTEC infections are not frequent but, when they occur, they are frequently associated with HUS syndrome. In piglets, VTEC strains are responsible for oedema disease up to two weeks after weaning [74].

1.2. Adherence of EPEC, EHEC and VTEC strains

Pathogenicity of EPEC infection can be tentatively divided into three stages [31]: (1) initial adherence and colonisation of the intestine; (2) translocation of bacterial signals into the eukaryotic cells via a type III secretion system that cause cytoskeleton rearrangements in enterocytes; and (3) intimate adherence of bacteria to eukaryotic cells by specific proteins, the intimins (coding by the *eae* gene). Steps 2 and 3 together cause the formation of A/E lesions. For EHEC infection, a fourth step consists of the production of verotoxins. For VTEC infection, steps 2 and 3 do not exist and verotoxins are produced after intestinal colonisation.

The molecular biology of the production of A/E lesions and the action of verotoxins is today quite well described and understood, even if research remains to be performed to fully understand the in vivo pathogenesis of those strains. On the contrary, the step of intestinal colonisation via specific adhesins initiating the interaction between the bacteria and host tissues and anchoring the bacteria onto the surface of the enterocytes is actually still poorly understood for many strains [120]. The importance of initial adherence in the infection resides in the following: (1) adherence is the first contact between bacteria and intestinal cells without which the other steps cannot occur, (2) adherence is the basis of host specificity for a lot of pathogens, (3) adhesins represent a good target for the development of a specific vaccinal prophylaxis [25].

The aim of this review is to describe the initial adhesins of the EPEC, EHEC and VTEC strains (Tab. I). During the last few years, several new adhesins and putative colonisation factors have been described, especially in EHEC strains [64, 65, 78, 107, 127]. First, this review will describe pathotype (EPEC, EHEC or VTEC) specific adhesins. Then, adhesins present in more than one of the three pathotypes will be described.

2. EPEC SPECIFIC ADHESINS

2.1. Bfp adhesin

2.1.1. Description

In 1991, Gìron et al. [41] showed that EPEC strains express rope-like bundles of filaments, termed bundle-forming pili (BFP), which create a network of fibres that bind the individual bacteria together. An antiserum against BFP reduces the capacity of EPEC to infect cultured epithelial cells. The *bfpA* gene codes for the main subunit. It is part of an operon of 14 genes: *bfpA* (the pre-pilin), *bfpB* (a lipoprotein), *bfpC* (a bitopic cytoplasmic membrane protein), *bfpD* (a hexameric cytoplasmic ATPase), *bfpE* (a polytopic cytoplasmic membrane protein), *bfpF* (a putative cytoplasmic nucleotide-binding protein), *bfpG* (an unknown function protein that interacts with bfpB), *bfpH* (an unknown function protein that is perhaps not expressed), *bfpI* (a pre-pilin-like protein), *bfpJ* (a pre-pilin-like protein), *bfpK* (a pre-pilin-like protein), *bfpL* (a protein localised with both the inner and outer membranes), *bfpP* (a pre-pilin peptidase) and *bfpU* (an unknown function protein that interacts with *bfpB*) [28, 30, 100, 103]. This operon is situated on a 50–70 MDa plasmid called EPEC Adherence Factor (EAF).

Table I. Distribution of the main adhesins present in EPEC, EHEC and VTEC strains.

Adhesin	Present in pathotypes	Host
Bfp	EPEC	Human, dog
AF/R1	EPEC	Rabbit
AF/R2	EPEC	Rabbit
Ral	EPEC	Rabbit
F18	VTEC	Pig
Saa	VTEC	Human, cattle, sheep
EibG	VTEC	Human
Spf	EPEC, EHEC	Human, cattle
Efa1	EPEC, EHEC	Human, cattle
ToxB	EPEC, EHEC, VTEC	Human, cattle
Lpf	EPEC, EHEC, VTEC	Human, cattle
F9	EPEC, EHEC	Human, cattle
Paa	EPEC, EHEC	Human, pig, cattle
Iha	EPEC, EHEC, VTEC	Human, pig, cattle
AIDA	EPEC, VTEC	Human, pig

The presence of the *bfpA* gene has been searched for in human and animal EPEC, EHEC and VTEC strains but also in *Salmonella* sp. serotypes [23, 25, 41, 42, 55, 99, 122]. The gene was only detected in a subclass of human EPEC strains and in a few EPEC strains from dogs, but not in other EPEC, EHEC and VTEC strains. Human EPEC strains harbouring the EAF plasmid are called "typical-EPEC" strains (t-EPEC) and other human EPEC strains that do not carry EAF plasmid are known as "atypical-EPEC" strains (a-EPEC) [119].

2.1.2. Involvement in the adherence

The plasmid containing the Bfp operon is responsible for the localised adherence (LA) phenotype on cell culture [3]. The complementation of a non-adherent *E. coli* strain with the EAF plasmid leads to an increase in adherence to epithelial cells. In 1985, Levine et al. [63] had already observed the importance of the plasmid during infection in vivo. EPEC strain E2348/69 (containing the EAF plasmid) and its mutant cured of EAF plasmid was inoculated into 10 volunteers. Diarrhoea occurred in nine out of the 10 volunteers who ingested the parental strain but in only 2 of the 9 who took the mutant. The *bfp* cluster is regulated by environmental signals such as temperature, calcium and ammonium [86]. These findings support the pathogenic role of BFP by the initiation of BFP production in the small intestine, but not in the colon and external habitats.

Adherence studies on mouse and human eukaryotic cells have indicated that BFP play an important role in the cell-type-dependent adherence of t-EPEC [111]. Indeed, the adherence of the bacteria is lower on mouse-derived cells than on human-derived cells. BFP are also implicated in biofilm formation. Moreira et al. [72] demonstrated that the genes encoding BFP are expressed during biofilm formation and that mutants that do not express BFP form more diffuse biofilms than does the wild type strain.

The Bfp adhesin contributes to the attachment of the bacteria to eukaryotic cells but does not seem to be necessary to cause disease in humans. Indeed, the a-EPEC strains (that do not carry the *bfpA* gene) are more and more implicated in diarrhoea outbreaks in both developed and developing countries. Various other adhesins (f.i. Paa, LpfAO113, Iha, Ehx, ToxB, LdaG) are present in a-EPEC strains [45] and play probably the same role as Bfp in adherence. Nevertheless, Bfp adhesin seems to be host specific considering that the *bfpA* gene is mostly found in strains isolated from humans. This host specificity could be explained by the fact that the *bfp* cluster is regulated by environmental signals [86] and that human intestines differ from animal intestines [43].

2.2. REPEC adhesins in rabbit EPEC strains

2.2.1. Description

Three different rabbit-specific adhesins exist in rabbit EPEC strains (REPEC): Adhesive factor/Rabbit 1 (AF/R1) [12], Adhesive factor/Rabbit 2 (AF/R2) [38], Ral [1].

The operon of AF/R1 adhesin is composed of seven genes: *afrA* (the structural subunit), *afrB* (an usher protein), *afrC* (a chaperone), *afrD* (an adhesin), *afrE* (an unknown function protein), *afrR* and *afrS* (two transcriptional regulators). *afrA*, *afrB* and *afrC* genes are required

for the expression of the pilus and for the adherence to the host cells and *afrD* and *afrE* genes are only required for the adherence to the host cells [22]. Penteado et al. [83] and Dow et al. [33] studied the prevalence of the AF/R1 adhesin and respectively found the adhesin in 0% and 4.7% of the REPEC (only found in O103:H2 serotype strains).

Fiederling et al. [38] cloned the *afr2* operon and showed that the *afr2G* gene (the major subunit) is homologous to *clpG* from the CS31A adhesin and *faeG* from the K88 (F4) adhesin. Penteado et al. [83] and Dow et al. [33] studied the prevalence of the AF/R2 adhesin and respectively found the adhesin in 83.3% and 23.3% of the REPEC of O103:H2, O132:H2, O153:H7, O126:H-serotypes.

Ral adhesin is coded by an operon situated on the 95 kb-pRAP plasmid (REPEC adherence plasmid) and is homologous to K88, CS31A and AF/R2 adhesins. The operon is composed of seven genes: *ralC*, *ralF* and *ralH* (three putative minor subunits); *ralG* (one putative major subunit); *ralI* (an unknown function protein); *ralD* and *ralE* (two chaperones). Dow et al. [33] studied the prevalence of the Ral adhesin and found that this adhesin is present in 35% of the REPEC of O153:H7, O15, O132:H2, O49:H2, O145 serotypes.

2.2.2. Involvement in the adherence

AF/R1 was first described by Berendson et al. [12]. In their experiments, they infected sections of human, guinea pig, rat and rabbit small intestines with an REPEC positive for AF/R1 and its deleted mutant and they detected the adherence by indirect immunofluorescence technique. The piliated REPEC strain fully adhered only to the rabbit section and only a few colonies of the non-piliated REPEC strain adhered to the small intestine section in comparison with the wild type. Rafiee et al. [87] identified a rabbit ileal microvillus membrane sialoglycoprotein complex with subunits of 130 and 140 kDa as a receptor(s) for AF/R1 fimbriae localised on the rabbit small intestine [94].

AF/R2 was first described in an REPEC O103 strain by Pillien et al. [85]. This adhesin gives the capacity of diffuse adherence (DA) to rabbit enterocytes and HeLa cells and there is a loss of pathogenicity after inoculation of deleted mutants into the rabbit.

Mutants deleted in the Ral operon show a 10 times lower colonisation of the rabbit intestine and a decrease in the severity of the disease in vivo [1]. Krejany et al. [58] showed that the adherence on the intestinal rabbit loop is lost with a mutant deleted in Ral adhesin.

As for BFP, REPEC adhesins appear to play a role in the attachment of bacteria to eukaryotic cells and seem to be host specific. AF/R1 binds specifically to a sialoglycoprotein complex. This receptor could vary among species as it has already been shown previously [87].

3. VTEC SPECIFIC ADHESINS

3.1. F18 adhesin

3.1.1. Description

In 1995, Rippinger et al. [91] designated two variants (F18ab and F18ac) that correspond to the related fimbrial types F107 [13], 2134P [76] and 8813 [95] of verotoxigenic and enterotoxigenic *E. coli* isolated, respectively, from porcine postweaning diarrhoea and oedema disease. The F18ab variant is mostly associated to the VTEC strains while the F18ac variant is mostly associated to the ETEC strains [26]. This fimbria is a long flexible filament with a maximum 4.6 nm diameter. Mainil et al. [69] showed that the F18 gene is localised on the same plasmid as the adhesin involved in diffuse adherence (AIDA) in *E. coli* isolated from piglets. The operon coding for this fimbria is composed of five genes: *fedA*, *fedB, fedC*, *fedE* and *fedF* [47, 48, 98]. The backbone is built from the major subunit, FedA (15.1 kDa); but this subunit is not sufficient for recognising the F18 receptor [47]. FedE (15.9 kDa) and FedF (30.1 kDa) are two minor subunits and are essential for fimbrial adherence [48, 98]. Smeds et al. [98] showed that the FedF protein plays the role of the adhesin of the F18 fimbriae. Indeed, anti-FedF antibodies, unlike anti-FedE serum, are able to inhibit *E. coli* adherence to porcine enterocytes. Moreover, subunit FedF is highly conserved among F18+ *E. coli*

isolates (from different countries and from the two different variants) [109]. FedB (86 kDa) and FedC (23.4 kDa) act respectively in the biosynthesis of the pili and as a chaperone. Several laboratories studied the distribution of the F18 adhesin in a collection of strains. Zweifel et al. [133] searched for the *fedA* gene in 31 VTEC strains isolated from healthy pigs at slaughter and only one strain was positive for this gene. Cheng et al. [26] looked for the presence of F18ab and F18ac variants in VTEC, VTEC/ETEC and ETEC strains isolated from diarrheic piglets. They found F18ab and F18ac adhesins in respectively 62% and 0% of VTEC, in 62.5% and 33% of VTEC/ETEC strains and in 4% and 8% of ETEC strains. Osek et al. [81] found the *fedA* gene in 2.7% of strains isolated from diarrheic piglets and in 2.2% of strains isolated from healthy piglets.

3.1.2. Involvement in the adherence

Several studies have shown that this adhesin allows adherence to microvilli of piglet enterocytes [76, 95, 129, 131]. The F18 receptor (F18R) plays an important role in the VTEC/ETEC infection. Piglets can possess (F18R+) or not the receptor (F18R−) and only those piglets that do possess the receptor are subject to infection with F18+ *E. coli* [40]. The F18R status of pigs is genetically determined [14] and, recently, Coddens et al. [27] have shown that the expression of the F18 receptor is positively correlated with the presence of histo-blood group antigens, that its levels rise with increasing age during the first 3 weeks after birth and that F18 receptor expression is maintained in older pigs (3–23 weeks old). Several laboratories have tried to develop vaccines against F18 fimbria to prevent post-weaning diarrhoea in piglets [15, 35, 110, 123].

3.2. Saa and EibG

3.2.1. Description

In 2001, Paton et al. [82] isolated a gene, named *saa* (STEC autoagglutinating adhesin), from the megaplasmid of an *eae*-negative O113:H21 VTEC strain (98NK2) responsible for an outbreak of HUS in Australia. In the 98NK2 strain, the protein is 516 amino-acids long, including four copies of a 37-aa direct repeat sequence, and is localised in the outer membrane of the cell. Saa produced by other VTEC strains vary in size as a consequence of variation in the number of copies of a 37-aa repeat unit. In 2006, Lucchesi et al. [67] found the existence of 5 variants based on this variation in the number of repeat units present in the 3′ coding region. Saa exhibits 24–27% of identity with two outer membrane proteins, YadA of *Yersinia enterocolitica* (a plasmid-encoded outer membrane protein implicated in epithelial cell adherence and invasion) and Eib of *E. coli* (*E. coli* immunoglobulin-binding protein) [82]. Several laboratories have studied the distribution of the *saa* gene in a collection of strains [18, 24, 50, 80, 112, 132]. The *saa* gene was found in VTEC strains isolated from cattle, humans, sheep and food. There always exists a negative correlation between the presence of the *saa* gene and the gene coding for intimin (*eae* gene).

In 2006, Lu et al. [66] identified a new gene designated *eibG* through the screening of transposon-mutagenised O91 *E. coli.* The gene encodes a 508-amino-acid protein that presents similarity to Eib proteins (*E. coli* immunoglobulin-binding protein). Lu et al. [66] examined the distribution of *eibG* in human O157, O26, O111 and O91 strains. All *eae*-positive and *saa*-positive strains were found to be negative for *eibG*.

3.2.2. Involvement in the adherence

Paton et al. [82] showed that the introduction of the *saa* gene cloned into a plasmid increases 9.7 fold the adherence of *E. coli* JM109 to HEp-2 and a semilocalised adherence pattern. Mutagenesis of this gene in the O113:H21 strain reduces the adherence significantly. The *saa* gene encodes an auto-agglutinating adhesin. In 2008, Toma et al. [114] showed that *saa*-positive VTEC strains exhibit differential binding properties to HEp-2 and Caco-2 cells. On the 32 strains studied for their adherence to epithelial cells in the absence or presence of D-mannose, 13 strains were sensitive to the presence of this sugar. Moreover, a VTEC strain (in which adherence was mannose

resistant) was deleted in the *saa* gene and its adherence to epithelial cells was not significantly decreased compared to the wild type, suggesting that multiple adherence mechanisms are present in *saa*-positive VTEC strains.

EibG is a new immunoglobulin-binding protein and acts as an adhesin in certain strains of VTEC. A mutant deleted in the *eibG* gene was constructed and its adherence phenotype was studied on Hep-2 cells. The mutant did not adhere to epithelial cells and the chain-like adherence pattern (CLA pattern) was restored after the transformation of the mutant with a plasmid carrying only *eibG*. Therefore, Lu et al. [66] suggested that the *eibG* gene is responsible for the CLA pattern.

Saa is present in several species and do not seem to be host specific but is present only in *eae*-negative, *stx*-positive strains and interestingly in *eibG*-negative strains. VTEC strains do not carry the *eae* gene and thus may compensate for this lack by a multiple adherence mechanism.

4. ADHESINS PRESENT IN MORE THAN ONE OF THE THREE PATHOTYPES

4.1. Spf adhesin

4.1.1. Description

The first fimbria to have been described in EHEC strains was part of a cluster called *sfp* (sorbitol-fermenting protein) encoded by a large plasmid in sorbitol-fermenting O157 strains [20]. This fimbria has similarities with the *pap* gene, which codes for the P-fimbriae in uropathogenic *E. coli* strains. The cluster is divided into six genes: *sfpA* (the major pilin), *sfpH*, *sfpC*, *sfpD*, *sfpJ*, and *sfpG* (the adhesin). Several studies showed that *sfpA* is present only in sorbitol-fermenting O157 strains (EHEC and EPEC strains isolated from humans with diarrhoea and HUS) [21, 39, 49]. In 2006, Lee et al. [61] detected *spfA* in sorbitol-fermenting O157 strains isolated from cattle. Recently, Bielaszewska et al. [16] detected the entire cluster in EHEC O165:H25/NM strains isolated from cattle and humans and they suggested that this cluster is acquired independently by EHEC O165:H25 and sorbitol-fermenting EHEC O157:NM.

4.1.2. Involvement in the adherence

A mutant deleted in the *sfp* gene is not more able to agglutinate erythrocytes. The expression of Sfp and the adherence to Caco-2 and HCT8 cells are increased in anaerobic conditions that imitate intestinal conditions [75]. It has, therefore, been suggested that Sfp production is induced under conditions resembling those of the natural site of infection and that Spf adhesin plays an important role in the adherence of sorbitol-fermenting strains.

4.2. ToxB and Efa1 adhesins

4.2.1. Description

In 2000, Nicholls et al. [78] identified a locus required for the adherence in vitro of an EHEC strain of serogroup O111:H- by transposon mutagenesis. This factor was called Efa1 for "EHEC Factor for Adherence". The *efa1* locus has a size of 9 669 bp and is situated on the pathogenicity island O122 [53]. The *efa1* locus is strongly associated with non-O157 EHEC and EPEC strains and a truncated variant of 1 299 pb is present in O157 EHEC strains [4, 44, 84]. Efa1 has 97.4% homology in amino acid and 99.9% identity in nucleic sequence with Lymphocyte Inhibitory FactorA (LifA), described in EPEC strain E2348/69, which codes for the lymphostatin. This toxin inhibits the proliferation of lymphocytes and the production of interleukin 2 and 4 [54].

In 2001, Tatsuno et al. [107] identified *toxB* on the pO157 plasmid in Sakai O157 EHEC strain. The *toxB* locus is 9.5 kb long and shares homology with the *efa1* locus (28% of nucleic acid sequence and 47% of amino acid sequence) and the gene coding for the toxin B from *Clostridium difficile* (20% of similarities in amino-acid sequence). Tozzoli et al. [118] looked for the presence of the entire *toxB* sequence in EHEC and EPEC strains of several serogroups by PCR and hybridisation. The complete *toxB* sequence is present in all O157 EHEC strains, in about 50% of EHEC O26

strains, in a few EHEC O118 and O123 strains and in a few EPEC O26 and O86 strains but not in O111 and O103 strains. They also suggested the existence of a polymorphism of *toxB* genes among the different *E. coli* serogroups. Finally, Tatarczak et al. [106] detected the *toxB* gene in VTEC strains from humans and cattle.

4.2.2. Involvement in the adherence

The mutant deleted in Efa1 is deficient in adherence on CHO (Chinese Hamster Ovary) cells. Shedding studies on calves aged 4 to 11 days with wild type and mutant strains show that the colonisation of the intestine is influenced by Efa1 for O5 and O111 EHEC strains [101]. In spite of the high homology between *lifA* and *efa1*, mutants deleted in *lifA* produce the A/E lesion and LA.

ToxB contributes to the adherence by the stimulation of the production of EspA, EspB (two translocator proteins) and translocated intimin receptor (Tir). Indeed, a pO157-cured strain of O157 Sakai has its adherence decreased and the expression level of the effectors EspA, EspB and Tir in this strain also decreased. The adherence is restored with the complementation of the pO157-cured strain of O157Sakai with a mini-pO157 plasmid composed of the *toxB* and *ori* regions. Stevens et al. [102] have shown that ToxB influences the expression of the LEE but not intestinal colonisation in sheep and calves in contrast with Tir.

Regarding the prevalence results [4], both genes are present in the majority of the EHEC and EPEC strains and are mostly absent in non pathogenic *E. coli* strains. Therefore, they might play an important role in the adherence of EPEC and EHEC strains.

4.3. Long polar fimbriae

4.3.1. Description

Long polar fimbria (LPF) was first described in *Salmonella enterica* serovar Typhimurium [6]. Then five homologues were described in *E. coli* strains. Two variants were found in O157 EHEC strains: *lpfA1* (localised in pathogenicity island OI141) and *lpfA2* (localised in pathogenicity island OI154). Doughty et al. [32] identified an *lpf* homologue in O113:H21 VTEC strain (called $lpfA_{O113}$) localised in pathogenicity island OI154. Toma et al. [112] identified another variant in O26:H11 EHEC strain (called $lpfA_{O26}$) localised in pathogenicity island OI141. Finally, Tatsuno et al. [108] identified an *lpf* homologue in EPEC strain E2348/69; however, this Lpf adhesin does not appear to be implicated in the adherence in this strain. Toma et al. [113] studied the distribution of *lpfA1, lpfA2,* $lpfA_{O113}$ and $lpfA_{O26}$ among a collection of *E. coli* strains. *lpfA1* and *lpfA2* are found in most O157 EHEC strains and O145 EHEC strains and in a few EPEC and ETEC strains [112, 113]. $lpfA_{O113}$ and $lpfA_{O26}$ are found in strains of different serogroups and pathotypes (VTEC, EPEC, EHEC, EAEC, ETEC) [4].

4.3.2. Involvement in the adherence

Baumler et al. [6] have shown that Lpf facilitates the attachment of the bacteria to murine Peyer patch cells. Torres et al. demonstrated that the adherence to HeLa cells decreases when *lpfA1* and *lpfA2* are mutated [115, 117]. $LpfA_{O113}$ enhances the adherence of O113:H21 VTEC strains on CHO-K1 cells [32]. The LPF is present in a wide range of strains isolated from different sources and belonging to different seropathotypes. Toma et al. [113] suggested that the acquisition of these genes in specific lineage of *E. coli* has probably contributed to the emergence of a pathogen from a typically commensal organism.

4.4. F9 fimbrial adhesin

4.4.1. Description

A novel potential fimbrial operon called F9 has been identified by transposon mutagenesis with O26 and O157 strains [34, 121]. This operon is present in most EHEC strains in all serogroups.

4.4.2. Involvement in the adherence

It has been shown that F9 promotes colonisation in vivo in calves. When complemented in K12, the binding of the bacteria is increased in bovine epithelial cells. However, the complementation of an O157 strain reduces the

adherence. This phenomenon is probably due to a physical competition with the type III secretion system present in O157 strains. A deletion of the operon in O157 strains reduces the shedding of the bacteria but the colonisation of the rectum is still present. Low et al. [64] concluded that F9 is not responsible for the rectal tropism of O157 strains but that it may be involved in the colonisation of other intestinal sites.

4.5. Paa adhesin

4.5.1. Description

Paa (Porcine attaching- and effacing associated) adhesin was first identified in a porcine EPEC strain (PEPEC) by transposon mutagenesis [2, 5]. The *paa* gene of 753 pb encodes a 27.6 kDa protein and is localised on the chromosome. Studies of the distribution in enteric *E. coli* strains have revealed that *paa* is present, on the one hand, in EHEC O157:H7 and O26, and in dog, rabbit, and pig EPEC isolates, and to a lesser extent in human EPEC strains and, on the other hand, in ETEC strains. In 2007, Leclerc et al. [60] studied *paa* in O149 ETEC strains. *paa* in ETEC is carried by high molecular weight plasmids and all *paa*-positive strains possess *estB*, *elt*, *astA* and *faeG* genes (coding respectively for the heat-stable enterotoxin B, the heat-labile enterotoxin, the enteroaggregative heat-stable enterotoxin and a part of F4 operon) and more than half also carry the *estA* gene (coding for heat-stable enterotoxin A). Moreover, they suggested that *paa* from ETEC and EPEC/EHEC strains could be derived from a common ancestor because *paa* from ETEC strains and *paa* from EPEC/EHEC strains contain IS signatures.

4.5.2. Involvement in the adherence

The transposon mutagenesis performed by An et al. [2] suggested that Paa plays a role in the A/E mechanism. Indeed, no A/E lesions were induced using a mutant deficient in the *paa* gene and the adherence phenotype was restored after the complementation of the *paa* mutant [5]. In addition, anti-Paa antibodies reduce the proportion of intact villi showing intimate adherence. In vivo, *eae*-positive and *paa*-negative mutants induce less severe or no A/E lesions in piglets that in the end developed no diarrhoea or delayed-onset diarrhoea.

4.6. Iha adhesin

4.6.1. Description

Tarr et al. [105] described an outer membrane protein similar to Iron-Regulated Gene A (IrgA) from *Vibrio cholerae* in cosmid obtained from the O157:H7 strain. This protein was called Iha for "IrgA Homologue Adhesin". This protein is 67 kDa in the O157:H7 strain and 78 kDa in laboratory *E. coli*. The *iha* gene is found in EPEC, EHEC and VTEC strains from humans, cattle and pigs [4, 104, 106, 112]. The *iha* gene is also present in 39% of *E. coli* isolated from patients with urosepsis [51] and it has been shown to be a virulence factor in urinary tract infection-associated strains (UTI) [52].

4.6.2. Involvement in the adherence

The adherence of mutants deleted in *iha* on eukaryotic cells is decreased and the *iha* gene provides an adherence of K12 strains to HeLa and MDBK cells [105]. Rashid et al. [89] and Leveillé et al. [62] showed that the transcription of *iha* is repressed by iron with a direct interaction between Ferric Uptake Regulation protein (Fur) and Iha. Moreover, Iha represents a Fur-regulated catecholate siderophore receptor in UTI strains. Therefore, Iha may be a dual-function virulence factor: the adherence to the host cell and the siderophore receptor activity.

4.7. AIDA adhesin

4.7.1. Description

Benz et al. [7–10] studied an O126:H27 EPEC strain implicated in infantile diarrhoea and presenting a DA phenotype. They showed that this DA phenotype is mediated by a 6 kb DNA fragment present in a 100 kb plasmid. This 6 kb fragment codes for an adhesin, called AIDA-I. The *aida-I* locus is composed of two

genes (1) *aah* an autotransporter adhesin heptosyltransferase encoding AAH protein, which modifies AIDA-I adhesin, and (2) *aidA*, the AIDA, consisting of AIDA-I (orfB) and AIDAc (orfBc). In 2001, Niewerth et al. [79] detected the *aida-I* locus in a porcine VTEC strain involved in oedema disease. The *aida-I* locus is localised on the same plasmid as the gene coding for the F18 adhesin in porcine strains [69]. In 2007, Zhao et al. [130] showed that the *aida-I* locus is occasionally present in porcine strains involved in post weaning diarrhoea and that a transfer between human and porcine strains is possible.

4.7.2. Involvement in the adherence

AIDA-I is involved in the DA phenotype but is also involved in bacteria aggregation and in biofilm formation [90, 97]. There is an intercellular interaction between AIDA-I-AIDA-I but also AIDA-I-Antigen43 (an autotransporter protein), which lead to the aggregation of cells. Several studies have focussed on the receptor of the AIDA-I adhesin. Laarmann et al. [59] found that AIDA-I recognises an integral membrane glycoprotein in HeLa cells as receptor of 119 kDa called gp119. Fang et al. [36] hypothesised the existence of a receptor in porcine intestinal mucus and found two proteins of 65 and 120 kDa (p65 and p120), that bind with high affinity to purified AIDA-I adhesion. In addition, *aida-I* positive *E. coli* binds to these proteins with higher affinity than do *aida-I* negative mutants. Recently, Benz et al. [11] showed that the environmental factors (different growth conditions) and the genetic backgrounds of the strain significantly influence the transcription activity of the genes.

4.8. Other non-specific miscellaneous adhesins

With the sequencing of the O157 strain [44, 84], several fimbriae were identified in silico. Low et al. [65] studied the distribution of 16 fimbrial gene clusters in *E. coli* and the expression of these fimbriae in different conditions. In those 16 identified fimbriae, 4 are specific to O157 strains in comparison with the K12 strain and 4 fimbriae correspond to previously studied adhesins (LpfA1, LpfA2, Curli and Type 1 fimbriae).

Calcium binding Antigen 43 Homologue (Cah) is a protein described in the O157 strain and is homologous to antigen 43 and AIDA-I [116]. This gene is 2 850 pb long and is present in duplicate in O157 strain EDL933. A K12 strain complemented with *cah* produces two proteins (one outer membrane protein and one heat extract protein) and shows the capacity to autoaggregate. In O157, Cah protein participates in biofilm formation and also binds to calcium. This protein is implicated more in autoaggregation properties than in binding to eukaryotic cells.

Recently, several other adherence factors have been described. First, Xicohtencatl-Cortes et al. described the Haemorrhagic Coli Pilus (HCP). HCP is found in EHEC and EPEC strains [4]. The inactivation of the main subunit (*hcpA* gene) in O157:H7 EHEC reduces adherence to cultured human intestinal and bovine renal epithelial cells and to porcine and bovine gut explants [127]. They also found that in addition to promoting bacterial attachment to host cells, HCP also plays a role in the invasion of epithelial cells, in the haemagglutination of rabbit erythrocytes, in biofilm formation, in the specific binding to laminin and fibronectin of the host cells, and in twitching motility [128]. Then, in 2008, Wells et al. [125] described a novel autotransporter protein, called EhaA (EHEC autotransporter) implicated in adherence and biofilm formation in the O157:H7 strain. Finally, Ferreira et al. [37] described the implication of the *pst* operon (the phosphate-specific transport system) on the adherence to host cells in an EPEC strain. Indeed, in the absence of *pst* there is a decrease in the expression of the main EPEC adhesins and a reduction in bacterial adherence to epithelial cells in vitro.

5. CONCLUDING REMARKS

Considering that adherence is the basis of host specificity for a lot of pathogens and that ruminants (especially cattle) are considered to represent the main reservoir of EHEC and

VTEC strains for humans, we could wonder about the distribution of the EPEC, EHEC and VTEC adhesins among humans and animals. Based on different prevalence studies, we can affirm that, except for a few adhesins (Bfp, REPEC and F18 adhesins), EPEC, EHEC and VTEC adhesins do not seem to be host specific. There are several possible explanations. First, the host specificity is based on other factors. This host specificity may be based upon another adhesin not yet discovered or another property such as the following: (i) differences in the sequences of genes coding for some adhesins present in human and bovine strains, resulting in host and tissue tropism, as already described in other families of fimbrial (P family) or afimbrial (AFA family) adhesins [12, 35]; (ii) variation in the expression of some adhesin-encoding genes according to the growth environment (bovine or human intestines; intestinal segments; age of the host; etc.), as observed for other genes [29]; and/or (iii) properties other than adherence such as an intermediate metabolism, which allows the bacteria to be better adapted to a bovine intestinal environment, such as the young calf intestine [34, 121]. Second, there is no actual host specificity and the strains carrying specific adhesins (Bfp, REPEC and F18 adhesins) could represent a subgroup of strains specifically adapted to one host and/or one environment. Therefore, the other EPEC, EHEC and VTEC strains could be considered as only one group that carry several adhesins without any specificity and acting together with the aim to attach the bacteria to the host cells. Thus, it is not surprising that the bacteria could have different adhesin profiles that are not linked to one specific host or to one specific pathotype.

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2. Objectives

Enterohaemorrhagic *Escherichia coli* strains (EHEC) represent an important problem for public health in developed countries. EHEC strains can infect human beings; ruminant faeces soil vegetal and animal foodstuffs (ruminants are considered to be healthy carriers), which, if consumed, can cause food poisoning with diarrhoeas. EHEC are generally accompanied by haemorrhagic colitis (HC) with occasional occurrences of potentially lethal renal sequelae (HUS). In the veterinary field, several serogroups of EHEC strains (e.g., O5, O26, O111, O118) are directly associated with digestive disorders in two-week to two-month-old calves, causing economic losses due to growth delay and calves weakness.

Pathogenicity of EHEC strains is probably divided in three stages:

1. The bacteria colonise the intestine via specific adhesins;
2. The bacteria produces a signal that causes cytoskeleton's rearrangement in enterocytes and intimate adherence of bacteria to eukaryotic cells by specific proteins, the INTIMIN adhesins;
3. Production of Shiga toxins (Stx), responsible for HC and HUS.

For EPEC infection, strains do not produce Stx (step 3); for VTEC infection, step 2 is omitted.

For many strains, the initial adherence via specific adhesins (step 1) is poorly understood. Still, its importance is significant, since 1) adherence is the first contact between bacteria and intestinal cells (without it, the infection cannot process any further); 2) adherence is the basis of host specificity for a lot of pathogens; 3) adhesins represent a good target for the development of a specific vaccinal prophylaxis.

The specific aim of this study on O26 EHEC and EPEC is twofold. First, we aim at identifying the factors of O26 EHEC strains implicated in initial attachment, in host specificity (human or cattle), or in both. Second, we wish to identify host-specific sequences by comparing EHEC strains genomes isolated from humans and from cattle.

This work is divided into four steps.

First, we collected new O26 EHEC, EPEC and VTEC strains from veal calves and wild cervids and studied the phylogenetic relationship of our O26 *E. coli* strains collection by Pulsed Field Gel Electrophoresis (PFGE) (chapter 3).

Secondly, we analysed the distribution of known adherence factors (presence of putative adhesins described in literature and polymorphisms in *tir*, *eae* and *tccP2* genes) in a collection of O26 EHEC and EPEC strains to highlight putative host specificity of these factors (chapter 4).

Thirdly, we searched for new virulence factors implicated in host specificity or in initial adherence by comparing a bovine O26 EHEC strains and a human O26 EHEC strain with Suppressive Subtractive Hybridisation method (chapter 5).

Fourthly, we comprehensively compared human and bovine strains genomes by Whole Genome PCR Scanning and multiple PCR-based IS fingerprint methods (chapter 6).

3. O26 *E. coli* strains

3.1. Description of existing collections

In the different sections of this thesis, we refer to a total of 90 strains of serogroup O26, which are either EHEC (n=53), EPEC (n=30), or non-EHEC/non-EPEC (n=7). Strains have been isolated from bovines (n=50), humans (n=38), piglets (n=1), or birds (n=1). They originate from the USA, Ireland, UK, Belgium, France, The Netherlands, Italy, Japan, Switzerland and Brazil. Their pathotypes (EPEC or EHEC) and O26(H11) serotype were checked by PCR for *stx1*, *stx2*, *eae*, *wzx-wzyO26*, *fliCH11* and *EHEC-hlyA* genes (Tables 2 and 3).

Table 2: PCR used to characterise strains' pathotype and serotype.

Primer name	Sequence (5' to 3')	Target gene	Annealing temp. (°C)	Amplicon size (bp)	Reference
B52	AGGCTTCGTCACAGTTG	*eae*	50	570	(China *et al.*, 1996)
B53	CCATCGTCACCAGAGGA				
B54	AGAGCGATGTTACGGTTTG	*stx1*	50	388	(China *et al.*, 1996)
B55	TTGCCCCCAGAGTGGATG				
B56	TGGGTTTTTCTTCGGTATC	*stx2*	50	807	(China *et al.*, 1996)
B57	GACATTCTGGTTGACTCTCTT				
wzx-wzyO26-F	AAATTAGAAGCGCGTTCATC	*wzxO26*	56	596	(Durso *et al.*, 2005)
wzx-wzyO26-R	CCCAGCAAGCCAATTATGACT				
EHEC-hlyA-F	ACGATGTGGTTTATTCTGGA	*EHEC-hlyA*	58	165	(Fagan *et al.*, 1999)
EHEC-hlyA-R	CTTCACGTGACCATACATAT				

3.2. Phylogeny

We performed two phylogenetic analyses on most of the strains: a Pulsed Field Gel Electrophoresis (PFGE) and a phylogenetic group determination (A, B1, B2 or D).

The PFGE was performed as previously described (Cobbaut *et al.*, 2009, Ooka *et al.*, 2009). PFGE profiles, using *Xba*I as a restriction enzyme, were obtained for 74 of the 90 strains. Others strains did not present any restriction profile for *Xba*I or were not tested. The 74 distinct electrophoresis profiles were used for the dendrogram construction (Dice coefficient, with an optimisation and position tolerance of 1%, Unweighted Pair Group Method with Arithmetic

Mean-UPGMA) (Figure 9). The dendrogram showed seven clusters, assuming a cutoff of 45% of similarity. When we applied a cutoff of 80% of similarity, we found 47 different clusters, indicating the strains high genetic variability.

Classical phylogenetic analyses revealed that *E. coli* strains could belong to four main phylogenetic groups (A, B1, B2 and D) (Herzer *et al.*, 1990). In 2000, Clermont and collaborators developed a multiplex PCR based on the detection of three genes to classify *E. coli* strains in these groups (Clermont *et al.*, 2000). Therefore, we tested our collection of strains with this triplex PCR. Most of the strains belonged to the B1 phylogenetic group (Table 3).

3.3. Search for new strains isolated from veal calves and wild animals

3.3.1. Preamble

To obtain new EPEC, EHEC, and VTEC strains from ruminants, we collected fresh faeces from veal calves and wild cervids in Belgium. We collected two samples in a veal facility located in northern Belgium, in January and May 2008. This industry provides veal meat, mainly to European countries. Regarding faeces from free-ranging deer (*Capreolus capreolus* and *Cervus elaphus*), we collected samples in southern Belgium during the hunting season (from October to December), in 2008 and 2009. To that avail, we collaborated with the *Surveillance Network of Wildlife Disease* from the University of Liège, and more specifically with Pr. Annick Linden. While collecting new strains for further studies, we carried out a prevalence survey, with molecular typing and antibiotic sensitivity of the strains, for both populations. We report these analyses in articles 2 and 3 of this thesis.

3.3.2. Article 2: "Prevalence, molecular typing and antibiotic sensitivity of enteropathogenic, enterohaemorrhagic and verotoxigenic Escherichia coli isolated from veal calves"

WETENSCHAP

Prevalence, molecular typing, and antibiotic sensitivity of enteropathogenic, enterohaemorrhagic, and verotoxigenic *Escherichia coli* isolated from veal calves

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ABSTRACT

Cattle are considered to be an important reservoir of enterohaemorrhagic *Escherichia coli* (EHEC) and verotoxigenic *Escherichia coli* (VTEC) strains that can cause disease in humans, and numerous studies of the prevalence of these strains in cattle (focusing mainly on dairy and beef cattle) have been carried out in different regions of Europe, Asia, and America. To date, only a few studies of veal calves have been published focusing on EHEC strains belonging to the O157 serogroup EHEC, whereas EHEC and VTEC can belong to hundreds of different serotypes (many of which are as dangerous to humans as the O157:H7 EHEC, such as strains of the O26, O91, O103, O111, O113 and O145 serogroups). The aim of this study was to investigate the presence of enteropathogenic *Escherichia coli* (EPEC), EHEC, and VTEC strains in veal calves in Belgium and to characterize the positive isolates (serogroups, virulence-associated factor-encoding genes and antibiotic resistance profiles). The prevalence of EPEC, EHEC, and VTEC strains in faecal samples from veal calves in Belgium was found to be 11.7% (6.5% of the calves were found to be positive for EPEC strains, 2.6% for EHEC, and 3.9% for VTEC strains). No O157:H7 EHEC strain was identified, but three calves were found to carry strains belonging to the O26 and O111 serogroups. The results of antibiotic sensitivity tests showed a high level of resistance (83% of strains were resistant or intermediate resistant to five or more antibiotics of the 13 tested antibiotics), which might be caused by the frequent use of antibiotics in veterinary practice.

SAMENVATTING

Prevalentie, moleculaire typering en antibioticagevoeligheid van enteropathogene, enterohemorrhagische en verotoxigene* Escherichia coli *geïsoleerd uit vleeskalveren

Rundvee wordt gezien als belangrijk reservoir van enterohaemorrhagische Escherichia coli *(EHEC)- en verotoxigene* Escherichia coli *(VTEC)-stammen die mensen ziek kunnen maken. In verschillende regio's in Europa, Azië en Amerika is veelvuldig onderzoek gedaan naar het voorkomen van deze stammen bij rundvee (waarbij de nadruk vooral lag op melk- en vleeskoeien). Tot op heden zijn echter slechts enkele studies verschenen bij vleeskoeien. Hierbij lag de nadruk op de EHEC-stammen uit de O157-serogroep EHEC. Maar zowel EHEC als VTEC komen voor in honderden verschillende serotypes, waarvan er veel net zo gevaarlijk zijn voor mensen als de O157 :H7 EHEC, zoals de serogroepen O26, O91, O103, O111, O113 en O145.*

Dit onderzoek had als doel om de aanwezigheid van enteropathogene Escherichia coli *(EPEC)-, EHEC- en VTEC-stammen in vleeskalveren in België in kaart te brengen en de positieve isolaten te karakteriseren (op serogroep, genen voor virulentiegeassocieerde factoren en antibioticaresistentieprofielen). De prevalentie van EPEC-, EHEC- en VTEC-stammen in ontlastingsmonsters van vleeskalveren in België bedroeg 11,7 procent (6,5 procent van de kalveren bleek positief te zijn voor EPEC-stammen, 2,6 procent was positief voor EHEC-stammen en 3,9 procent was positief voor VTEC-stammen). Er werd geen O157 :H7 EHEC-stam geïdentificeerd, maar bij drie kalveren werden stammen aangetroffen uit de O26- en O111-serogroepen. De resultaten van de antibioticagevoeligheidstesten lieten een hoog resistentieniveau zien (83 procent van de stammen waren resistent of gemiddeld resistent tegen vijf of meer van de dertien onderzochte antibiotica). De hoge resistentie wordt mogelijk veroorzaakt door het veelvuldige gebruik van antibiotica in de diergeneeskundige praktijk.*

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This work was done in the Laboratory of Bacteriology of the Faculty of Veterinary Medicine (University of Liège, Liège, Belgium) and the results of this work were presented in the PEN (Pathogenic *E. coli* Network) meeting "Epidemiology & Transmission of Pathogenic *E. coli*" in Stockholm (Sweden). Marjorie Bardiau is a PhD fellow of the "Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture" (FRIA). This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (contract RF 6172). Lastly, the authors thank the owner and the veterinarian of the veal farm, who made it possible for us to perform this study.

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC), enterohaemor-

rhagic *Escherichia coli* (EHEC), and verotoxigenic *Escherichia coli* (VTEC) represent three important classes of enteric pathogens that can cause enteritis and enterotoxaemia in humans and animals. These pathogens are defined on the basis of two main virulence properties (28). The main virulence property of EPEC strains is the production of a specific histological and ultrastructural lesion called an "attaching and effacing lesion" (A/E lesion), characterized by the loss (= effacement) of the microvilli of the enterocytes as a consequence of cytoskeleton rearrangements initiated by type III-secreted (T3S) bacterial effectors, and by the intimate (<10 nm) attachment of the bacteria to host enterocytes, via an interaction between an outer membrane protein named intimin and one of the T3S effectors called Tir (for Translocated Intimin Receptor) (25). The main virulence property of VTEC strains is the production of verotoxins (VTS) (or Shiga-like toxins, STXS or SLTS), which are lethal to eukaryotic cells both in vitro (Vero, HeLa, and/or MDBK cells) and in vivo (endothelial cells), by blocking protein synthesis (19). EHEC strains share the main virulence properties of EPEC and VTEC strains: the production of A/E lesions and VTS (STXS). Today, EHEC strains are considered to have evolved from EPEC strains through the acquisition of bacteriophages carrying stx genes encoding for SLT (30, 37).

In contrast to their limited importance in developed countries, EPEC strains are a major cause of infant diarrhoea in developing countries, often associated with high mortality rates (8). EPEC strains infect mainly infants under 2 years of age. EPEC strains are also associated with diarrhoea in most domestic animal species. In bovines, EPEC strains are associated with diarrhoea in 1- to 8-week-old calves (9, 27). The diarrhoea is a consequence of the production of the A/E lesion and of the ensuing inflammatory response of the host (8).

EHEC strains can cause different intestinal and extra-intestinal syndromes in humans: undifferentiated diarrhoea, haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), and thrombotic thrombocytopaenic purpura (TP) (29). In developed countries (USA, Canada, UK, France, Japan, etc) EHEC strains are responsible for individual human cases, and for small-to-large outbreaks (13, 24, 31-33, 35). Human infection can occur via consumption of vegetable and other foodstuffs contaminated by faeces from ruminants (mainly cattle), which may be asymptomatic healthy carriers (6, 15). Nevertheless, some EHEC strains are also responsible for undifferentiated diarrhoea in young calves of up to 3 months of age (23, 27, 34).

VTEC strains cause clinical syndromes mainly in humans and piglets but can also be isolated from a wide range of domestic and wild animals, which are healthy asymptomatic carriers (38). VTEC infections are not common in humans but, when they happen, they are frequently associated with HUS. VTEC strains are also responsible for oedema disease in piglets, which occurs up until 2 weeks after weaning (26). While in most cases the source of human infection is foodstuffs contaminated with ruminant faeces, human and porcine VTEC strains are different and no cross-contamination has been reported.

Cattle thus represent an important reservoir of EHEC and VTEC strains that can cause disease in humans (5, 7, 16, 23). Consequently, numerous studies of their prevalence in cattle have been carried out in different regions of Europe, Asia, and America (3, 14, 18, 20, 21, 36), focusing mainly on dairy and beef cattle. To date, only five studies of veal calves have been published, which focused their search on EHEC strains belonging to the O157 EHEC (4, 11, 12, 14, 17), whereas EHEC and VTEC can belong to hundreds of different serotypes, many of which are as dangerous to humans as the O157:H7 EHEC, such as strains of the O26, O91, O103, O111, O113 and O145 serogroups.

The aim of this study was to investigate the presence of EPEC, EHEC, and VTEC strains in veal calves in Belgium using polymerase chain reaction assays (PCRS) targeting the genes coding for intimin adhesin (eae) and for VTS (vt1, vt2, vt2c). The positive isolates were further characterized by PCR for other virulence-associated factor-encoding genes (EHEC-hlyA, bfp) and for five of the most important somatic serogroups (O26, O103, O111, O145, and O157). They were also tested for their antibiotic resistance profiles against 13 frequently used antibiotics.

MATERIALS AND METHODS

Collection of specimens and isolation of *E. coli* strains

All the samples were collected between January and May 2008 on one commercial farm [?company] producing approximately 120,000 veal calves per year. A first group of samples (G1) consisted of rectal swabs or faecal samples from veal calves aged 1 to 20 weeks. The second group of samples (G2) consisted of jejuno-ileal content from 6-month-old veal calves taken at the time of slaughter.

The samples were inoculated onto Gassner agar plates and incubated for 18 hours at 37 °C. Subsequently, five lactose-positive colonies per calf were picked up and transferred into LB broth with 0.1% tryptophan. Bacteria were grown for 8 hours at 37 °C, and Kovacs reagent was added to detect indole production. Indole-positive isolates were stored at -80 °C in 20% glycerol until further characterization.

Genotypic characterization

DNA extraction was carried out as described previously by China et al. (10). Briefly, a pure bacterial culture was grown for 8 hours at 37 °C in LB broth with slight agitation. Then 300 µl was centrifuged for 1 min at 13,000 rpm and the supernatant was discarded. After the addition of 50 µl of sterile water, the suspension was boiled for 10 min. Afterwards, the suspension was centrifuged for 1 min at 13,000 rpm and the supernatant was stored at -20 °C.

All PCR conditions for the detection of the eae, vt1, vt2, wzxO26, fliCH11, rfbO157, wzxO111, wzxO103, wzxO145, vt2c, bfpA, and EHEC-hlyA genes have been described

Primer name	Sequence (5' to 3')	Target gene	Annealing temp. (°C)	Amplicon size (bp)	Reference
B52	AGGCTTCGTCACAGTTG	eaeA	50	570	(China et al., 1996)
B53	CCATCGTCACCAGAGGA				
B54	AGAGCGATGTTACGGTTTG	slt-I	50	388	(China et al., 1996)
B55	TTGCCCCCAGAGTGGATG				
B56	TGGGTTTTTCTTCGGTATC	slt-II	50	807	(China et al., 1996)
B57	GACATTCTGGTTGACTCTCTT				
wzx-wzyO26-F	AAATTAGAAGCGCGTTCATC	wzxO26	56	596	(Durso et al., 2005)
wzx-wzyO26-R	CCCAGCAAGCCAATTATGACT				
fliC-H11-F	ACTGTTAACGTAGATAGC	fliCH11	56	224	(Durso et al., 2005)
fliC-H11-R	TCAATTTCTGCAGAATATAC				
wzxO157-F	CGGACATCCATGTGATATGG	rfbO157	60	259	(Paton & Paton, 1998)
wzxO157-R	TTGCCTATGTACAGCTAATCC				
wzxO111-F	TAG AGA AAT TAT CAA GTT AGT TCC	wzxO111	62	406	(Paton & Paton, 1998)
wzxO111-R	ATA GTT ATG AAC ATC TTG TTT AGC				
wzxO103-F	TTGGAGCGTTAACTGGACCT	wzxO103	57	321	(Fratamico et al., 2005)
wzxO103-R	GCTCCCGAGCACGTATAAG				
wzxO145-F	CCATCAACAGATTTAGGAGTG	wzxO145	59	609	(Feng et al., 2005)
wzxO145-R	TTTCTACCGCGAATCTATC				
slt-IIc-F	GCGGTTTTATTTGCATTAGT	slt-IIc	52	124	(Osek, 2003)
slt-IIc-R	AGTACTCTTTTCCGGCCACT				
bfpA-F	AATGGTGCTTGCGCTTGCTGC	bfpA	56	326	(Gunzburg et al., 1995)
bfpA-R	GCCGCTTTATCCAACCTGGTA				
EHEC-hlyA-F	ACGATGTGGTTTATTCTGGA	EHEC-hlyA	58	165	(Fagan et al., 1999)
EHEC-hlya-R	CTTCACGTGACCATACATAT				

Table 1: primers used in this study.

previously (Table 1). All PCR products were separated by 1.5% agarose gel electrophoresis. Gels were stained with SYBR Green and were visualized under UV light.

A Fisher's exact test was performed to assess statistical differences ($p<0.01$) between the different groups of animals.

Antibiotic susceptibility tests

Susceptibility tests were carried out on the positive isolates for the eae, vt1, and/or vt2 genes, using the disc diffusion method of Bauer et al. (2) on Mueller-Hinton agar (Oxoid,). Zones of inhibition were measured (in millimetres) after overnight incubation at 37 °C and were interpreted according to the CLSI (Clinical and Laboratory Standards Institute) (CLSI, 2008). Thirteen antibiotics used on the farm were tested: cefuroxime (30 µg), ceftiofur (30 µg), ampicillin (10 µg), neomycin (5 µg), enrofloxacin (5 µg), the combination of trimethoprim-sulfamethoxazole (1.25 µg-23.75 µg), tetracycline (30 µg), (Becton Dickinson), florfenicol (30 µg), flumequin (30 µg) (Oxoid), tylosin (150 µg), and the combination of lincomycin-spectinomycin (15 ²g-200 µg) (Neo-Sensitabs).

RESULTS

Prevalence of EPEC, EHEC and VTEC strains in veal calves (Table 2)

One hundred and ninety-five strains of *E. coli* (g1) isolated from the faeces of 39 diarrhoeic and non-diarrhoeic calves (G1) aged between 1 and 20 weeks of age, and 190 strains (g2) isolated from intestinal content of 38 non-diarrhoeic 6-month-old calves (G2) were examined for virulence factors. According to the PCR results, 11.7% of the calves were carriers of one of the three pathotypes (6.5% of the calves were found positive for EPEC strains, 2.6% for EHEC, and 3.9% for VTEC strains). The percentage of carriers did not differ between G1 (5+/39) and G2 (4+/38), between diarrhoeic (1+/8) and non-diarrhoeic (8+/69) calves, or between Belgian Blue (1+/30) and Black and White calves (8+/47) (Fisher Exact Test, $p<0.01$). Eighteen of the isolates from 9 different calves were positive with the multiplex PCR for eae, vt1, and vt2 genes: 5 isolates were eae+vt1+ (EHEC); 3 isolates were vt2c+ (VTEC); 1 isolate was vt1+ (VTEC); and 9 isolates were eae+ (EPEC). The five EHEC strains were isolated from two calves and the four VTEC strains from 3 calves in the G1 group (Table 2). The nine EPEC strains were isolated from the four calves in the G2 group and from one calf in the G1 group.

Typing of the PCR-positive isolates (Table 2)

Of the most frequent EHEC serogroups (O157, O26, O111, O103, and O145), the O26 and O111 serogroups were identified in 5 isolates (all were eae+ and vt1+) and 1 isolate (vt1+), respectively, but no isolates belonging to the O157, 0145 or 0103 serogroup were detected. All isolates of the O26 serogroup were positive for the fliCH11 gene.

All of the EPEC, EHEC, and VTEC isolates tested negative with the PCR for the bfp gene, and 11 strains (the five O26 EHEC, the O111 VTEC and five EPEC) tested positive with the PCR for the EHEC-hlyA gene, all but one isolated from five calves from the G1 group (Table 2).

Antibiotic susceptibility tests (Table 2)

Of the 18 EPEC, EHEC, and VTEC isolates, none was sensitive or resistant to all of the thirteen antibiotics tested. One isolate showed intermediate resistance to one antibiotic and two isolates showed intermediate resistance to two antibiotics, and these three strains were sensitive to all of the other antibiotics tested. Fifteen strains were resistant to up to three of the thirteen antibiotics tested.

DISCUSSION

Cattle are considered to be a major reservoir of EPEC, EHEC, and VTEC strains (6, 7, 16, 22). Numerous studies of their prevalence in cattle have been carried out in different regions of Europe, Asia, and America (3, 14, 18, 20, 21, 36), focusing mainly on dairy and beef cattle. To date, only five other studies of veal calves have been published and focused on EHEC strains belonging to the O157 EHEC (4, 11, 12, 14, 17), whereas EHEC and VTEC can belong to hundreds of different serotypes, many of which are as dangerous to humans as the O157:H7 EHEC, such as strains of the O26, O91, O103, O111, O113, and O145 serogroups. The aim of this study was to determine the prevalence of EPEC, EHEC, and VTEC strains in veal calves in Belgium. To our knowledge, it is the first study of this type on veal calves in Belgium.

The prevalence of EPEC, EHEC, and VTEC strains in faecal samples from Belgian veal calves was 11.7%: 2.6% of the calves were positive for EHEC strains, 6.5% for EPEC, and 3.9% for VTEC strains. No O157:H7 EHEC strain was identified, but three calves (3.9% of the calves) were found to carry strains belonging to the "gang of five" serogroups (O157, O26, O111, O103, O145), which are frequently associated with human disease: five EHEC isolates belonged to the O26 serogroup and one VTEC isolate belonged to the O111 serogroups. Although infrequently infected, veal calves could be considered as a potential vector of EHEC and VTEC strains that can infect humans. However, these O26 and O111 strains may also be more cattle-specific and cause diarrhoea in veal calves. Indeed, EHEC strains belonging to some serogroups, including O26, O111, and O118, are also responsible for undifferentiated diarrhoea in young calves up to 3 months of age (23).

The other EPEC and VTEC strains identified did not belong to these five serogroups dangerous for humans. There are two possibilities: (1) the other serogroups do not infect humans, or do so only occasionally, and so there is a low potential risk of zoonosis, or (2) the other serogroups represent pathogenic serogroups that can lead to human infections. The 2007 annual report of the European Union on zoonotic agents (1) supports the second hypothesis. In 2007, 29% of reported confirmed VTEC cases in humans concerned untyped or strains of untypeable serogroups that did not belong to the gang of five (O157, O26, O111, O103, and O145). However, comparison of the percentage of VTEC strains found in veal calves (6.5% in our study), the percentage of VTEC found on beef carcasses in Belgium (0.4% of 1611 carcasses in 2007), and the percentage of reported confirmed VTEC cases in humans (0.4 cases per 100,000 habitants in 2007) (Anonymous, 2009) shows that food safety practices are well applied in Belgian slaughterhouses and that the zoonotic risk is limited in Belgium.

The results of the antibiotic sensitivity tests were disappointing in view of the high percentage of resistance, which might be explained by the frequent use of the tested antibiotics in veterinary practice. In total, 83% of the strains were multiresistant (resistant to more than two antibiotics) and, in our case, these multiresistant strains were resistant or showed intermediate resistance to five or more antibiotics of the 13 antibiotics tested. On average, strains were resistant to 4.5 ± 2.2 antibiotics and showed intermediate resistance to 1.4 ± 1.3 antibiotics, with 28% of the strains showing resistance or intermediate resistance to eight or more antibiotics. A challenge would be to reduce the use of antibiotics and to use them only when it is strictly necessary, to avoid the emergence of multiresistant pathogenic strains.

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Population	Calf n°	Strain n°	slt	eae	Serogroup	EHEC-hlyA	bfp	Antibiotic resistance pattern
G1	11	11.2	1	+	O26:H11	+	-	A: R; T: R; L/S: I; TTC: R; T/S: R
	11	11.4	1	+	026:H11	+	-	A: R; T: R; L/S: I; TTC: R; T/S: R
	15	15.3	2c	-	NI	-	-	A: R; N: R; T: R; TTC: R; T/S: R
	15	15.5	2c	-	NI	-	-	N: R; T: R; TTC: R; T/S: R
	18	18.1	-	+	NI	+	-	A: R; CX: I; T: R; L/S: I; TTC: R; FF: I; T/S: R
	18	18.3	-	+	NI	+	-	A: R; T: R; L/S: I; TTC: R; FF: I; T/S: R
	18	18.4	-	+	NI	+	-	A: R; T: R; L/S: I; TTC: R; FF: I; T/S: R
	18	18.5	-	+	NI	+	-	A: R; T: R; L/S: I; TTC: R; FF: I; T/S: R
	20	20.1	1	+	O26:H11	+	-	T:I
	20	20.2	1	+	O26:H11	+	-	CX: I; T: I
	20	20.3.1	1	+	O26:H11	+	-	CX: I; T: I
	20	20.3.2	2c	-	NI	-	-	N: R; T: R; TTC: R; T/S: R
	25	25.1	1 and 2	-	O111	+	-	N: R; T: R; TTC: R; T/S: R
G2	59	59.4	-	+	NI	-	-	A: R; T: R; TTC: R; T/S: R
	63	63.5	-	+	NI	-	-	A: R; N: R; T: I; TTC: R; T/S: R; F: R
	68	68.3	-	+	NI	-	-	A: R; CX: I; T: R; TTC: R; T/S: R
	68	68.4	-	+	NI	-	-	A: R; T: R; TTC: R; T/S: R; F: R; E: R
	70	70.2	-	+	NI	+	-	A: R; N: R; T: I; L/S: R; TTC: R; T/S: R

Table 2: results obtained for each strain (NI: not identified; A: ampicillin; CX: cefuroxim; E: enrofloxacin; FF: florfenicol; F: flumequin; L/S: lincomycin/spectinomycin; N: neomycin; T: tylosin; T/S: trimethoprim/sulfamethoxasol; TTC: tetracyclin; R: resistant; I: intermediate).

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3.3.3. Article 3: "Enteropathogenic (EPEC), enterohaemorrhagic (EHEC) and verotoxigenic (VTEC) Escherichia coli in wild cervids"

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ORIGINAL ARTICLE

Enteropathogenic (EPEC), enterohaemorragic (EHEC) and verotoxigenic (VTEC) *Escherichia coli* in wild cervids

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antibiotic susceptibility, EHEC, EPEC, prevalence, VTEC, wildlife.

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Abstract

Aims: The aim of this study was to investigate the presence of enteropathogenic (EPEC), enterohaemorragic (EHEC) and verotoxigenic (VTEC) *Escherichia coli* strains in free-ranging wild ruminants in Belgium and to characterize the positive isolates (serogroups and virulence-associated factor-encoding genes).

Methods and Results: *Escherichia coli* strains isolated from faeces of wild cervids were characterized by PCR targeting genes coding for the main virulence properties of EPEC, EHEC and VTEC strains. The prevalence rate of these pathogenic strains in faecal samples obtained from the wild ruminants was found to be 15%. No pathogenic isolate was found to belong to the O157, O26, O111, O103 or O145 serogroups. Moreover, a new gene, *eibH*, showing 88% identity with *eibG* was detected in VTEC strains.

Conclusions: The results reveal that wild ruminants could be considered as a potential source of VTEC and EPEC infection for humans and possibly also for domestic ruminants.

Significance and Impact of the Study: Our study suggests the potential risk of transmission of VTEC, EHEC and EPEC strains from wild ruminants to humans via the consumption of venison and to domestic ruminants because of sharing of the same pasture. Indeed, many serogroups other than O157 EHEC have also been shown to be responsible for outbreaks in humans in several countries, and studies focusing solely on O157:H7 EHEC tend to underestimate this risk of transmission.

Introduction

Enteropathogenic (EPEC), enterohaemorragic (EHEC) and verotoxigenic (VTEC) *Escherichia coli* represent three important classes of enteric pathogens that can cause enteritis and enterotoxaemia in humans and animals. They are characterized by two main virulence properties: (i) the production of histological and ultra-structural lesions called 'attaching and effacing lesions' (A/E lesions) and (ii) the production of Shiga toxins (Stx) (also called verotoxins) (Nataro and Kaper 1998). A/E lesions are characterized firstly, by the effacement of the enterocyte microvilli, as a consequence of cytoskeleton rearrangements initiated by type III-secreted (T3S) bacterial effectors and secondly, by the intimate (<10 nm) attachment of the bacteria to the host enterocytes, via the interaction between an outer membrane protein called intimin (encoded by the *eae* gene) and one of the T3S effectors called Tir (after translocated intimin receptor) (Chen and Frankel 2005; Spears *et al.* 2006). The Shiga toxins are lethal for eukaryotic cells both *in vitro* (Vero, HeLa and/or MDBK cells) and *in vivo* (endothelial cells), because of their effect of blocking protein synthesis (Konowalchuk *et al.* 1977). The Stx toxins belong to one of two families: Stx1 or Stx2. There are several variants of these toxins, particularly within the Stx2 family (Mainil and Daube 2005).

The main virulence property of EPEC strains is the production of A/E lesions, causing the occurrence of an inflammatory reaction and of diarrhoea (Moon *et al.* 1983).

EPEC strains are a major cause of infantile diarrhoea in developing and developed countries and are responsible for thousands of deaths worldwide (Chen and Frankel 2005; Ochoa *et al.* 2008). They are also associated with diarrhoea in most domestic animals species. In cattle, they are associated with diarrhoea in young calves from 1 to 8 weeks of age (China *et al.* 1998).

The main virulence property of VTEC strains is the production of Stx. VTEC strains cause clinical syndromes mainly in humans and piglets. But they can also be isolated from a wide range of domestic and wild animals, which may be asymptomatic healthy carriers (Wieler and Bauerfeind 2003). VTEC infections are less common in humans but, when they do occur, they are frequently associated with haemolytic uraemic syndrome (HUS) (Mainil and Daube 2005). VTEC strains are also responsible for oedema disease in piglets up to 2 weeks after weaning (Moxley 2000).

EHEC strains share the same main virulence properties as the EPEC and VTEC strains, that is the production of A/E lesions and Stx. Nowadays, EHEC strains are considered to have evolved from EPEC strains through the acquisition of bacteriophages encoding Stx (Reid *et al.* 2000) (Wick *et al.* 2005). EHEC strains can cause various syndromes in humans: undifferentiated diarrhoea, haemorrhagic colitis and HUS (Raffaelli *et al.* 2007) and have been associated with significant disease outbreaks in developed countries (USA, Canada, United Kingdom, France, Japan, etc.) in recent years (Stirling *et al.* 2007; Sonoda *et al.* 2008). In several cases, disease has been shown to occur via the consumption of vegetal and animal foodstuffs contaminated by ruminant faeces (mainly cattle) (Erickson and Doyle 2007). Some EHEC strains have also been shown to be responsible for undifferentiated diarrhoea in young calves up to 3 months of age (Mainil and Daube 2005).

Domestic ruminants (especially cattle) are considered to be the main reservoir of EHEC strains for human infection (Hancock *et al.* 2001; Mainil and Daube 2005). Nevertheless, wild ruminants are also considered to represent a potential source of infection for humans and possibly also for domestic ruminants (Pierard *et al.* 1997; Rabatsky-Ehr *et al.* 2002; Simpson 2002). Most studies focus on O157 EHEC strains (Fischer *et al.* 2001; Renter *et al.* 2001; Kemper *et al.* 2006; Heuvelink *et al.* 2008), despite the fact that EHEC strains can belong to a number of different serotypes, many of which are as dangerous to humans as the O157:H7 EHEC, such as the O26, O103, O111 and O145 strains (Campos *et al.* 2004). Only a few studies on wild ruminants do not focus their research on O157 EHEC strains (Asakura *et al.* 1998; Fukuyama *et al.* 1999; Sanchez *et al.* 2009).

The aim of this study was to investigate the presence of EPEC, EHEC and VTEC strains in free-ranging wild ruminants in Belgium using PCR targeting genes coding for the main virulence properties, that is A/E lesions and Stx. The positive isolates were further characterized by PCR for other virulence-associated factor-encoding genes and for five of the most important EHEC somatic antigens (O26, O103, O111, O145 and O157). The positive isolates were also tested for their antibiotic resistance profiles against eight frequently used antibiotics in bovine veterinary medicine.

Materials and methods

Collection of faecal samples and isolation of *Escherichia coli* strains

Through a targeted surveillance programme, the rectal contents of 133 free-ranging wild cervids (*Cervus elaphus* and *Capreolus capreolus*) were collected during the 2008 and 2009 hunting seasons (from 1 October to 31 December) in Wallonia (southern part of Belgium) (Table 1). Individual postmortem examination involved the determination of sex, age (through tooth eruption patterns), body weight and body condition.

The faeces were inoculated onto Gassner agar plates (Merck, Whitehouse Station, NJ, USA) and incubated for 18 h at 37°C. Subsequently, three lactose-fermenting colonies per sample were randomly picked up and transferred into Luria–Bertani (LB) broth (Invitrogen, Carlsbad, CA, USA) with 0·1% tryptophan (Sigma-Aldrich, St Louis, MO, USA). Bacteria were grown for 8 h at 37°C, and Kovacs reagent (Merck) was then added to detect indol production (underlining the action of tryptophanase). Only the isolates that were positive in the indol test were stored at −80°C in 20% glycerol until further characterization.

Genotypic characterization

DNA extraction was carried out by a boiling method as described previously by China *et al.* (1996). Briefly, a pure bacterial culture was grown for 8 h at 37°C in LB broth with slight agitation. Three hundred microlitres of culture was centrifuged for 1 min at 13 000 rev min^{-1}, and the supernatant was discarded. After adding 50 μl of sterile water, the suspension was boiled for 10 min. Afterwards, the suspension was centrifuged for 1 min at 13 000 rev min^{-1}, and the supernatant was stored at −20°C.

Genotypic characterization by PCR (Table 2) was performed in three steps: (i) detection of the *eae* gene coding for the intimin adhesin and of the *stx1* and *stx2* genes coding for Stx toxins (China *et al.* 1996); (ii) detection of

Table 1 Distribution of hunter-killed wild cervids sampled [juvenile, <1 year of age; subadult, 1–2 years of age; adults, >1 year of age (*Capreolus capreolus*) or >2 years of age (*Cervus elaphus*)]

	Number of animals	Age group	Number of animals	Gender	Number of animals
Cap. capreolus	52	Juvenile	15	Female	9
				Male	6
		Adult	37	Female	24
				Male	13
C. elaphus	81	Juvenile	26	Female	14
				Male	12
		Subadult	15	Female	10
				Male	5
		Adult	40	Female	18
				Male	22

Table 2 Primers used in this study

Primer name	Sequence (5′–3′)	Target gene	Annealing temp. (°C)	Amplicon size (bp)	References
B52	AGGCTTCGTCACAGTTG	*eae*	50	570	China *et al.* (1996)
B53	CCATCGTCACCAGAGGA				
B54	AGAGCGATGTTACGGTTTG	*stx1*	50	388	China *et al.* (1996)
B55	TTGCCCCCAGAGTGGATG				
B56	TGGGTTTTTCTTCGGTATC	*stx2*	50	807	China *et al.* (1996)
B57	GACATTCTGGTTGACTCTCTT				
wzx-wzyO26-F	AAATTAGAAGCGCGTTCATC	*wzxO26*	56	596	Durso *et al.* (2005)
wzx-wzyO26-R	CCCAGCAAGCCAATTATGACT				
wzxO157-F	CGGACATCCATGTGATATGG	*rfbO157*	60	259	Paton and Paton (1998)
wzxO157-R	TTGCCTATGTACAGCTAATCC				
wzxO111-F	TAG AGA AAT TAT CAA GTT AGT TCC	*wzxO111*	62	406	Paton and Paton (1998)
wzxO111-R	ATA GTT ATG AAC ATC TTG TTT AGC				
wzxO103-F	TTGGAGCGTTAACTGGACCT	*wzxO103*	57	321	Fratamico *et al.* (2005)
wzxO103-R	GCTCCCGAGCACGTATAAG				
wzxO145-F	CCATCAACAGATTTAGGAGTG	*wzxO145*	59	609	Feng *et al.* (2005)
wzxO145-R	TTTCTACCGCGAATCTATC				
bfpA-F	AATGGTGCTTGCGCTTGCTGC	*bfpA*	56	326	Gunzburg *et al.* (1995)
bfpA-R	GCCGCTTTATCCAACCTGGTA				
EHEC-hlyA-F	ACGATGTGGTTTATTCTGGA	*EHEC-hlyA*	58	165	Fagan *et al.* (1999)
EHEC-hlya-R	CTTCACGTGACCATACATAT				
saa-F	CGTGATGAACAGGCTATTGC	*saa*	50	119	Jenkins *et al.* (2003)
saa-R	ATGGACATGCCTGTGGCAAC				
eibGa-F	ATTTCTTTATGAGTGTGAGGTGTTG	*eibG*	51	552	This study
eibGa-R	CTGTCAGCAATTAAAACTCGAAGTT				
eibGb-F	ATCGGCTTTCATCGCATCAGGAC	*eibG*	60	548	Lu *et al.* (2006)
eibGb-R	CCACAAGGCGGGTATTCGTATC				
eibGc-F	TGTAAAAGACAGTGTTGAGCAACT	*eibG*	51	569	This study
eibGc-R	CGATGAAAGCCGATTGTTTTAA				
SubHCDF	TATGGCTTCCCTCATTGCC	*subA*	60	556	Paton and Paton (2005)
SubSCDR	TATAGCTGTTGCTTCTGACG				

the *bfpA*, *saa* and *eibG* genes coding for other adhesins and of *subA* and *EHEC-hlyA* coding for other toxins produced by different EPEC, EHEC and/or VTEC strains, in the positive isolates; and (iii) genotypic serotyping of the positive isolates. In addition, two new PCRs were designed during this study for the amplification of the 5′ end and of the 3′ end of the *eibG* gene (Table 2). All PCR products were separated by electrophoresis in 1·5% agarose gels. Gels were stained with SYBR Green (Roche Diagnostics Corporation, Basel, Switzerland) and

visualized under UV light. A Fisher's exact test was performed to assess the statistical differences ($P < 0{\cdot}01$) between the different groups of wild cervids (Table 1).

DNA sequencing

DNA fragments were purified using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Sequencing of the two DNA strands was performed by the dideoxynucleotide triphosphate chain termination method with a 3730 ABI capillary sequencer and a BigDye Terminator kit version 3.1 (Applied Biosystems, Carlsbad, CA, USA) at GIGA (Groupe Interdisciplinaire de Génoprotéomique Appliquée, University of Liège, Belgium). Sequence analysis was performed using Vector NTI 10.1.1 (Invitrogen, Carlsbad, CA, USA).

Antibiotic susceptibility tests

Susceptibility tests were carried out on the positive isolates for the *eae*, *stx1* and/or *stx2* genes. The tests were performed by the disc diffusion method of Bauer *et al.* (1966) on Mueller–Hinton agar (Oxoid, Hampshire, UK) (Bauer *et al.* 1966). Zones of inhibition were measured (in millimetres) after overnight incubation at 37°C and were interpreted according to the CA-SFM (Comité de l'Antibiogramme de la Société Française de Microbiologie) (Anonymous 2003). Eight antibiotics used widely in bovine veterinary medicine were tested: ceftiofur (30 μg); enrofloxacin (5 μg); the association of trimethoprim–sulfamethoxazole (1·25–23·75 μg); oxytetracycline (30 μg); spectinomycin (100 μg); gentamicin (10 μg); the association of amoxicillin/clavulanic acid (30 μg) (Becton Dickinson, Franklin Lakes, NJ); and florfenicol (30 μg) (Oxoid).

Results

Presence of VTEC, EHEC and EPEC strains

Thirty-seven isolates (37/399, 9·3%) tested positive with at least one of the three initial PCRs: six isolates (1·5%) tested positive with the PCR for the *eae* gene only (EPEC); nine isolates (2·3%) with the PCR for the *stx1* gene only (VTEC); 20 isolates (5%) with the PCR for the *stx2* genes only (VTEC); and two isolates (0·5%) with the PCRs for the *stx1* and *stx2* genes only (VTEC). No isolate tested positive with the PCRs for both the *eae* and the *stx* genes (EHEC). The number of positive isolates varied from one to all three colonies per animal tested (Table 3).

The PCR-positive *E. coli* were isolated from 20 cervids (20/133, 15%). The percentage of carriers did not statistically differ between *C. elaphus* (11/81, 13·6%) and *Cap. capreolus* (9/52, 17·3%), between male (9/58, 15·5%) and female (11/75, 14·7%) animals or between

Table 3 Results obtained for each animal [juvenile, <1 year of age; subadult, 1–2 years of age; adults, >1 year of age (*Capreolus capreolus*) or >2 years of age (*Cervus elaphus*)]

Species	Animal no.	No. of positive strains	Sex	Age	*stx*	*eae*	*eibH*
Cap. capreolus	A09/305	2+/3	F	Adult	1	–	–
	A09/311	1+/3	F	Adult	–	+	–
	A09/325	2+/3	M	Adult	1 and 2	–	–
	A09/332	1+/3	M	Adult	–	+	–
	A09/335	1+/3	F	Juvenile	2	–	–
	A09/337	2+/3	M	Adult	2	–	+
	A09/378	2+/3	M	Adult	2	–	+
	CH09/03	1+/3	M	Juvenile	2	–	–
	CH09/90	3+/3	F	Adult	1	–	–
C. elaphus	A09/299	3+/3	F	Adult	2	–	–
	A09/313	2+/3	F	Adult	2	–	+
	A09/315	2+/3	F	Juvenile	2	–	–
	A09/340	3+/3	F	Adult	2	–	–
	A09/345	2+/3	F	Juvenile	–	+	–
	A09/347	1+/3	M	Juvenile	2	–	–
	A09/371	3+/3	M	Adult	1	–	–
	A09/386	1+/3	F	Subadult	–	+	–
	A10/42	1+/3	M	Juvenile	1	–	–
	C09/73	1+/3	M	Adult	2	–	–
	C09/149	1+/3	F	Adult	–	+	–
		2+/3			2	–	+

adult (13/77, 16·9%) and juvenile/subadult (7/56, 12·5%) animals (Fisher's exact test, $P < 0·01$).

Of those 20 cervids, five were found to be carrying EPEC, four were carrying *stx1* + VTEC, 11 were carrying *stx2* + VTEC, and one was carrying *stx1* + /*stx2* + VTEC. One cervid (C09/149) was carrying two different types of strains: two *stx2* + VTEC and one EPEC strain.

Typing of the 37 PCR-positive isolates

All 37 PCR-positive isolates tested negative with the PCRs for the *bfpA*, *saa* and *subA* genes, and only one *stx2* + VTEC from a roe deer tested positive with the PCR for the *EHEC-hlyA* gene (Table 3).

On the other hand, eight *stx2* + VTEC (40% of the *stx2* + VTEC and 26% of all VTEC) isolated from four adult cervids (36·4% of cervids carrying stx2 + VTEC and 25% of cervids carrying VTEC) tested positive with the PCR for the middle of the *eibG* gene using primers eibGb-F and eibGb-R. These amplicons were sequenced for further identification and comparison. The eight amplicon sequences had 100% identity with each other, but only 74% identity with the *eibG* gene (AB255744). Therefore, the 5′ end and the 3′ end of the *eibG* gene were amplified using two other pairs of primers (eibGa-F/eibGa-R and eibGc-F/eibGc-R: Table 2) and were sequenced to obtain the sequence of the entire *eibG*-like gene (1527 bp). The sequence obtained showed 88% identity with the *eibG* gene (AB255744). The accession numbers of the nucleotide sequence of the *eibG*-like gene, called *eibH*, are HM114306, HM114307, HM114308 and HM114309.

Genotypic serotyping

No EPEC or VTEC isolate was found to be positive with the PCRs for the tested serogroups (O157, O26, O111, O103 and O145).

Antibiotic susceptibility tests

Two isolates (both VTEC) were sensitive to all the tested antibiotics, and only 10·8% of the isolates (two EPEC and two VTEC) were intermediate or resistant to more than three of the eight antibiotics (Table 4 and Fig. 1).

All 37 EPEC and VTEC isolates were sensitive to the association of trimethoprim–sulfamethoxazole to enrofloxacin and to gentamicin, but 24·3, 21·6 and 8·1% were, respectively, intermediate to florfenicol, to tetracycline and to ceftiofur. Conversely 81·1% of isolates were intermediate or resistant to the association of amoxicillin–clavulanic acid, and 35·1% were resistant to spectinomycin.

Table 4 Results obtained for each strain for the antibiotic resistance pattern

Species	Animal no.	Strain no.	Antibiotic resistance pattern
Capreolus capreolus	A09/305	A09/305.1	AMC, FF, TTC: I
		A09/305.2	AMC, C: I
	A09/311	A09/311.1	AMC, FF, TTC: I
	A09/325	A09/325.1	AMC: I
		A09/325.2	AMC: I
	A09/332	A09/332.1	FF: I
	A09/335	A09/335.1	AMC: I
	A09/337	A09/337.1	AMC: I
		A09/337.2	AMC: I
	A09/378	A09/378.1	AMC: I
		A09/378.2	FF: I
	CH09/03	CH09/03.1	AMC: I
	CH09/90	CH09/90.1	AMC: I; Sp: R
		CH09/90.2	AMC, FF, TTC: I
		CH09/90.3	AMC, C, TTC: I; Sp: R
Cervus elaphus	A09/299	A09/299.1	AMC: I
		A09/299.2	AMC: I
		A09/299.3	AMC: I
	A09/313	A09/313.1	AMC: I
		A09/313.2	/
	A09/315	A09/315.1	AMC, FF: I; Sp: R
		A09/315.2	AMC, FF, TTC: I; Sp: R
	A09/340	A09/340.1	AMC, C: I
		A09/340.2	AMC, TTC: I
		A09/340.3	AMC, TTC: I
	A09/345	A09/345.1	AMC, FF, TTC: I; Sp: R
		A09/345.2	AMC, FF, TTC: I; Sp: R
	A09/347	A09/347.1	AMC: I
	A09/371	A09/371.1	AMC: I; Sp: R
		A09/371.2	AMC: I; Sp: R
		A09/371.3	Sp: R
	A09/386	A09/386.1	AMC: I; Sp: R
	A10/42	A10/42.1	/
	C09/73	C09/73.1	Sp: R
	C09/149	C09/149.1	AMC, Sp: R
		C09/149.2	AMC: I
		C09/149.3	Sp: R

AMC, amoxicillin/acid clavulanic; C, ceftiofur; FF, florfenicol; Sp, spectinomycin; TTC, tetracycline; R, resistant; and I, intermediate.

Discussion

Because wild ruminants can represent a source of EHEC or VTEC infection for humans, and possibly also for domestic ruminants (Pierard *et al.* 1997; Simpson 2002; Miko *et al.* 2009), the purpose of this study was to determine the prevalence of EHEC, VTEC and EPEC strains in free-ranging wild ruminants in Belgium during two consecutive hunting seasons. Unlike previous studies (Fischer *et al.* 2001; Renter *et al.* 2001; Kemper *et al.* 2006; Heuvelink *et al.* 2008), we did not focus on the O157:H7 EHEC serotype. The overall prevalence rate of healthy carriers of

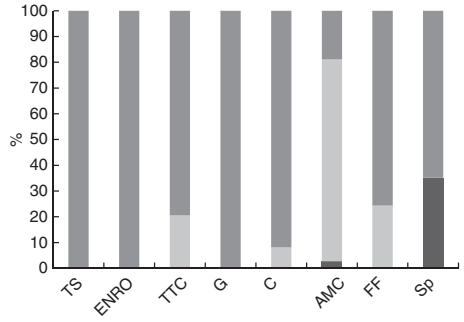

Figure 1 Representation of the percentage of sensitive, intermediate and resistant strains to the tested antibiotics. AMC, amoxicillin/acid clavulanic; C, ceftiofur; ENRO, enrofloxacin; FF, florfenicol; G, gentamicin; Sp, spectinomycin; TS, trimethoprim/sulfamethoxasol; and TTC, tetracycline. (▩) Sensitive; (▩) intermediate and (▩) resistant.

EPEC, VTEC and EHEC strains among wild cervids in the south of Belgium was found to be 15%, or 20 of 133 animals. Of these 20 animals, four were carriers of EPEC, 15 of VTEC and one animal a carrier of EPEC and VTEC; none of the animals was found to carry an EHEC strain. To our knowledge, this is the first such broad study in Belgium, and only a few such studies have been performed worldwide (Asakura *et al.* 1998; Fukuyama *et al.* 1999; Sanchez *et al.* 2009).

In 2009, Sánchez *et al.* found a prevalence of 23·9% of VTEC *sensu stricto* strains in wild ruminants in Spain. This study as well as ours suggested that the prevalence of VTEC and EPEC strains in wild ruminants can be high. Moreover, our technical approach (picking three lactose-fermenting colonies for each faecal sample) lacks sensitivity, and the 'real' prevalence is probably higher. Therefore, both studies suggest that prevalence studies should not focus solely on O157:H7 EHEC (Fischer *et al.* 2001; Renter *et al.* 2001; Kemper *et al.* 2006; Heuvelink *et al.* 2008). Such restricted studies are interesting in demonstrating a potential source of O157:H7 EHEC infection for humans. However, they tend to underestimate the potential risk of transmission of other VTEC, EHEC and EPEC strains from wild ruminants to humans via the consumption of venison and to domestic ruminants owing to the sharing of the same pasture. Indeed, many serogroups other than O157 EHEC have also been shown to be responsible for outbreaks in humans in several countries (Bettelheim 2007; Miyajima *et al.* 2007), and about one quarter of confirmed EHEC/VTEC cases in humans are caused by untyped or untypeable strains (Anonymous 2010). In some countries, strains that do not belong to the 'gang of five' (O157, O26, O111, O103 and O145) have been shown to be responsible for a high proportion of human cases (e.g. 66% in Germany and 44% in Sweden) (Anonymous 2010). It is also worth noting that 11 (69%) of the wild cervids in the present study were found to carry VTEC strains that are PCR positive for a gene encoding one Stx2 toxin, of which two variants (Stx2 and Stx2c) are the most potent in humans and are frequently responsible for HUS (Bonnet *et al.* 1998).

The other typing results of the 31 VTEC isolates were negative for the *subA* and *saa* gene (as was demonstrated in (Sanchez *et al.* 2009) for the *saa* gene), and only one isolate was positive for the *EHEC-hlyA* (unlike in (Sanchez *et al.* 2009). However, eight isolates from four adult cervids (A09/337, A09/378, A09/313 and C09/149) were found here to harbour an *eibG-like* gene, called *eibH*, which is 88% homologous to the published *eibG* gene sequence (AB255744). To date, the *eibG* gene has only been found in human VTEC *sensu stricto* strains (Lu *et al.* 2006). This new variant is perhaps specific to wild and/or domestic ruminants. To confirm this hypothesis, the distribution of the *eibG-like* gene needs to be studied in other VTEC strains from wild and domestic ruminants, from humans and from other potential reservoirs (wild and domestic pigs, birds, cats, dogs, etc.).

All the five EPEC isolates identified in the present study are 'atypical EPEC' or a-EPEC, because they do not harbour any *bfpA* genes. This is in full agreement with the observation that Bfp is only produced in human strains, with the exception of a few strains from dogs and cats (Goffaux *et al.* 2000; Chen and Frankel 2005). Nevertheless, current studies suggest that a-EPEC strains are emerging pathogens that are becoming even more frequent than the typical EPEC strains in humans in both developing and developed countries (Hernandes *et al.* 2009). If this is confirmed in the future, we cannot exclude animal EPEC strains as potential zoonotic pathogens.

As expected, the results of the antibiotic sensitivity testing of the 37 VTEC and EPEC isolates showed a much lower sensitivity in comparison with *E. coli* isolates from cattle (Martin *et al.* 2007; Srinivasan *et al.* 2007). Indeed, in the present study, only 21·6% of the isolates isolated from wild ruminants were found to be resistant (intermediate or resistant) to more than two antibiotics. Moreover, these isolates did not show a strong resistance to the antibiotics; most of them were intermediate to the antibiotics. Additionally, some *E. coli* isolated from the same animal presented different antibiotic susceptibility profiles. This could be explained by the fact that these isolates (intermediate or resistant) are at the limit of being sensitive. The fact that wild animals are normally not in close contact with antibiotics could explain their low level of resistance (Thaller *et al.* 2010). The remaining

antibiotic resistance level could be explained by: (i) the exposure to antibiotics or related antimicrobial chemicals in their food intake (through complementary food in winter); (ii) the transmission of resistant strains from cattle to wild ruminants because of their close contact; and/or (iii) the persistence of antibiotics in the environment.

More similar studies need to be performed on wild and farmed cervids in different countries, and future research should also focus on: (i) collecting more data on carrier prevalence of non-O157 EHEC, of VTEC and of a-EPEC; (ii) evaluating the pathogenic potential of these strains in wild cervids, if any; (iii) analysing the risk of transmission to humans and to domestic ruminants; but also (iv) studying the possible contamination of wild cervids by domestic ruminants.

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4. Distribution of already described adherence factors

4.1. Preamble

During the last ten years, many new adhesins have been described in EPEC, EHEC and VTEC strains, more especially after whole genome sequencing of different strains (Low *et al.*, 2006). Additionally, a few of them are host specific for human or animal isolates (e.g. BFP and human t-EPEC strains, F18 and porcine VTEC strains, AF/R1, AF/R2, RAL and Rabbit EPEC strains). Moreover, Tir/Intimin mechanism explanations have evolved over the last few years, Bono *et al.* has revealed the association of some polymorphisms with isolate sources (bovine or human) (Bono *et al.*, 2007). With our objective in mind, namely to study the host specificity that potentially exists in well-known adherence factors (adhesins and Tir/Intimin), we performed two experiments:

1) We studied the distribution of 27 putative adhesins in O26 EPEC and EHEC strains, isolated from bovines and humans, in order to ascertain whether some are host specific (Article 4);
2) We studied polymorphisms of *eae*, *tir* and *tccP2* genes existing in O26 EPEC and EHEC strains, isolated from bovines and humans, to determine whether some are host specific (Article 5).

4.2. Article 4: "Putative adhesins of enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *Escherichia coli* of serogroup O26 isolated from humans and cattle"

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Putative Adhesins of Enteropathogenic and Enterohemorrhagic *Escherichia coli* of Serogroup O26 Isolated from Humans and Cattle▽

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Enterohemorrhagic *Escherichia coli* (EHEC) strains are responsible for food poisoning in developed countries via consumption of vegetal and animal food sources contaminated by ruminant feces, and some strains (O26, O111, and O118 serogroups) are also responsible for diarrhea in young calves. The prevalence of 27 putative adhesins of EHEC and of bovine necrotoxigenic *E. coli* (NTEC) was studied with a collection of 43 bovine and 29 human enteropathogenic (EPEC) and EHEC strains and 5 non-EPEC/non-EHEC (1 bovine and 4 human) O26 strains, using specific PCRs. Four "groups" of adhesins exist, including adhesins present in all O26 strains, adhesins present in most O26 strains, adhesins present in a few O26 strains, and adhesins not present in O26 strains. The common profile of EHEC/EPEC strains was characterized by the presence of *loc3*, *loc5*, *loc7*, *loc11*, *loc14*, *paa*, *efa1*, *iha*, $lpfA_{O26}$, and $lpfA_{O113}$ genes and the absence of *loc1*, *loc2*, *loc6*, *loc12*, *loc13*, *saa*, and *eibG* genes. Except for the $lpfA_{O26}$ gene, which was marginally associated with bovine EHEC/EPEC strains in comparison with human strains ($P = 0.012$), none of the results significantly differentiated bovine strains from human strains. One adhesin gene (*ldaE*) was statistically ($P < 0.01$) associated with O26 EHEC/EPEC strains isolated from diarrheic calves in comparison with strains isolated from healthy calves. *ldaE*-positive strains could therefore represent a subgroup possessing the specific property of producing diarrhea in young calves. This is the first time that the distribution of putative adhesins has been described for such a large collection of EHEC/EPEC O26 strains isolated from both humans and cattle.

Enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *Escherichia coli* strains represent two important classes of enteric pathogens that cause diarrhea in humans and animals. They have in common the ability to produce a histopathological lesion on enterocytes, called an "attaching and effacing" lesion. The intimate attachment of the bacteria to enterocytes and the localized effacement of microvilli are the main characteristics of the attaching and effacing lesion (26).

EPEC strains are an important cause of infant diarrhea in developing countries and are often associated with high mortality rates (8). Human EPEC strains are subdivided into classical (type 1) and nonclassical (type 2) strains on the basis of the production of bundle-forming pili or the presence of the encoding genes. Nonclassical EPEC strains are also present in different animal species. In bovines, nonclassical EPEC strains are associated with diarrhea in young calves of up to 3 months of age (9).

EHEC strains are considered to have evolved from EPEC strains through the acquisition of bacteriophages encoding Shiga toxins (Stxs) (31, 45). EHEC strains cause several clinical syndromes in humans (mainly in children and elderly people), such as diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura. These have been responsible for large outbreaks in many developed countries, especially Japan, the United States, and the United Kingdom (26). Transmission can occur via consumption of vegetal and animal foodstuffs contaminated by ruminant feces (mainly cattle) (7). Some EHEC strains are also responsible for undifferentiated diarrhea in young calves of up to 3 months of age (24).

EPEC and EHEC strains can belong to more than 1,000 O:H serotypes. In EHEC infections, O157:H7 is the main serotype responsible for several outbreaks and sporadic cases of hemorrhagic colitis and hemolytic-uremic syndrome, but non-O157 serogroups (such as O26, O145, O111, and O103) can also be associated frequently with severe illness in humans (5, 35). Though most, if not all, EHEC serogroups are carried by healthy animal ruminants, a few are associated with diarrhea in calves (O5, O26, O111, O118, etc.). Human and animal EPEC strains also belong to a series of O antigenic groups, including O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (6). Thus, several serogroups are present in both pathotypes (EHEC and EPEC) and can infect both humans and cattle. Although classical EPEC strains have always been regarded as host specific, EHEC strains have not, and the actual situation regarding nonclassical EPEC strains remains unknown.

The first step in EPEC and EHEC infection is the initial adherence of bacteria to intestinal cells. This adherence step could be the basis for any host specificity via the production of colonization factors, such as the bundle-forming pilus adhesin of classical human EPEC strains.

Low et al. analyzed 14 putative fimbrial gene clusters revealed by the EHEC O157:H7 Sakai sequence (21). Of these 14 putative fimbriae, several had already been described under other names, including LpfA1 (42), LpfA2 (43), F9 (20), type 1 fimbriae (32) (34), and curli fimbriae (30). Long polar fimbria (Lpf)-encoding genes had also been described previously, including $lpfA_{O26}$ and $lpfA_{O113}$, described by Toma et al. (41) and Doughty et al. (12), respectively.

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In addition, other putative adhesins have been described, as follows: a 67-kDa adherence-conferring protein (Iha) similar to *Vibrio cholerae* IrgA confers the capacity to adhere to epithelial cells in a diffuse pattern (38); Efa1 (EHEC factor for adherence), described by Nicholls et al. (27), mediates the binding of bacteria to CHO cells in vitro; ToxB, a protein encoded by a gene located on the 93-kb pO157 plasmid, is required for full adherence of the EHEC O157:H7 Sakai strain (39); Saa is an autoagglutinating adhesin identified in locus of enterocyte effacement-negative verotoxigenic *E. coli* strains (29); EibG is a protein responsible for the chain-like adherence phenotype of Saa-negative verotoxigenic *E. coli* strains (22); Paa (porcine attaching and effacing-associated) adhesin, described by An et al. (1), is involved in the early steps of the adherence mechanism of porcine EPEC strains (2); and the hemorrhagic coli pilus (HCP), whose inactivation of the main subunit (*hcpA* gene) reduces adherence to cultured human intestinal and bovine renal epithelial cells and to porcine and bovine gut explants, was observed in EHEC O157:H7 (46).

The aim of this study was to establish the prevalence in bovine and human EPEC and EHEC strains belonging to the O26 serogroup of a total of 23 putative adhesins previously described for EHEC strains and of four fimbrial and afimbrial adhesins associated with bovine necrotoxigenic *E. coli* (NTEC) (36). The presence of these genes was correlated, on the one hand, with the source of isolation, and on the other hand, with EHEC/EPEC virulence factors (*eae*, stx_1, stx_2, and EHEC *hlyA*).

MATERIALS AND METHODS

Bacterial strains. A total of 77 strains of serogroup O26 isolated in the United States, Ireland, Belgium, France, Japan, and Brazil were studied (Table 1) and included 5 non-EPEC/non-EHEC strains, 28 EPEC strains, and 44 EHEC strains. Forty-four strains were isolated from the feces or intestines of young calves (1 non-EPEC/non-EHEC, 18 EPEC, and 25 EHEC strains), and 33 strains were isolated from humans (4 non-EPEC/non-EHEC, 10 EPEC, and 19 EHEC strains). Most of the strains had been described previously (37), but their pathotype (EPEC or EHEC) and serotype O26:H11 status were confirmed by PCR for the stx_1, stx_2, *eae*, EHEC *hlyA*, $wzx\text{-}wzy_{O26}$, and $fliC_{H11}$ genes (Table 1) (10, 13, 15).

The following positive controls were used: strains EH017 and EH383 (for the 14 putative fimbrial gene clusters, *toxB* gene, *paa* gene, *iha* gene, and *hcpA* gene), strain 239KH89 (for the *afa8-E* gene), strain 25KH9 (for the *f17A* gene), and strain 31A (for the *clpG* and *clpE* genes). The nonpathogenic strain HS (O9:H4) was used as a negative control.

PCR. All primers used in this study are listed in Table 2. DNA templates were prepared by boiling, as previously described (10). For PCRs, the following mixture was used: 1 U of *Taq* DNA polymerase (New England Biolabs), 5 μl of a mixture with a 2 mM concentration of each deoxynucleoside triphosphate, 5 μl of 10× ThermoPol reaction buffer [20 mM Tris-HCl (pH 8.8, 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100], 5 μl of each primer (10 μM), and 3 μl of DNA template in a total volume of 50 ml. All PCR conditions have been described previously (Table 2), and the annealing temperatures are listed in Table 2. Some PCRs were performed in duplicate to confirm the results.

DNA sequencing. The DNA fragments were purified using a NucleoSpin Extract II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Sequencing of the two DNA strands was performed by the dideoxynucleotide triphosphate chain termination method with an ABI 3730 capillary sequencer and a BigDye Terminator kit, version 3.1 (Applied Biosystems), at the Groupe Interdisciplinaire de Génoprotéomique Appliquée (University of Liège, Belgium). Sequence analysis was performed using Vector NTI 10.1.1 (Invitrogen).

Statistical analysis. Fisher's exact test was performed to assess statistical differences ($P < 0.01$).

RESULTS

Distribution of putative EPEC and EHEC adhesin-encoding genes. The distribution of putative EPEC and EHEC adhesin-encoding genes is shown in Table 3. The following genes were detected in the majority of the strains: *loc3* (in 100% of the strains), *loc14* (in 100% of the strains), *loc5* (in 99% of the strains), *loc7* (in 99% of the strains), *loc8* (in 83% of the strains), *loc11* (in 97% of the strains), *efa1* (in 92% of the strains), *toxB* (in 79% of the strains), *paa* (in 97% of the strains), *iha* (in 92% of the strains), $lpfA_{O26}$ (in 94% of the strains), and $lpfA_{O113}$ (in 95% of the strains).

In addition, a few strains were positive for some adhesins that have not yet been described for EPEC/EHEC O26 strains, including *hcpA* (four strains), *loc4* (two strains), *loc9* (three strains), and *loc10* (two strains). These amplicons were sequenced for further identification and comparison. The four *hcpA* amplicon sequences had 100% identity with the *hcpA* gene. The two *loc4* amplicon sequences were 69% identical to the *loc4* gene of the positive control and 100% identical to the *ybgD* gene of *Shigella*, coding for a putative fimbrial subunit-like protein. Nevertheless, the two usher protein-encoding genes (*loc4 usher1* and *loc4 usher2* genes) associated with the *loc4* fimbriae were not detected in those two strains. The three *loc9* amplicon sequences were identical to the amplicon sequence of the positive control. The two usher protein-encoding genes and the fimbrial subunit-encoding gene (*loc9 usher1*, *loc9 usher2*, and *loc9* fimbrial subunit genes) were also detected in the three strains by PCR. One of the two *loc10* amplicon sequences was identical to the *loc10* gene, whereas the second one had only 74% identity. The latter sequence was 99% identical to the *yfcV* gene, coding for a putative fimbrial subunit of *E. coli* UTI89. However, the two usher protein-encoding genes (*loc4 usher1* and *loc4 usher2* genes) associated with the *loc10* fimbriae were detected in neither strain. Three genes, *loc1*, *loc2*, and *loc6*, were not detected in any of the strains.

Distribution of putative NTEC adhesin-encoding genes. Fimbrial and afimbrial adhesins (Afa8-E, F17A, ClpG, and ClpE) associated with bovine NTEC strains had previously been used as search targets for 24 EHEC and EPEC strains of serogroup O26 (36). We extended the adhesin search to 77 strains. Four strains were *afa8-E* positive, 2 strains were *f17A* positive, 1 strain was *clpG* positive, and 18 strains (25%) were *clpE* positive. These amplicons were also further identified and compared after being sequenced. The *afa8-E* and *f17A* amplicon sequences were identical to the amplicon sequences of the respective positive controls. One *clpE* amplicon sequence was 100% identical to the *clpE* gene. The strain carrying this sequence was also positive for the *clpG* gene. The other 17 *clpE* amplicon sequences had 100% identity with each other, 90% identity with the *clpE* gene, and 100% identity with the *ldaE* gene, coding for the chaperone of the "locus for diffuse adherence," described by Scaletsky et al. (33). Eleven of the 17 strains carrying these sequences were also positive for the *ldaG* gene, coding for the main subunit of the locus for diffuse adherence.

Distribution of putative adhesins according to source of isolation (cattle or humans), geographical origin, and pathotype. Several adhesin-encoding genes (*loc8*, *loc11*, *efa1*, *toxB*, *iha*, $lpfA_{O26}$, $lpfA_{O113}$, and *ldaE*) were statistically associated

TABLE 1. Serotypes, sources of isolation, geographical origins, and pathotypes of tested strains

Strain	Host	Status of host[a]	Origin	EPEC or EHEC	Presence of adhesin gene or variant (for *stx*)				
					eae	*stx*	EHEC *hlyA*	*wzx-wzy*$_{O26}$	*fliC*$_{H11}$
TC3108	Cattle	Healthy	United States	EHEC	+	1	−	+	+
TC3109	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3117	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3180	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3269	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3273	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3302	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3305	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3375	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3380	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3629	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3630	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3631	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3632	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3656	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC3657	Cattle	Healthy	United States	EHEC	+	1	+	+	+
TC6169	Cattle	Diarrheic	United States	EHEC	+	1	+	+	+
4276	Cattle	Diarrheic	Ireland	EHEC	+	1	+	+	+
A39	Cattle	?	?	EHEC	+	1	+	+	+
357S89	Cattle	Diarrheic	Belgium	EHEC	+	1	+	+	+
379S89	Cattle	Diarrheic	Belgium	EHEC	+	1	+	+	+
122	Cattle	?	?	EHEC	+	1	+	+	+
63	Cattle	?	?	EHEC	+	1	+	+	+
A14	Cattle	?	?	EHEC	+	1	+	+	+
331S89	Cattle	Diarrheic	Belgium	EHEC	+	1	+	+	+
EH031	Human	Diarrheic	Belgium	EHEC	+	1	+	+	+
EH182	Human	Diarrheic	Belgium	EHEC	+	1	+	+	+
EH193	Human	Diarrheic	Belgium	EHEC	+	2	+	+	+
EH296	Human	Diarrheic	Belgium	EHEC	+	2	+	+	+
EH298	Human	Diarrheic	Belgium	EHEC	+	2	+	+	+
EH196	Human	Diarrheic	Belgium	EHEC	+	2	+	+	+
EH284	Human	Diarrheic	Belgium	EHEC	+	1	+	+	+
EH322	Human	Diarrheic	Belgium	EHEC	+	1	+	+	+
EH324	Human	Diarrheic	Belgium	EHEC	+	1	+	+	+
TC5710	Human	HUS	United States	EHEC	+	1 and 2	−	+	+
TC5711	Human	HUS	United States	EHEC	+	1	+	+	+
TC6168	Human	Diarrheic	United States	EHEC	+	1	−	+	+
02/113	Human	?	France	EHEC	+	1	+	+	+
99/109	Human	?	France	EHEC	+	2	+	+	+
03/151	Human	?	France	EHEC	+	1	+	+	+
03/139	Human	?	France	EHEC	+	1	+	+	+
99/145	Human	?	France	EHEC	+	1	+	+	+
99/147	Human	?	France	EHEC	+	1	+	+	+
11368	Human	Diarrheic	Japan	EHEC	+	1	+	+	+
TC1988	Human	?	Brazil	EPEC	+	−	+	+	+
TC3145	Cattle	Healthy	United States	EPEC	+	−	+	+	+
TC3486	Cattle	Healthy	United States	EPEC	+	−	+	+	+
TC3748	Cattle	Healthy	United States	EPEC	+	−	+	+	+
TC4004	Cattle	Healthy	United States	EPEC	+	−	+	+	+
TC4219	Cattle	Healthy	United States	EPEC	+	−	+	+	+
TC4221	Cattle	Healthy	United States	EPEC	+	−	+	+	+
TC3848	Cattle	Healthy	United States	EPEC	+	−	+	+	+
TC659	Cattle	Diarrheic	United States	EPEC	+	−	+	+	+
333KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	+	+	+
334KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	−	+	+
351KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	−	+	+
352KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	+	+	+
631KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	−	+	+
C15333	Cattle	?	?	EPEC	+	−	−	+	+
331KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	−	+	+
335KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	−	+	+
355KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	−	+	+
354KH91	Cattle	Diarrheic	Belgium	EPEC	+	−	−	+	+
TC6165	Human	Diarrheic	United States	EPEC	+	−	+	+	+
TC6166	Human	Diarrheic	United States	EPEC	+	−	+	+	+
TC6167	Human	Healthy	United States	EPEC	+	−	−	+	+
03/178	Human	?	France	EPEC	+	−	+	+	+
00/054	Human	?	France	EPEC	+	−	+	+	+
00/106	Human	?	France	EPEC	+	−	−	+	+
00/113	Human	?	France	EPEC	+	−	−	+	+
02/145	Human	?	France	EPEC	+	−	−	+	+
02/057	Human	?	France	EPEC	+	−	−	+	+
T282	Cattle	Diarrheic	United States	Non-EPEC/non-EHEC	−	−	−	+	+
00/103	Human	?	France	Non-EPEC/non-EHEC	−	−	−	+	+
00/130	Human	?	France	Non-EPEC/non-EHEC	−	−	−	+	+
03/023	Human	?	France	Non-EPEC/non-EHEC	−	−	−	+	+
C4071	Human	?	France	Non-EPEC/non-EHEC	−	−	−	+	+

[a] HUS, hemolytic-uremic syndrome.

TABLE 2. Primers used in this study

Primer	Sequence (5′ to 3′)	Target	Annealing temp (°C)	Amplicon size (bp)	Reference
loc1-F	CGACAACGTTGATGTTTAGC	*loc1* main subunit	48	300–500	21
loc1-R	GCCTTTTGTAACAGGATTGC				
loc2-F	GGTATGCATAGCGTTACC	*loc2* main subunit	42	300–500	21
loc2-R	CTGCTGGCAAATCTTATGC				
loc3-F	GCGGTACAATTCACTTTGAAGG	*loc3* main subunit	53	300–500	21
loc3-R	CATTTGCTTGCCCTGCTGATGC				
loc4-F	GCCATATCTCTACTATTCGC	*loc4* main subunit	43	300–500	21
loc4-R	GTTATCCATCTGTTCCATCC				
loc5-F	CTGTGGTATGTGCAACGTCC	*loc5* main subunit	51	300–500	21
loc5-R	CCCCGTAGCGATATAATCAAC				
loc6-F	CCTACAGTCACTTTTCAGGG	*loc6* main subunit	44	300–500	21
loc6-R	GATTAATTAGAGGTAGCTCAGG				
loc7-F	CTTCATTTAATCAGGCAGCC	*loc7* main subunit	47	300–500	21
loc7-R	GAGTACCATACTGTGTAATATTTGC				
loc8-F	GGTGATGAATCAGTAACGACC	*loc8* main subunit	48	300–500	21
loc8-R	GTGCCATCAATCAAGTCGG				
loc9-F	CACCATGTACATTTGTCGC	*loc9* main subunit	46	300–500	21
loc9-R	CAGTACGTCACTGCTATCTCC				
loc10-F	GCTGCAACAATGGTAATGGG	*loc10* main subunit	53	300–500	21
loc10-R	GTAATCTGGAAGGTCGTGTTGGC				
loc11-F	CTTTTCGCAGGTAATGCCG	*loc11* main subunit	50	300–500	21
loc11-R	GATTTCGGATGCTTCAACG				
loc12-F	GTGGTATCGCAATCTTCC	*loc12* main subunit	42	300–500	21
loc12-R	GGTAAAGTAGAGAACCG				
loc13-F	GATTGTAGGAGCATTAGCG	*loc13* main subunit	45	300–500	21
loc13-R	CTATCGATCTGACTCAATGCC				
loc14-F	GTCGTTGCTGCCAATGTTTGC	*loc14* main subunit	48	300–500	21
loc14-R	GAAATGTAGCGAAGTAGAGCC				
LpfA-O26-F	GTT CTG TTT GCC TTA TCT GC	$lpfA_{O26}$	52	509	40, 41
LpfA-O26-R	TAA GTC AGG TTG AAG TCG AC				
LpfA-O113-F	ATGAAGCGTTAATATTATAG	$lpfA_{O113}$	50	573	28
LpfA-O113-R	TTATTTCTTATATTCGAC				
efa1-F	TAA GCG AGC CCT GAT AAG CA	*efa1*	55	630	17
efa1-R	CGT GTT GCT TGC CTT TGC				
toxB-F	ATACCTACCTGCTCTGGATTGA	*toxB*	55	602	41
toxB-R	TTCTTACCTGATCTGATGCAGC				
afa8-E-F	CTAACTTGCCATGCTGTGACAGTA	*afa8-E*	65	302	36
afa8-E-R	TTATCCCCTGCGTAGTTGTGAATC				
f17A-F	GCAGAAAATTCAATTTATCCTTGG	*f17A*	55	537	36
f17A-R	CTGATAAGCGATGGTGTAATTAAC				
clpE-F	GGTCAGGCCTGGGTGGACAATATC	*clpE*	58	240	36
clpE-R	GCGATAGAACAGTTTCAGCTTCGT				
clpG-F	GGGCGCTCTCTCCTTCAAC	*clpG*	55	403	36
clpG-R	CGCCCTAATTGCTGGCGAC				
paa-F	TCAGAACAATCTGCTCTGGCTA	*paa*	52	413	19
paa-R	CACGTAGTCTGGCGCTATTTC				
iha-F	CAAATGGCTCTCTTCCGTCAATGC	*iha*	59	925	36
iha-F	CAGGTCGGGGTTACCAAGT				
saa-F	CGTGATGAACAGGCTATTGC	*saa*	50	119	18
saa-R	ATGGACATGCCTGTGGCAAC				
eibG-F	ATCGGCTTTCATCGCATCAGGAC	*eibG*	60	?	22
eibG-R	CCACAAGGCGGGTATTCGTATC				
B52	AGGCTTCGTCACAGTTG	*eaeA*	50	570	10
B53	CCATCGTCACCAGAGGA				
B54	AGAGCGATGTTACGGTTTG	*sltI*	50	388	10
B55	TTGCCCCCAGAGTGGATG				
B56	TGGGTTTTTCTTCGGTATC	*sltII*	50	807	10
B57	GACATTCTGGTTGACTCTCTT				
EHEC-hlyA-F	ACGATGTGGTTTATTCTGGA	EHEC *hlyA*	58	165	15
EHEC-hlyA-R	CTTCACGTGACCATACATAT				
wzx-wzyO26-F	AAATTAGAAGCGCGTTCATC	$wzx\text{-}wzy_{O26}$	56	596	13
wzx-wzyO26-R	CCCAGCAAGCCAATTATGACT				
fliC-H11-F	ACTGTTAACGTAGATAGC	$fliC_{H11}$	56	224	13
fliC-H11-R	TCAATTTCTGCAGAATATAC				
G98-F	TCGCTAGTTGCTGACAGATTT	*hcpA*	49	?	46
G99-R	AATGTCTGTTGTGTGCGACTG				
ldaG-F	ATGAAAAAGACACTATTAGCACTGG	*ldaG*	48	?	33
ldaG-R	TGAATCTCCCAGCCAAAA				
loc4U1-F	CCTCTATGTCGCACCACAC	*loc4 usher1*	48	300–500	21
loc4U1-R	GTCGGCGTCATTGTATTTG				
loc4U2-F	GCTCAATCAAAAAGGCGTC	*loc4 usher2*	46	300–500	21
loc4U2-R	CCGTTATTCCACGTTGAG				
loc9U1-F	GGATAATAATCCTGGTGAGTG	*loc9 usher1*	46	300–500	21
loc9U1-R	CACTTTGCTTGCTCGCAC				
loc9U2-F	GGAACCGCGATCAGTTTA	*loc9 usher2*	46	300–500	21
loc9U2-R	GCTGTCCATCGGATCTTA				
loc9fim-F	GCATTGAAGTTTAACTGGTG	*loc9* fimbrial subunit	44	300–500	21
loc9fim-R	CCTGGTTTATCTGTTTTCC				
loc10U1-F	GATGACGATGTTATCAACGG	*loc10 usher1*	48	300–500	21
loc10U1-R	GAAGAAACCGCCTTCCAC				
loc10U2-F	GGAGTTATGTCAATGCCT	*loc10 usher2*	42	300–500	21
loc10U2-R	GGATCCCAGTTGATGTCG				

TABLE 3. Distribution of putative adhesin genes

Strain type	No. of strains tested	No. (%) of strains positive for gene by PCR												
		loc1	*loc2*	*loc3*	*loc4*	*loc5*	*loc6*	*loc7*	*loc8*	*loc9*	*loc10*	*loc11*	*loc12*	*loc13*
Total	77	0 (0)	0 (0)	77 (100)	2 (3)	76 (99)	0 (0)	76 (99)	64 (83)	3 (4)	2 (3)	75 (97)	0 (0)	0 (0)
Bovine strains	44	0 (0)	0 (0)	44 (100)	1 (2)	43 (98)	0 (0)	43 (98)	38 (86)	2 (5)	1 (2)	44 (100)	0 (0)	0 (0)
Human strains	33	0 (0)	0 (0)	33 (100)	1 (3)	33 (100)	0 (0)	33 (100)	26 (79)	1 (3)	1 (3)	31 (94)	0 (0)	0 (0)
stx_1-positive strains	38	0 (0)	0 (0)	38 (100)	1 (3)	38 (100)	0 (0)	37 (97)	30 (79)	2 (5)	0 (0)	38 (100)	0 (0)	0 (0)
stx_2-positive strains	5	0 (0)	0 (0)	5 (100)	0 (0)	5 (100)	0 (0)	5 (100)	4 (80)	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)
stx_1- and stx_2-positive strains	1	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)
EPEC strains	28	0 (0)	0 (0)	28 (100)	0 (0)	27 (96)	0 (0)	28 (100)	27 (96)	0 (0)	1 (4)	28 (100)	0 (0)	0 (0)
Non-EPEC/non-EHEC strains	5	0 (0)	0 (0)	5 (100)	1 (20)	5 (100)	0 (0)	5 (100)	2 (40)	1 (20)	0 (0)	3 (600)	0 (0)	0 (0)
EHEC strains	44	0 (0)	0 (0)	44 (100)	1 (2)	44 (100)	0 (0)	43 (98)	35 (80)	2 (5)	1 (2)	44 (100)	0 (0)	0 (0)
Belgian strains	21	0 (0)	0 (0)	21 (100)	0 (0)	20 (95)	0 (0)	21 (100)	17 (81)	0 (0)	1 (5)	21 (100)	0 (0)	0 (0)
Brazilian strains	1	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
French strains	16	0 (0)	0 (0)	16 (100)	1 (6)	16 (100)	0 (0)	16 (100)	12 (75)	1 (6)	0 (0)	14 (88)	0 (0)	0 (0)
Irish strains	1	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Japanese strains	1	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
American strains	32	0 (0)	0 (0)	32 (100)	0 (0)	32 (100)	0 (0)	32 (100)	27 (84)	1 (3)	1 (3)	32 (100)	0 (0)	0 (0)
Strains of unknown origin	5	0 (0)	0 (0)	5 (100)	1 (20)	5 (100)	0 (0)	4 (80)	5 (100)	1 (20)	0 (0)	5 (100)	0 (0)	0 (0)
European strains	38	0 (0)	0 (0)	38 (100)	1 (3)	37 (97)	0 (0)	38 (100)	30 (79)	1 (3)	1 (3)	36 (95)	0 (0)	0 (0)
Diarrheic bovine strains	16	0 (0)	0 (0)	16 (100)	0 (0)	15 (94)	0 (0)	16 (100)	15 (94)	1 (6)	1 (6)	16 (100)	1 (6)	0 (0)
Healthy bovine strains	23	0 (0)	0 (0)	23 (100)	0 (0)	23 (100)	0 (0)	23 (100)	18 (78)	0 (0)	0 (0)	23 (100)	0 (0)	0 (0)

with the EHEC/EPEC strains in comparison with the non-EPEC/non-EHEC strains ($P < 0.01$), and the $lpfA_{O26}$ gene was marginally statistically associated with bovine EHEC/EPEC strains in comparison with human strains ($P = 0.012$). Moreover, the *ldaE* gene was statistically associated with the bovine EHEC/EPEC strains isolated from diarrheic calves in comparison with the other bovine strains ($P < 0.01$) but not in comparison with the human EHEC/EPEC strains. The *f17A*, *afa8-E*, and *clpG*/*clpE* genes were detected only in the EPEC strains isolated from diarrheic calves in Belgium, though this association was not statistically significant due to the small number of positive strains. For the other adhesins, no relationship was observed between the source (cattle or humans), the geographical origin, or the pathotype of the strains and their prevalence.

DISCUSSION

Studies on the prevalence of putative EHEC adhesins have focused mostly on O157:H7 strains and more rarely on a few non-O157 strains. However, non-O157:H7 serogroups (such as O26, O145, O111, and O103) are frequently associated with severe illness in humans (5), and in many countries, O26 strains are the second most prevalent serogroup of EHEC strains (4). Moreover, O26 strains possess the particularity of producing disease in both humans and calves (23). Evidence also exists that human and bovine EHEC O26 strains are heterogeneous, leading to the hypothesis that at least some of them may be host specific. The step involving the initial adherence of bacteria to intestinal cells could be the basis of such host specificity, as is the case with other pathogenic *E. coli* strains and virulence factors, such as F18a of porcine verotoxigenic *E. coli*, AF/R1 and AF/R2 of rabbit enteropathogenic *E. coli*, F4 and F6 of porcine enterotoxigenic *E. coli*, etc. (16, 25). Therefore, 77 EHEC and EPEC O26 strains recovered from different sources (human or bovine) in different countries were tested by PCR for the presence of genes coding for 27 putative adhesins previously described or used as search targets for EHEC and EPEC strains (these previous attempts had either not involved O26 strains or used only a limited number of those strains). This is the first time that the distribution of so many putative adhesin-encoding genes has been described for such a large collection of EPEC/EHEC O26 strains.

According to the PCR results, the following four "groups" of adhesin genes exist: adhesin genes present in all O26 strains (*loc3* and *loc14*), adhesin genes present in most O26 strains (*loc5*, *loc7*, *loc8*, *loc11*, *efa1*, *toxB*, *paa*, *iha*, $lpfA_{O26}$, and $lpfA_{O113}$), adhesin genes present in a few O26 strains (*loc4*, *loc9*, *loc10*, *afa8-E*, *f17A*, *ldaE*, *clpE*/*clpG*, and *hcpA*), and adhesin genes not present in O26 strains (*loc1*, *loc2*, *loc6*, *loc12*, *loc13*, *saa*, and *eibG*). The common adhesin profile of EHEC/EPEC O26 strains is therefore characterized by the presence of *loc3*, *loc5*, *loc7*, *loc11*, *loc14*, *paa*, *efa1*, *iha*, $lpfA_{O26}$, and $lpfA_{O113}$ genes and the absence of *loc1*, *loc2*, *loc6*, *loc12*, *loc13*, *saa*, and *eibG* genes. Interestingly, the *loc8*, *loc11*, *afa8-E*, *f17A*, *ldaE*, *efa1*, *toxB*, *iha*, $lpfA_{O26}$, and $lpfA_{O113}$ genes were more frequent in EHEC/EPEC strains than in other strains. Also, several strains were found to be positive for some adhesin genes that have so far not been described for EPEC/EHEC O26 strains, such as *hcpA*, *loc4*, *loc9*, *loc10*, *afa8-E*, *f17A*, and *clpE*/*clpG*.

Nevertheless, none of the adhesins studied was significantly associated with bovine or human strains ($P > 0.01$). On the other hand, the *ldaE* gene was found to be statistically associated with EHEC/EPEC O26 strains isolated from diarrheic calves in comparison with strains isolated from healthy calves. These *ldaE*-positive strains may therefore represent a subgroup possessing the specific property of producing diarrhea in young calves (without presuming their capacity to cause disease in humans).

Since not all EHEC/EPEC strains isolated from diarrheic calves are positive for *ldaE*, the capacity of the other strains to cause diarrhea in young calves must be based upon another property, such as (i) other, rarer adhesins (*afa8-E*, *f17A*, *clpG*/*clpE*, etc.); (ii) the existence of differences in the sequences of genes coding for some adhesins present in human and bovine strains, resulting in host and tissue tropism, as already described for other families of fimbrial (P family) and afimbrial (AFA family) adhesins (3, 16), which can be detected only after sequencing of the whole encoding genes; (iii) variation in

TABLE 3—*Continued*

No. (%) of strains positive for gene by PCR													
loc14	*efa1*	*toxB*	*afa8-E*	*f17A*	*ldaE*	*clpE/clpG*	*paa*	*iha*	*saa*	*eibG*	$lpfA_{O26}$	$lpfA_{O113}$	*hcpA*
77 (100)	71 (92)	61 (79)	4 (5)	2 (3)	17 (22)	1 (1)	75 (97)	71 (92)	0 (0)	0 (0)	72 (94)	73 (95)	4 (5)
44 (100)	43 (98)	37 (84)	4 (9)	2 (5)	11 (25)	1 (2)	43 (98)	43 (98)	0 (0)	0 (0)	44 (100)	43 (98)	3 (7)
33 (100)	28 (85)	24 (73)	0 (0)	0 (0)	6 (18)	0 (0)	32 (97)	28 (85)	0 (0)	0 (0)	28 (85)	30 (91)	1 (3)
38 (100)	38 (100)	36 (95)	0 (0)	0 (0)	3 (8)	0 (0)	38 (100)	38 (100)	0 (0)	0 (0)	38 (100)	38 (100)	1 (3)
5 (100)	5 (100)	5 (100)	0 (0)	0 (0)	0 (0)	0 (0)	5 (100)	5 (100)	0 (0)	0 (0)	5 (100)	5 (100)	0 (0)
1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)
28 (100)	27 (96)	19 (68)	4 (14)	2 (7)	14 (50)	1 (4)	27 (96)	26 (93)	0 (0)	0 (0)	27 (96)	27 (96)	2 (7)
5 (100)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	4 (80)	1 (20)	0 (0)	0 (0)	1 (20)	2 (40)	1 (20)
44 (100)	44 (100)	41 (93)	0 (0)	0 (0)	3 (7)	0 (0)	44 (100)	44 (100)	0 (0)	0 (0)	44 (100)	44 (100)	1 (2)
21 (100)	21 (100)	16 (76)	4 (19)	2 (10)	7 (33)	1 (5)	20 (95)	20 (95)	0 (0)	0 (0)	21 (100)	20 (95)	3 (14)
1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)
16 (100)	11 (69)	9 (56)	0 (0)	0 (0)	3 (19)	0 (0)	15 (94)	11 (69)	0 (0)	0 (0)	11 (69)	13 (81)	1 (6)
1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)
1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)
32 (100)	31 (97)	29 (91)	0 (0)	0 (0)	3 (9)	0 (0)	32 (100)	32 (100)	0 (0)	0 (0)	32 (100)	32 (100)	0 (0)
5 (100)	5 (100)	4 (80)	0 (0)	0 (0)	4 (80)	0 (0)	5 (100)	5 (100)	0 (0)	0 (0)	5 (100)	5 (100)	0 (0)
38 (100)	33 (87)	26 (68)	4 (11)	2 (5)	10 (26)	1 (3)	36 (95)	32 (84)	0 (0)	0 (0)	33 (87)	34 (89)	0 (0)
16 (100)	15 (94)	11 (69)	4 (25)	2 (13)	7 (44)	1 (6)	15 (94)	15 (94)	12 (75)	0 (0)	16 (100)	15 (94)	3 (19)
23 (100)	23 (100)	22 (96)	0 (0)	0 (0)	0 (0)	0 (0)	23 (100)	23 (100)	19 (83)	0 (0)	23 (100)	23 (100)	0 (0)

the expression of some adhesin-encoding genes according to the growth environment (bovine or human intestine, intestinal segment, age of the host, etc.), as observed for other genes (11); or (iv) properties other than adherence, such as intermediate metabolism, which allows bacteria to be better adapted to a bovine intestinal environment, such as the young calf intestine (14, 44).

In conclusion, the answer to the question of host specificity of bovine and human EHEC/EPEC O26 strains may simply be that several subgroups of strains exist depending on the presence or absence of one or several properties allowing the pathogens to colonize (or hampering them from doing so) one specific host intestine (young calf, adult cattle, and/or human intestine) and allowing them to cause diarrhea. Only adherence experiments with enterocytes from humans and bovines and/or in vivo challenge of young calves with wild-type strains and mutants would bring final answers to these questions.

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4.3. Article 5: "Study of polymorphisms in *tir* and *eae* genes in enterohaemorrhagic and enteropathogenic *Escherichia coli* of serogroup O26"

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Study of polymorphisms in *tir*, *eae* and *tccP2* genes in enterohaemorrhagic and enteropathogenic *Escherichia coli* of serogroup O26

Marjorie Bardiau, Sabrina Labrozzo and Jacques G Mainil*

Abstract

Background: Enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* are responsible for food poisoning (enteritis and enterotoxaemia) in humans in developed countries. Cattle are considered to be an important reservoir of EHEC and EPEC strains for humans. Moreover, some of the strains, belonging to the O26, O111, O118 serogroups, for example, are also responsible for digestive disorders in calves. The Translocated intimin receptor (Tir), the intimin (Eae) and the Tir-cytoskeleton coupling protein (TccP) represent three virulence factors implicated in the intimate attachment of the bacteria to the eukaryotic cell. Major variants have already been described for these genes among the different serogroups but minor variations have not often been studied. In this study, we examined the polymorphisms of the *tir*, *eae* and *tccP2* genes of O26 strains (EPEC and EHEC isolated from bovines and from humans) with the aim to determine whether these polymorphisms are host specific or not.

Results: Of the 70 tested strains, 10 strains (14% of the strains) presented one or several polymorphisms in the *tir* and *eae* genes, which have never previously been described. Concerning *tccP2* detection, 47 of the 70 strains (67% of the strains) were found to be positive for this gene. Most of the strains were found to possess *tccP2* variants described in strains of serogroup O26. Nevertheless, three strains had *tccP2* genes respectively described in strains of serogroup O111, O103 and O55. Moreover, none of the polymorphisms was statistically specific to the bovine or the human isolates. Nevertheless, the two major variants of *tccP2* were statistically associated with the pathotype (EPEC or EHEC).

Conclusions: In conclusion, *tir* and *eae* gene polymorphisms were found not to be numerous and not to be predominantly synonymous. Moreover, no difference was observed between human and bovine strains regarding the presence of polymorphisms. Finally, some *tccP2* variants appeared to be pathotype specific. Further investigations need to be performed on a larger number of strains in order to confirm this specificity.

Background

Enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* represent two important classes of enteric pathogens. EPEC strains belonging to different serogroup (e.g. 026, 055, 086, 0111, O128) are a major cause of infant diarrhoea in many countries and are also associated with diarrhoea in most domestic animal species [1,2]. These strains can be classified into two groups: typical-EPEC strains (t-EPEC), harbouring a specific plasmid named EPEC Adherence Factor (EAF plasmid), and atypical-EPEC strains (a-EPEC), which do not carry this specific EAF plasmid. EHEC strains have been responsible for individual cases, and small to large outbreaks in developed countries [3-8]. O157:H7 is the main serotype responsible for human illness in several countries. Nevertheless non-O157 serogroups can also be associated frequently with severe disease in humans and O26 serogroup represent the second more important serogroup in Europe [9-11]. Syndromes caused in humans are diverse: undifferentiated diarrhoea, haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TP) [12]. Transmission often occurs via consumption of foodstuffs contaminated by faeces from ruminants (mainly cattle), which can be asymptomatic healthy carriers [13,14].

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Nevertheless, several serogroups of EHEC strains (e.g. O26, O111, O118) are also associated with diarrhoea in calves [15-18].

EPEC and EHEC share four stages in their pathogenicity: (1) colonisation of the intestine by specific adhesins, (2) translocation of a signal into the enterocyte by the type III secretion system (T3SS) of the bacteria and integration of the Translocated intimin receptor (Tir) into the host cell membrane, (3) intimate adhesion of bacteria to eukaryote cells by specific adhesins (intimins) that bind to Tir, and (4) actin polymerization after Tir phosphorylation. These four stages allow the bacteria to produce a specific lesion called an "attaching and effacing (A/E) lesion" [1]. Furthermore, as well as using the Tir phosphorylation pathway, some strains (EPEC 2 strains and the vast majority of non-O157 EHEC strains) are able to utilize the T3SS effector TccP2 (Tir-cytoskeleton coupling protein 2) to trigger actin polymerization, which leads to the formation of a pedestal characteristic of the A/E lesion [19].

Sequence variations in the Tir receptor-, intimin adhesin- and TccP2 effector-encoding genes (*tir*, *eae* and *tccp2*) have been described between EHEC and EPEC strains, and these can lead to major or minor polymorphisms (variants) of the encoded proteins [20-24]. Major variants of Tir and intimin are related, to some extent, to the serogroups of the EHEC and EPEC strains, whereas minor variants can exist within a serogroup for the same major variant, although these have not often been defined [25,26]. EHEC and EPEC strains belonging to the O26 serogroup classically produce the beta major variant of Tir and intimin, but their minor variants have not been studied [26,27]. Only two major variants of TccP have been described that are related to the pathotype of the strain [19]. EHEC and EPEC strains of O26 serogroup produce the TccP2 variant with six minor variants identified [23,24].

The purposes of this study were (1) to investigate the polymorphism of the *tir*, *eae* and *tccP2* genes between O26 EPEC and EHEC strains isolated from bovines and from humans; and (2) to determine whether these polymorphisms are specific to bovine or human strains.

Results

Detection of *tir*, *eae* and *tccP2* genes

All the tested strains of serogroup O26 were found to possess β type *eae* and *tir* genes. Moreover, of the 70 tested strains, 10 strains (14% of the strains) presented one or several polymorphisms in these two genes. None of the polymorphic strains possessed polymorphism in both *eae* and *tir* genes. Concerning *tccP2* detection, 47 of the 70 strains (67% of the strains) were positive for this gene. Most of the strains possessed *tccP2* variants described in strains of serogroup O26. Three strains had *tccP2* genes respectively described in strains of serogroup O111, O103 and O55.

Polymorphisms in the *eae* gene

For the *eae* gene, four polymorphisms were detected in nucleotide positions 255 (G > A), 1859 (C > T), 2415 (A > T) and 2772 (C > T) in *eae* β gene reference strain 14I3, (accession number FJ609815) and five unique *eae* β genotypes were defined (Table 1). The "classical" genotype (strain 14I3 sequence) was represented by 93% (65 +/70) of the strains and the four other genotypes were represented by only one or two strains. Even though there was no statistical significance ($p = 0.078$), all the strains that presented polymorphism were bovine EPECs. One polymorphism was non-synonymous and gave one genotype different in the amino-acid (AA) sequence: valine was coded in place of alanine in AA position 620. This AA is situated in the D0 Ig-like domain.

Polymorphisms in the *tir* gene

For the *tir* gene, five polymorphisms were detected in nucleotide positions 133 (T > G), 571 (insertion of GATACAAAG), 939 (G > A), 1080 (G > T) and 1302 (C > T) in *tir* β genes reference strain 95ZG1 (accession number AF070068) and four unique *tir* β genotypes were defined (Table 2). Interestingly, one polymorphism (position 939) was found to be present in all the strains. One genotype was represented by 93% (65+/70) of the strains, and the other three genotypes were represented by only one or two strains. Two polymorphisms were found to be non-synonymous and gave three different genotypes in the AA sequences: for the first polymorphism, serine was coded in place of alanine in AA position 45; for the second polymorphism, three AA (TKE) were inserted into AA position 191. These two polymorphisms were situated in the N-terminal part of the gene. Nevertheless, when we compared polymorphisms regarding the host and the pathotype (EPEC or EHEC), none was found to be specific to the bovine or the human isolates ($p < 0.05$) or to EPEC or EHEC pathotype.

Polymorphisms in the *tccP2* gene

For the *tccP2* gene, seven genotypes (Table 3) were detected in the collection. All had been previously described [23,24]. The *tccP2* variant described in reference strain 11368 (accession number AB253564) was found to be present in 34% (24+/70) of the strains. The *tccP2* variant described in reference strain EC38/99 (accession number AB275131) was present in 17% (12 +/70) of the strains. *tccP2* variants described in reference strains 12009 and CB00225 (accession number AB253581 and AB275122 respectively) were both

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Table 1 *eae* β gene polymorphism (aa: amino acid, A: alanine, V: valine)

	Number of strains			Polymorphism 1 (S) 255 G => A	Polymorphism 2 (NS) 1859 C => T 620 aa: A =>V	Polymorphism 3 (S) 2415 A => T	Polymorphism 4 (S) 2772 C => T
	Human	Bovine					
	0	1	Genotype 1	+	-	-	-
	0	2	Genotype 2	+	+	-	-
	0	1	Genotype 3	-	-	+	-
	0	1	Genotype 4	-	-	-	+
	28	37	Genotype 5	-	-	-	-
Total	**28**	**42**					

present in 6% (4+/70) of the strains. Three *tccP2* variants described in reference strains ED411, ED71 and 5905 (accession number AB253567, AB253576 and AB356001 respectively) were represented by only one strain each. None of the variants was found to be specific to the bovine or the human isolates ($p < 0.05$). Nevertheless, the two major variants were statistically associated with the pathotype ($p < 0.01$): the *tccP2* gene AB275131 was statistically associated with the EPEC strains in comparison with the EHEC strains and the *tccP2* gene AB253564 was statistically associated with the EHEC strains in comparison with the EPEC strains.

Discussion

The Tir receptor (encoded by the *tir* gene) and the intimin adhesin (encoded by the *eae* gene) are both implicated in the adherence of the EPEC and EHEC strains to eukaryotic cells via the binding of the intimin to the Tir receptor (previously translocated to the host cell). The A/E lesion is then produced and is characterized by the loss of microvilli and intimate attachment of the bacteria to the host cell. Moreover, non-O157 strains can utilize TccP2, as well as Tir, to trigger actin polymerization during the production of the A/E lesion [19]. There are variations in the *eae*, *tir* and *tccP2* gene sequence and many variants have been described [20-22]. Nevertheless small variations (polymorphisms) inside the same variants have not often been described. In 2007, Bono *et al.*[25] studied the polymorphism of *tir* and *eae* genes in O157 strains and associated two tir polymorphisms with the isolate source (bovine or human). With this in mind, we performed the present work to study the polymorphism of the *tir*, *eae* and *tccP2* genes existing in O26 EPEC and EHEC strains isolated from bovines and from humans with a view to determinate whether these polymorphisms are specific to bovine or human strains in the O26 serogroup.

tccP2 variants were found to be present in 67.1% of the tested strains. This is not surprising regarding the results obtained by Ooka *et al.* and Ogura *et al.*, who respectively found the *tccP2* gene in 82.3% of O26 a-EPEC strains and in 71.4% of O26 EHEC strains [23,24]. It is possible that the negative isolates use only the Tir phosphorylation pathway or that they utilize another unknown pathway. Moreover, the distribution of *tccP2* variants appears to be specific to the pathotype. In our study, *tccP2* variant (accession number AB253564) originally described in the O26 EHEC 11368 reference strain was found to be statistically associated to EHEC strains in our study and *tccP2* variant (accession number AB275131) originally described in O26 a-EPEC EC38/99 reference strain was found to be statistically associated

Table 2 *tir* β gene polymorphism (aa: amino acid, A: alanine, S: serine, T: threonine, K: lysine, E: glutamic acid)

	Number of strains			Polymorphism 1 (S) 1080 G => T	Polymorphism 2 (S) 1302 C => T	Polymorphism 3 (NS) 133 T => G 45 aa: S => A	Polymorphism 4 (NS) insertion1 571 GATACAAAG 191 aa: TKE	Polymorphism 5 (S) 939 G => A
	Human	Bovine						
	0	2	Genotype 1	+	-	-	-	+
	2	0	Genotype 2	-	+	-	+	+
	1	0	Genotype 3	-	-	+	-	+
	25	40	Genotype 4	-	-	-	-	+
Total	**28**	**42**						

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Table 3 *tccP2* gene polymorphism

Accession number of *tccP2* variant (variant described in serogroup)	Positive isolates in				
	Total	EHEC	EPEC	Bovine	Human
AB253564 (O26)	**24+/70**	21+/44	3+/26	17+/42	7+/28
AB275131 (O26)	**12+/70**	2+/44	10+/26	9+/42	3+/28
AB275122 (O26)	**4+/70**	0+/44	4+/26	4+/42	0+/28
AB253581 (O013)	**4+/70**	4+/44	0+/26	0+/42	4+/28
AB253567 (O26)	**1+/70**	1+/44	0+/26	0+/42	1+/28
AB253576 (O55)	**1+/70**	1+/44	0+/26	1+/42	0+/28
AB356001 (O111)	**1+/70**	0+/44	1+/26	0+/42	1+/28

to a-EPEC strains. However, further studies need to be performed in order to confirm this pathotype specificity. If this association appears to be confirmed, it could be used as a tool to study, among other things, O26 EPEC strains (isolated from patients or from calves) in order to determine if these strains are "real" O26 EPEC strains or O26 EHEC strains that have lost *stx* genes[28].

In comparison with O157 strains, O26 strains do not possess a large number of polymorphisms in the *tir* gene (only four different genotypes were revealed by our study and the major one was represented by 92.8% of the strains in comparison with ten different genotypes revealed by the study of Bono *et al.* with the major one represented by 68.6%). By contrast, *eae* polymorphisms are, in both studies, very limited. Bono *et al.* explained this difference in polymorphism frequency (between *eae* and *tir* genes) among O157 strains by the fact that both genes have evolved under a different kind of selective pressure. The difference in *tir* polymorphism frequency between O157 and O26 strains could also be explained by a different kind of selective pressure between both serogroups. Currently, we know that O157 EHEC strains and O26 EHEC and EPEC strains possess two different actin signalling pathways [19]. The O157 EHEC strains use only the TccP adaptor to induce actin polymerization and the O26 EHEC and EPEC strains can use two other pathways: the TccP2 adaptor and the phosphorylation of Y474 Tir residue. Therefore, it is not surprising that *tir* polymorphisms are more frequent in O157 EHEC strains than in O26 EHEC and EPEC strains.

Furthermore, the polymorphisms in *tir* and *eae* genes revealed by our study are mainly synonymous. For the *eae* gene, only one polymorphism was found to be non-synonymous (valine is coded in place of alanine in position 620) and this is situated in the D0 Ig-like domain. This polymorphism is not surprising and the consequences on the protein structure are probably nil for two reasons: firstly, in the *eae* ζ gene, valine is situated at this position and secondly, D0 is a divergent region that is not entirely conserved [29]. For the *tir* gene, two polymorphisms were found here to be non-synonymous and these are located near the amino terminus of Tir. This region is normally situated in the host cytosol after Tir translocation and is probably implicated in pedestral length, pedestral efficiency and translocation in the host cell [30].

Finally, concerning host specificity, in contrast to O157 strains [25], our study revealed that *tir* and *eae* polymorphisms are not associated with the host (human or bovine). In comparison to O157 strains, which seem to be host classifiable using nucleotide polymorphisms [31,32], we were unable to distinguish O26 strains. Several studies have suggested that O157 strains can be separated into two distinct lineages (lineages I and II), which appear to have distinct ecological characteristics, and which are associated with the host [33-36].

Conclusions

In conclusion, *tir* and *eae* genes of O26 EHEC and EPEC strains are well conserved. Polymorphisms are not numerous or predominantly synonymous. Moreover, no difference was observed between human and bovine strains regarding the presence of polymorphisms. Finally, *tccP2* variants appear to be pathotype specific. Further investigations need to be performed on a larger number of strains in order to confirm this specificity.

Methods

Bacterial strains

A total of 70 EHEC (n = 44) and EPEC (n = 26) strains of serogroup O26 isolated from bovine (n = 42) and humans (n = 28) and from diverse countries (USA, Ireland, Belgium, France, Japan and Brazil) were studied. Most of the strains had been described previously [37,38] and their pathotype (EPEC or EHEC) and serotype O26:H11 had been confirmed by PCR for *stx1*, *stx2*, *eae*, *wzx-wzy*$_{O26}$ and *fliC*$_{H11}$ genes [39-41].

PCR reaction

A 2941 pb segment of the *eae* gene, a 1559 pb segment of the *tir* gene and a 753 pb segment of *tccP2* gene were amplified by PCR, using respectively four pairs of primers, two pairs of primers and one pair of primers. All

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the primers used in this study and all the annealing temperatures are listed in Table 4. For PCR reactions, the following mixture was used: 1 U of *Taq* DNA polymerase (New England Biolabs, USA), 5 μl of 2 mM deoxynucleoside triphosphates, 5 μl of 10X ThermoPol Reaction Buffer (20 mM Tris-HCl (pH 8.8, 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 5 μl of each primer (10 μM), and 3 μl of a DNA template in a total volume of 50 μl.

DNA sequencing

The DNA fragments amplified were purified using the NucleoSpin Extract II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Sequencing of the two DNA strands was performed by the dideoxynucleotide triphosphate chain termination method with a 3730 ABI capillary sequencer and a BigDye Terminator kit version 3.1 (Applied Biosystems, USA) at the GIGA (Groupe Interdisciplinaire de Génoprotéomique Appliquée, Belgium). Sequence analysis was performed using Vector NTI 10.1.1 (Invitrogen, USA). DNA sequencing was performed three times.

Statistical analysis

A Fisher's exact test was performed to assess statistical differences.

Table 4 Primers used in this study (R = A+G, K = T+G, Y = C+T)

Primer name	Sequence (5' to 3')	Target gene	Annealing temp. (°C)	Amplicon size (bp)	Reference
B52	AGGCTTCGTCACAGTTG	*eaeA*	50	570	[39]
B53	CCATCGTCACCAGAGGA				
B54	AGAGCGATGTTACGGTTTG	*stx1*	50	388	[39]
B55	TTGCCCCCAGAGTGGATG				
B56	TGGGTTTTTCTTCGGTATC	*stx2*	50	807	[39]
B57	GACATTCTGGTTGACTCTCTT				
wzx-wzyO26-F	AAATTAGAAGCGCGTTCATC	wzx_{O26}	56	596	[41]
wzx-wzyO26-R	CCCAGCAAGCCAATTATGACT				
fliC-H11-F	ACTGTTAACGTAGATAGC	$fliC_{H11}$	56	224	[41]
fliC-H11-R	TCAATTTCTGCAGAATATAC				
B139	CRCCKCCAYTACCTTCACA	*tir* β	53	560	[27]
B140	GATTTTTCCCTCGCCACTA				
tir(591-1617)-F	TCCAAATAGTGGCGAGGGAA	*tir* β	54	1026	This study
tir(591-1617)-R	TTAAACGAAACGTGCGGGTC				
B73	TACTGAGATTAAGGCTGATAA	*eae* β	50	520	[27]
B137	TGTATGTCGCACTCTGATT				
eae(37-1142)-F	CGGCACAAGCATAAGCTAAA	*eae* β	51	1105	This study
eae(37-1142)-R	AGTTTACACCAACGGTCGCC				
eae(1001-2046)-F	TCCGCTTTAATGGCTATTTACC	*eae* β	50	1045	This study
eae(1001-2046)-R	TGCCTTCGCTGTTGTTTTAT				
eae(2319-2972)-F	GGCTCTGCAAAGAACTGGTT	*eae* β	50	653	This study
eae(2319-2972)-R	AGTCTCTATCAAACAAGGATACACG				
tccP2-F	ATGATAAATAGCATTAATTCTTT	*tccP2*	56	753	[24]
tccP2-R	TCACGAGCGCTTAGATGTATTAAT				

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Authors' contributions
MB conceived of the study, carried out the sequence alignment and drafted the manuscript. SL carried out the PCR reactions. JGM participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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5. Search for new virulence factors implicated in the host specificity and/or in the initial adherence

5.1. Preamble

Neither the respective prevalence of previously described adhesions, nor the polymorphisms in *tir, eae* and *tccP2* genes in O26 EPEC and EHEC, revealed host specificity. Consequently, we compared the whole genome of a bovine strain with a human strain using the Suppressive Subtractive Hybridisation (SSH) technique (Article 6). We aimed at selecting specific DNA fragment coding for virulence factors potentially implicated in the host specificity or in the initial adherence.

We briefly describe the SSH method first: it allows identifying genes that are present in one sample (the tester) but not in another (the driver). This technique goes through a seven–step process (Figure 10):

1. Extraction of the DNA genome of the two samples;
2. Digestion of the tester and the driver separately to form short blunt-ended fragments;
3. Ligation of the digested-tester DNA with two different adaptors in both pools;
4. First hybridisation between each ligated-tester DNA pool and the digested-driver DNA;
5. Second hybridisation between the two hybridised samples obtained from step 4;
6. PCR amplification of the differential expressed sequences;
7. Cloning of the differential expressed sequences.

In our case, we have subtracted a bovine O26 EHEC strain genome (the tester) from a human O26 EHEC strain genome (the driver). We amplified bovine strain specific sequences and studied both their specificity and their encoding sequences.

5.2. Article 6: "Comparison between a bovine and a human enterohaemorrhagic *Escherichia coli* strains of serogroup O26 by Suppressive Subtractive Hybridisation reveals the presence of atypical factors in EHEC and EPEC strains"

Comparison between a bovine and a human enterohaemorrhagic *Escherichia coli* strain of serogroup O26 by Suppressive Subtractive Hybridisation reveals the presence of atypical factors in EHEC and EPEC strains

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Running title : SSH between a bovine and a human O26 EHEC strains

Keywords

Enterohaemorrhagic *Escherichia coli*

Suppressive Subtractive Hybridization

Serogroup O26

PAI I_{CL3}

Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) strains are responsible for food poisoning in humans in developed countries via consumption of vegetal and animal foodstuffs contaminated by ruminant faeces. The clinical conditions caused by EHEC strains vary from undifferentiated diarrhoea to haemorrhagic colitis with, in a few cases, the appearance of the haemolytic uremic syndrome (HUS), which can lead to death. Most EHEC strains can be found in the gut of healthy ruminants, but some of the strains, belonging to O26, O111, O118 serogroups, for example, are also responsible for digestive disorders in calves. The aim of this research was to study the genomic differences between two EHEC strains of serogroup O26 isolated from a young calf and a human with diarrhoea, in order to identify specific sequences of the bovine strain that could be implicated in initial adherence or host specificity. No sequence implicated in host specificity was found during our study. Finally, several factors, not usually present in EHEC strains of serogroup O26, were identified in the bovine strain. One of them, the PAI I_{CL3} locus initially presented as a marker for LEE-negative VTEC strains, was found in 11.3% of EPEC and EHEC strains.

Introduction

In humans, enterohaemorraghic *Escherichia coli* (EHEC) is responsible for the production of diarrhoea, generally accompanied by haemorrhagic colitis (HC) with, in a few percent of cases, occurrence of renal sequelae (haemolytic uremic syndrome, HUS), which can lead to death. EHEC strains were recognized as a distinct class of pathogenic *E. coli* in 1983 after two outbreaks in the United States (Wells et al. 1983). Today, they represent a significant problem for public health in developed countries. Indeed, large outbreaks are caused by EHEC strains (Nataro and Kaper 1998) and transmission often occurs via consumption of vegetal and animal foodstuffs contaminated by faeces of adult ruminants (mainly cattle), which can be healthy carriers (Caprioli et al. 2005). The most common EHEC serotype is O157:H7, which causes disease worldwide, but other serogroups such as O26, O111, and/or O103 are also of high epidemiological importance in some countries (Brooks et al. 2005; Bettelheim 2007). In the veterinary field, different serogroups of EHEC strains (O5, O26, O111, O118, for example) are directly associated with diarrhoea in two week- to two month-old calves (Moxley and Francis 1986; Stordeur et al. 2000; Hornitzky et al. 2005). The consequences are economic losses due to a delay in growth and weakness of the calves.

Some pathogenic *E. coli* are host-specific, based upon the production of host-specific properties, in particular adhesins and colonization factors (for example, human typical EPEC, rabbit-EPEC, porcine-VTEC). However, the actual situation about the host specificity regarding those EHEC serogroups (e.g. O26 and O111) infecting both humans and young calves, and present in healthy adult ruminants, is unknown: do some isolates possess some degree of host-specificity or can all isolates in fact infect all the hosts? As with host-specific pathogenic *E. coli*, the basis of any host-specificity of those EHEC strains may be related to the production of

specific colonization factors, although such adhesins of EHEC strains have not yet been identified (Bardiau et al. 2009).

The aim of this study was (i) to explore the genomic differences, using suppressive subtractive hybridization (SSH), between two EHEC strains of serogroup O26, one isolated from a young calf and the other isolated from a human with diarrhoea, in order to identify specific sequences of the bovine strain; (ii) to analyse the bovine strain-specific sequences regarding their potential implication in adherence to epithelial cells; and (iii) to study the prevalence of these strain-specific sequences in a collection of human and bovine EHEC and EPEC strains.

Materials and methods

Bacterial strains

Subtractive suppressive hybridization was performed between the bovine EHEC strain 4276 of serogroup O26 isolated in Ireland from a diarrheic calf (Kerr et al. 1999) and the human EHEC strain 11368 of serogroup O26 isolated in Japan from a human suffering from diarrhoea (Ogura et al. 2009). The distribution of the specific sequences was investigated in additional EHEC (n=44) and EPEC (n=27) strains of serogroup O26 isolated from humans (n=27) and from cattle (n=44). Most of the strains have been described previously (Szalo et al. 2004; Bardiau et al. 2009) and their characteristics are described in the supplemental Table 1.

Pulsed Field Gel Electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis (PFGE) analyses were performed as already described (Cobbaut et al. 2009; Ooka et al. 2009) on most of the tested strains. In brief, bacterial cells were embedded in 1.8% Certified Low Melt Agarose (Bio-Rad Laboratories, Inc., Tokyo, Japan), lysed with a buffer containing 0.2% sodium deoxycholate, 0.5% N-

lauroylsarcosine, and 0.5% Brij-58, and treated with 100 µg/ml proteinase K. *Xba*I-digested genomic DNA was separated by using CHEF MAPPER (Bio-Rad Laboratories, Inc.) with 1% Pulsed Field Certified Agarose (Bio-Rad Laboratories, Inc.,) at 6.0 V/cm for 22 hours and 18 minutes with pulsed times ranging from 47 to 44.69 seconds. Size of each DNA band was estimated by Lane Analyser (ATTO Corp., Tokyo, Japan). The banding patterns were analysed using the Dice coefficient, with an optimization and position tolerance of 1%. Dendrograms were prepared by the Unweighted-Pair Group Method using arithmetic average Algorithm (UPGMA).

Suppressive subtractive hybridization

Genomic DNA was extracted from *E. coli* strain 4276 and *E. coli* strain 11368 using the cetyltrimethylammonium bromide procedure described by Ausubel *et al.* (Ausubel 1994). Subtractive hybridization was carried out using the PCR-Select Bacterial Genome Subtractive kit (Clontech) as recommended by the manufacturer. The bovine EHEC strain 4276 was the tester and the human EHEC strain 11368 was the driver. The PCR products obtained were cloned into the pGEM-T Easy Vector System (Promega), and transformed into *E. coli* JM109. The recombinant clones were plated onto LB plates containing ampicillin (100 µg/ml), 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 µg X-Gal/ml (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). White colonies containing recombinant plasmids with inserts were picked up, grown overnight at 37 °C in LB broth with ampicillin (100 µg/ml), and stored in a freezer (-20 °C) until further use.

DNA sequencing

The plasmid inserts were amplified by PCR with specific primers (nested primers 1 and 2R from the Clontech protocol) and the DNA fragments were purified using the NucleoSpin Extract II kit

(Macherey-Nagel) according to the manufacturer's instructions. Sequencing of the two DNA strands was performed by the dideoxynucleotide triphosphate chain termination method with a 3730 ABI capillary sequencer and a BigDye Terminator kit version 3.1 (Applied Biosystems) at the GIGA (Groupe Interdisciplinaire de Génoprotéomique Appliquée, University of Liège, Belgium). Sequence analysis was performed using Vector NTI 10.1.1 (Invitrogen). DNA sequences were further examined for homology with the National Center for Biotechnology Information (NCBI) BLASTN and BLASTX programs (http://www.ncbi.nlm.nih.gov/BLAST/). The expectation value of 0.001 was chosen as the cutoff.

DNA colony hybridization

Several EHEC strain 4276-specific sequences were chosen for extended analysis. Their distribution was searched for in the collection of 71 bovine and human EHEC and EPEC strains by DNA colony hybridization at 65 °C on Whatman 541 paper filters (Whatman), as previously described (Mainil et al. 1997). The DNA probes were derived by PCR from plasmid inserts obtained with SSH. The DNA probe fragments were purified using the NucleoSpin Extract II kit (Macherey-Nagel) according to the manufacturer's instructions and labelled with α^{32}P-dCTP (Perkin-Elmer) by random priming using the Ready-To-Go dCTP-labeling beads (Amersham Biosciences). Labelled DNA probes were purified with Microcon-YM30 spin columns (Millipore).

PCR reactions

All primers and PCR conditions used in this study have been described previously (China et al. 1996; Shen et al. 2004; Durso et al. 2005) (Supplemental Table 2). DNA extraction was carried out by a boiling method as described previously by China *et al.* (China et al. 1996). For the PCR reactions, the following mixture was used: 1 U of *Taq* DNA polymerase (New England Biolabs), 5 µl of 2 mM deoxynucleoside triphosphates, 5 µl of 10X ThermoPol Reaction Buffer (20 mM

Tris-HCl (pH 8.8, 25 °C), 10 mM KCl, 10 mM $(NH_4)2S0_4$, 2 mM $MgS0_4$, 0.1% Triton X-100), 5 µl of each primer (10 µM), and 3 µl of a DNA template in a total volume of 50 µl.

Statistical analysis

A Fisher's exact test was performed to assess statistical differences ($p<0.01$).

Results

Pulsed Field Gel Electrophoresis (PFGE)

PFGE profiles were obtained for 60 of the 73 tested strains. Others strains did not present any restriction profile for XbaI or could not be tested. The 60 distinct electrophoresis profiles were used for dendrogram construction (Supplemental Figure 1). The dendrogram showed five clusters, assuming a cutoff of 45% of similarity. When a cutoff of over 80% of similarity was adopted, 38 different clusters were found, indicating the high genetic variability among the strains.

Identification of the bovine EHEC strain 4276-specific genes in the subtractive library

A total of 1920 clones resulting from the suppressive subtractive hybridization process were obtained, of which 772 were randomly sequenced, resulting in 296 contigs after removal of redundant sequences. The specificity of the contigs to the bovine EHEC strain (strain 4276) was determined by a BLASTN search with the human EHEC strain (strain 11368) genome sequenced by Ogura *et al.* (Ogura et al. 2009). Of the 296 non-redundant DNA contigs, 115 contained genes different from those of the human EHEC strain (strain 11368).

Analysis of the bovine EHEC strain 4276-specific genes

BLASTN and BLASTX against the GenBank were searched for the 115 contigs specific to the bovine strain (Table 1 and Supplemental Table 3). Several groups of genes were revealed by more than one clone: colicin resistance genes, multiple antibiotic resistance region from *Salmonella enterica*, phages P1 and P7, pathogenicity island (termed PAI I_{CL3}) described in the VTEC O113:H21 *E. coli* CL3 (containing putative adhesins and haemolysins), genes from the genomic islands GEI 3.21 described in *E. coli* O111:H-, transposase from *Enterobacter cloacae*, *E. coli* and *Acinetobacter baumanii*, predicted type I restriction-modification enzyme from *E. coli* 0127:H6 E2348/69, DEAD/DEAH box helicase from *Nitromonas europea*, SNF2 family helicase from *E. coli* strain E24377A, plasmid pO111_2 from *E. coli* O111:H- and plasmid pSMS35_8 from *E. coli* SMS-3-5. BLASTN revealed six sequences that are not homologous to any annotated DNA sequences in GenBank. The other sequences were detected in only one clone and corresponded to genes specific to *Klebsiella pneumoniae, Pseudomonas aeruginosa, Citrobacter rotendium, Shigella sonnei, Erwinia sp., Desulfurispirillum indicum, Dickeya zeae, Pantoea ananatis* and several strains of *E. coli.*

Distribution of specific sequences in a collection of EHEC and EPEC isolates

Several sequences (in bold in Table 1 and Supplemental Table 3) were chosen for further characterization based upon the frequency of the contigs in the subtractive library or upon the putative involvement in adherence to the eukaryotic cells or in host specificity: genes from PAI I_{CL3}, four sequences with no homology, genes from P1 and P7 phages, genes from genomic island GEI 3.21, hypothetical proteins from E23477A strain, DEAD/DEAH box helicase from *Nitromonas sp.*, genes from *E. coli O111:H-* strain 11128, transposase from *Acinetobacter baumanii.*, ABC transporter from *Dickeya zeae*, and *avrA* genes from *E. coli* strain CB769. The regions of DNA homologous to that previously identified in the subtractive library were searched

for in EHEC and EPEC strains of serogroup O26 isolated from human and from cattle using DNA colony hybridization (Table 2) or using specific PCR for PAI I_{CL3} locus (Table 3). Statistical analyses were performed to assess differences in the presence of the fragments according to host specificity (human or bovine) and/or pathotype (EHEC or EPEC). Two sequences, both homologous to the genomic island GEI 3.21 from *E. coli* O111:H-, were statistically associated with EPEC strains in comparison with EHEC strains. One of the fragments homologous to P1 phage was statistically associated with EHEC in comparison with EPEC. The sequence homologous to the predicted type I restriction-modification enzyme from *E. coli O127:H6* strain E2348/69 was statistically associated with strains isolated from humans in comparison with strains isolated from bovines. All the other fragments were associated with neither pathotype nor host.

Distribution of the PAI I_{CL3} locus in human and bovine EPEC and EHEC strains

Shen *et al.* (Shen et al. 2004) first described the PAI I_{CL3} locus in the O113:H21 VTEC strain CL3. PAI I_{CL3} is a hybrid genomic region composed of genes similar to EDL933 (serotype O157:H7) O islands 122 and 48, to *Yersinia pestis*, to *Ralstonia solanacearum*, to *Pseudomonas syringae*, to *Fusobacterium nucleatum*, to *Bacillus subtilis*, to *Salmonella enterica*, and to *Sulfolobus tokodaii* (Table 3). To date, PAI I_{CL3} has been detected only in *eae*-negative VTEC strains associated with diseases in humans and never in any other pathogenic or commensal *E. coli* and it may therefore be used as a new marker for those strains (Girardeau et al. 2009). Since several genes of PAI I_{CL3} have been identified here in the bovine EHEC strain 4276 of serogroup O26, their distribution was studied with specific PCRs in the collection of human and bovine EHEC and EPEC strains.

Eight strains (3 human EPEC and 5 human and bovine EHEC strains) were found to be positive for several PCRs targeting different genes of the PAI I_{CL3} locus (Table 3). According to

their PFGE pattern, these eight strains are not closely related. Indeed, they are present in the five clusters revealed by the PFGE dendrogram with a similarity of 45% suggesting that these genes were horizontally acquired. No statistical difference was associated with the pathotype and/or the host origin ($p<0.01$). This genomic island can in fact be divided into four parts: two genomic segments (GS-I inserted and GS-II including two genes of OI-122) bordered by OI-48 segments either side (Shen et al. 2004). The eight strains were tested positive here with the PCRs for the 3 genes of GS-I and for all 6 genes of the two OI-48 segments. To verify that Z1640 gene is intact or not, we performed two PCRs: one PCR targeting the Z1640-1 and Z1640-3 sequences (using Z1640-F and Z1640-R primers) and one PCR targeting the Z1640-1 and S1 sequences (using Z1640-F and S1-bis-R primers). The eight strains were positive only with the Z1640/S1 PCR. On the other hand, only the S4 gene of GS-II was detected in all eight strains, while the other genes (including *S10* and *S11* genes of OI-122) were detected in none to 6 strains only.

Discussion

Several serogroups of enterohaemorrhagic *E. coli* strains (e.g. O5, O26, O111, O118) can infect both humans and calves, and can also be found in healthy cattle. Factors implicated in host specificity have been identified for some other pathogenic *Escherichia coli* strains, but not for EHEC strains. Such factors could be based on proteins intervening in the colonization stage (adhesins, for example). Therefore, we wanted to explore the genomic differences between a bovine EHEC strain of serogroup O26 and a human EHEC strain of serogroup O26 using suppressive subtractive hybridization (SSH) to identify specific sequences of the bovine strain. This study aimed to explore the potential implication in initial adherence or host specificity of the specific sequences.

In the SSH library, we obtained 115 unique fragments that were specific to the bovine strain. These fragments include sequences with homology to genes or pathogenicity islands (PAIs) present only in other specific *E. coli* pathotypes (e.g. VTEC) or other species (e.g. *Klebsiella, Nitromonas*), which are not known to be present in EHEC strains of serogroup O26. This heterogeneity supports the hypothesis of a horizontal acquisition of genomic regions from other pathogenic bacteria (Brzuszkiewicz et al. 2009; Juhas et al. 2009; Kelly et al. 2009). Moreover, it reflects the genomic plasticity of EHEC and/or *E. coli* strains. This finding supports the hypothesis of Mokady *et al.* (Mokady et al. 2005) suggesting that this variation in the genome contents of *E. coli* could indicate that its evolutionary strategy tends to create a mixed assortment of virulence factors coming from various pathogenic strains. This combination leads to a unique set of such factors, which helps the bacteria to better survive.

The PAI I_{CL3} locus, first described by Shen *et al.* (Shen et al. 2004) in the VTEC O113:H21 *E. coli* CL3, was found in 11.3% of the tested EHEC and EPEC strains of serogroup O26. These results are surprising when compared to those obtained by Girardeau *et al.* (Girardeau et al. 2009) suggesting that PAI I_{CL3} is unique to LEE-negative VTEC strains, and that this locus thus provides a new marker for such strains. We have reported here that the locus could also be present in *eae*-positive strains belonging to a major serogroup involved in human diseases. Girardeau *et al.* (Girardeau et al. 2009) have suggested that PAI I_{CL3} used to be present in most *E. coli* pathotypes but that many of these pathotypes have undergone extensive deletions (probably via homologous recombination between Insertion Sequences (IS) elements, which removed almost the entire locus). We can assume that our positive strains were not deleted for this locus. Another possible explanation is that these strains have recently acquired the PAI I_{CL3} locus via horizontal transfer, which hypothesis is supported by the fact that the PAI I_{CL3}-positive strains are not closely related.

Concerning host specificity, only one sequence appears to be statistically specific to human strains in comparison with bovine strains. Nevertheless, this sequence is only present in a few strains (7% of bovine strains and 33% of human strains) and therefore could not represent a host-specific marker. Moreover, three sequences were statistically associated to the pathotype (EHEC or EPEC) but these sequences were not present in more than half of the EPEC strains. However, host-specific factors could perhaps not be detected by SSH for one of the following reasons: 1) the subtraction is non-exhaustive and host-specific factors were not detected; 2) this host specificity is not based on the presence/absence of specific factors/genes; 3) there is no host specificity.

In conclusion, our findings support the hypothesis of the acquisition of genomic regions from other pathogenic bacteria (*E. coli* or others) by horizontal transfers and reflect the genomic plasticity of EHEC or even *E. coli* strains. This variation in the genome contents of *E. coli*, suggested as a evolutionary strategy to better survive by Mokady *et al.* (Mokady et al. 2005), could lead to serious problems in public health and to the emergence of highly virulent new strains if one strain could acquire several strong virulence systems from different pathogenic bacteria, as it was dramatically illustrated by the 2011 Shiga toxin-producing *E. coli* O104:H4 German outbreak (Denamur 2011; Rasko et al. 2011).

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Table 1. Results of the BLASTN against the GenBank searched for the 115 contigs specific to the bovine strains

Function	Number of non-redundant and specific contigs	BLASTN results	Species
Antibiotic resistance	9	Colicin resistance	*Escherichia coli*
	3	Multiple antibiotic resistance region	*Salmonella enterica, Klebsiella pneumoniae*
Mobile functions	7	**Transposase**	*Enterobacter cloacae, Escherichia coli, Acinetobacter baumanii*
	1	Excisionase	*Escherichia coli*
Genomic island	1	Genomic island GEI1.94	*Escherichia coli*
	1	Genomic island AGI-5	*Escherichia coli*
	7	**Genomic island GEI3.21**	*Escherichia coli*
Unknown function	14	**Hypothetical protein**	*Escherichia coli, Pseudomonas aeruginosa, Citrobacter rodentium*
	6	**No homology**	-
Adherence related	7	**Pathogenicity island I (PAI I_{CL3})**	*Escherichia coli*
	1	**Putative haemolysin/haemagglutinin**	*Citrobacter rodentium*
	1	espP	*Escherichia coli*
	1	tonB	*Shigella sonnei*
Metabolism	3	**Predicted type I restriction-modification enzyme, S subunit**	*Escherichia coli*
	2	N-6 DNA methylase	*Desulfurispirillum indicum, Dickeya zeae*
	1	Galactosyl transferase	*Erwinia sp.*
	4	**DEAD/DEAH box helicase**	*Nitrosomonas europaea, Pseudomonas aeruginosa*
	3	SNF2 family helicase	*Escherichia coli*
	2	**ABC transporter**	*Dickeya zeae, Nitrosomonas europaea*
Phage related	8	**Enterobacteria phage P7 or P1**	*Enterobacteria phage P7 or P1*
	1	Putative tail fiber assembly protein	*Shigella sonnei*
	1	**Putative phage repressor protein**	*Escherichia coli*
Other	1	FhaB	*Pantoea ananatis*
	1	**avrA**	*Escherichia coli*
	23	**Plasmid pO111_2**	*Escherichia coli*
	1	Plasmid pCRP3	*Citrobacter rodentium*
	3	Plasmid pSMS35_8	*Escherichia coli*
	1	Plasmid pHUSEC41-1	*Escherichia coli*
	1	Plasmid pO145-NM	*Escherichia coli*
TOTAL	**115**		

Table 2. Distribution of specific sequences with putative host specificity or interest in terms of potential adherence

	Number of strains	Number and percentage of strains found positive for																		
		LG100	Gamma D11	LG53	LG31	LG106	LG88	LG69	LG109	LG118	Epsilon G6	Alpha F3	Dzeta H6	*LG28*	*LG45*	*LG12*	Nu G3	LG2	Espilon E1	LG92	LG16
	Number of tested strains	No homology	No homology	No homology	No homology	Enterobacteria phage P7	**Recombination enhancement function**	Plasmid pO111_2	plasmid pO111_2	putative phage repressor protein	Hypothetical protein	Hypothetical protein	Hypothetical protein	ABC transporter	DEAD/DEAH box helicase	DEAD/DEAH box helicase	avrA	**Genomic island GEI3.21**	**Genomic island GEI3.21**	**predicted type I restriction-modification enzyme, S subunit**	Transposase
Total no. of strains	75	3	6	52	2	14	15	14	5	12	0	2	23	1	1	4	36	24	16	12	23
		4%	8%	69%	3%	19%	20%	19%	7%	16%	0%	3%	31%	1%	1%	5%	48%	32%	21%	16%	31%
Bovine strains	44	3	4	31	1	9	10	10	1	7	0	1	11	1	1	4	25	15	11	**3**	13
		7%	9%	70%	2%	20%	23%	23%	2%	16%	0%	2%	25%	2%	2%	9%	57%	34%	25%	**7%**	30%
Human strains	27	0	2	21	1	5	5	4	4	5	0	0	12	0	0	0	11	9	5	**9**	10
		0%	7%	78%	4%	19%	19%	15%	15%	19%	0%	0%	44%	0%	0%	0%	41%	33%	19%	**33%**	37%
EPEC strains	27	0	2	19	1	2	**1**	2	1	2	0	1	6	0	0	0	8	**15**	**12**	4	7
		0%	7%	70%	4%	7%	**4%**	7%	4%	7%	0%	4%	22%	0%	0%	0%	30%	**56%**	**44%**	15%	26%
EHEC strains	44	3	4	33	1	12	**14**	12	4	10	0	1	17	1	1	4	28	**9**	**4**	8	16
		7%	9%	75%	2%	27%	**32%**	27%	9%	23%	0%	2%	39%	2%	2%	9%	64%	**20%**	**9%**	18%	36%

Table 3. Distribution of the genes carried by the PAI I_{CL3} locus in the eight positive strains (B: bovine, H: human)

Similar protein (% identity)			Strain Pathotype Host Genes	4276 EHEC B	TC6165 EPEC H	TC6166 EPEC H	02/057 EPEC H	99/147 EHEC H	A39 EHEC B	63 EHEC B	A14 EHEC B
Z1635, unknown protein, *E. coli* EDL933 (97)			Z1635	+	+	+	+	+	+	+	+
Z1636, unknown protein, *E. coli* EDL933 (96)	OI-48		Z1636	+	+	+	+	+	+	+	+
Z1637, unknown protein, *E. coli* EDL933 (95)			Z1637	+	+	+	+	+	+	+	+
YPO2491, putative hemolysin activator, *Y. pestis* CO92 (64)			S1	+	+	+	+	+	+	+	+
RS02573, putative hemolysin activating-like protein, *R. solanacearum* (57)	GSI		S2	+	+	+	+	+	+	+	+
YPO2490, putative hemolysin (53)			S3	+	+	+	+	+	+	+	+
YPO0599, putative adhesin, *Y. pestis* CO92 (50)											
YPO2490, putative hemolysin (41)			S4	+	+	+	+	+	+	+	+
YPO0599, putative adhesin, *Y. pestis* CO92 (39)											
YPO0599, putative adhesin, *Y. pestis* CO92 (83)			S5	+	+	-	-	+	+	-	-
Y2435, putative transposase, *Y. pestis* KIM (38)			S6	+	+	-	-	+	+	+	+
TnpA, transposase, *P. syringae* (60)			S7	-	-	-	-	-	-	-	-
FN0835, hypothetical protein, *F. nucleatum* ATCC 25586 (27)			S8	-	-	-	-	-	-	-	-
YozI, unknown protein, *B. subtilis* (32)	GSII		S9	-	+	+	-	-	-	-	-
Z4322, unknown protein, *E. coli* EDL933 (94)		OI-122	S10	-	-	-	-	-	-	-	-
Z4321, unknown protein, *E. coli* EDL933 (98)			S11	-	+	+	-	-	-	+	+
Orf1, similarity with helicase, *S. enterica* (40)			S12	+	-	-	-	-	-	-	-
ST0071, hypothetical esterase *Sulfolobus tokodaii* (30)			S13	-	+	-	-	-	-	-	-
Y2679, hypothetical protein, *Y. pestis* KIM (39)			S14	-	-	-	-	-	+	+	+
Z1640, unknown protein, *E. coli* EDL933 (90)											
Z1641, unknown protein, *E. coli* EDL933 (96)			S15	+	+	+	+	+	+	+	+
Z1642, unknown protein, *E. coli* EDL933 (99)	OI-48										
Z1643, unknown protein, *E. coli* EDL933 (97)			Z1643	+	+	+	+	+	+	+	+
Z1644, unknown protein, *E. coli* EDL933 (98)			Z1644	+	+	+	+	+	+	+	+

6. Comprehensive genomic comparison of bovine and human strains

6.1. Preamble

We used the Whole Genome PCR Scanning method (WGPS) to concurrently study more strains, and compared the genome of ten EHEC strains of serogroup O26 isolated from bovines and humans (Article 7). We aimed at detecting host-specific genomic regions present in either human or bovine strains.

This method was developed by Ohnishi and collaborators to compare the whole genome of eight O157:H7 EHEC strains (Ohnishi *et al.*, 2002). The WGPS allows studying the genome structure diversity in closely-related strains. To be brief, the whole genome of each O26 strain was amplified with 565 pairs of PCR primers based on the sequence of the human O26 EHEC strain 11368 (Ogura *et al.*, 2009). Then, strain-to-strain differences were analysed by comparing their amplicons profile, that is, by comparing the presence or absence of amplicons, and the differences in amplicon sizes.

Additionally, the genome sequencing of the human O26 EHEC strain 11368 —used as a reference in the WGPS— has revealed the presence of 14 copies, all located on the chromosome of the IS element named IS*621*. The WGPS comparison results have underlined the important level of small-size structural polymorphisms (SSSPs) among the O26 EHEC strains in the IS*621* genomic regions. Therefore, we attempted to develop a new epidemiological method based on the variation in genomic location and copy numbers of IS*621* by a multiplex PCR printing method, originally developed for O157:H7 EHEC strains (Ooka *et al.*, 2009) (Article 8).

6.2. Article 7: "Genomic analysis of human and bovine enterohemorrhagic Escherichia coli strains of serogroup O26 using Whole Genome PCR Scanning"

Genomic analysis of human and bovine enterohemorrhagic *Escherichia coli* strains of serogroup O26 using Whole Genome PCR Scanning

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25 1. Introduction

26

27 Enterohemorrhagic *Escherichia coli* strains (EHEC) were first recognized as a distinct class
28 of pathogenic *E.coli* in 1983 after two outbreaks in the United States (18). Since that time, EHEC
29 strains have represented an important problem for public health and have been responsible of
30 large outbreaks in many developed countries like Japan, the United States, the United Kingdom,
31 Ireland, Germany, etc. (4, 6, 8, 10, 15, 16, 18). Moreover, transmission occurs via consumption
32 of vegetal and animal foodstuffs contaminated by ruminant feces (mainly cattle) (5). The most
33 common serogroup that causes disease worldwide is O157:H7, but other serogroups like O26,
34 O111, and O103 can also be responsible for an important percentage of disease in some country
35 (3, 4).

36 In humans, EHEC strains are characterized by the production of diarrhea that could lead
37 to hemorrhagic colitis (HC) with, in a few percent of the cases, apparition of the Hemolytic
38 Uremic Syndrome (HUS) and the Thrombotic Thrombocytopenic Purpura (TTP) that can
39 conduce to death. In veterinary field, several serogroups of EHEC strains (f.i. O26, O111, O118)
40 are directly associated with diarrhea in two-week to two-month old calves (7, 11). The
41 consequences are economic losses due to growth delay and calves weakness.

42 Several serogroups (f.i. O5, O26, O111, O118) can infect both humans and calves, and
43 can also be found in healthy cattle. If some pathogenic *E. coli* are considered to be host-specific
44 (f.i. classical EPEC, Rabbit-EPEC, Porcine-VTEC), the actual situation regarding EHEC remains
45 unknown. Specific factors (like adhesins or metabolism adapted genes) could be at the basis of
46 host specificity (human or calves) for EHEC strains.

47

48 The Whole Genome PCR Scanning is a method that was previously described by Ohnishi
49 *et al.* to compare the whole genome of eight O157 strains (12, 14). This method allows
50 investigating genome structure diversity in closely related strains. Moreover, Ben Zakour *et al.*
51 used this method on *Staphylococcus aureus* strains to study the genetic basis of their host
52 adaptation (2). In view to perform a more sensitive genomic comparison of O26 EHEC strains
53 and to detect host specific regions of human and bovine O26 EHEC strains, we compared five
54 human and five bovine strains by the Whole Genome PCR Scanning method.
55
56 2. Materials and methods
57
58 **Bacterial strains and genomic DNA extraction.**
59 A total of 10 EHEC strains of serogroup O26 isolated in the USA, Ireland, Belgium, France,
60 Japan and Italy were studied (Table 1). Five strains were isolated from human and five strains
61 were isolated from cattle or calves. The O26 EHEC strain 11368, isolated from a human, was
62 used as the reference in the WGPScanning. For the distribution of the potential host-specific
63 sequences, a total of 55 strains (38 EHEC and 17 EPEC; 31 isolated from bovine and 24 isolated
64 from human) of serogroup O26 isolated in the USA, Ireland, Belgium, France, Japan and Brazil
65 were studied. Most of the strains had been described previously (1, 17) but their pathotype and
66 serotype were confirmed by PCR for *stx1*, *stx2*, *eae, wzx-wzy*$_{O26}$ and *fliC*$_{H11}$ genes as previously
67 described (9). Bacteria were grown at 37° in Luria-Bertani medium. Genomic DNA was isolated
68 using the Genomic-tip 100/G and Genomic DNA buffer set (Quiagen) according to the
69 manufacturer instruction.
70
71

72 **Pulsed-field gel electrophoresis.**

73 Pulsed-field gel electrophoresis (PFGE) analyses were performed according to the method
74 described by Terajima et al. [51] with some modification. In brief, bacterial cells were embedded
75 in 0.9% Certified Low Melt Agarose (Bio-Rad Laboratories, Inc., Japan), lysed with a buffer
76 containing 0.2% sodium deoxycholate, 0.5% N-lauroylsarcosine, and 0.5% Brij-58, and treated
77 with 100 μg/ml proteinase K. *XbaI*-digested genomic DNA was separated by using CHEF
78 MAPPER (Bio-Rad Laboratories, Inc.) with 1% Pulsed Field Certified Agarose (Bio-Rad
79 Laboratories, Inc.,) at 6.0 V/cm for 22 h and 18 minutes with pulsed times ranging from 47 to
80 44.69 s. Sizes of each DNA band were estimated by Biogene (Vilber Lourmat, France). The
81 banding patterns were analyzed using the Dice coefficient, with an optimization and position
82 tolerance of 1%. Dendrograms were prepared by the Unweighted-Pair Group Method using
83 arithmetic average Algorithm (UPGMA).
84
85 **WGPScanning.**
86 The WGPScanning method was developed for O26 strains based on the same method applied for
87 O157 strains and previously described by Ohnishi *et al.* (14). Briefly, 565 pairs of PCR primers
88 were designed based on the genomic sequence of the O26 EHEC strain 11368 (13). These PCR
89 primers amplified 565 segments covering the whole genome. All PCR primers sequences are
90 listed in Supplemental Table 1. LA taq PCR kit (Takara Shuzo, Kyoto, Japan) was used to
91 perform Long PCR on 1 ng of genomic DNA. PCR conditions consist of 30 cycles of a two-step
92 amplification program: 20s at 98°C and 16 minutes at 69°C. Long PCR products were separated
93 by using Field-Inversed Gel Electrophoresis (FIGE) with 1% Pulsed Field Certified Agarose
94 (Bio-Rad Laboratories, Inc.,) and their sizes were estimated by Lane Analyzer.
95

Distribution of selected sequences in EHEC and EPEC strains.

The distribution of sequences of interest that could be specific to human or bovine and revealed by the WGPScanning method was investigated in a collection of EHEC and EPEC strains of serogroup O26. Long PCR was performed on 1 ng of genomic DNA using LA taq PCR kit (Takara Shuzo, Kyoto, Japan) in the same PCR conditions as described above for the WGPScanning. Results were visualized on Field Inversion Gel Electrophoresis (FIGE). A Fisher's exact test was performed to assess statistical differences ($p<0.05$).

3. Results

Phylogenetic analysis of O26 strains.

PFGE profiles were obtained for the reference, the 10 tested strains and one commensal *E. coli* strain 282. The distinct electrophoresis profiles were used for dendrogram construction (Figure 1). The dendrogram showed that profile similarities ranged from 53% to 97.5%. These results indicated that all the strains exhibited significant variation in the *XbaI*-digestion pattern. None of the strains are grouped together according to their host or country of origins.

Structural diversity of O26 strains.

The results of the LR-PCR reactions applied to the reference and the 10 tested strains are shown in Figure 2. In the reference strain 11368, all amplicons obtained were of the expected sizes. Nevertheless, three LR-PCR products could not be amplified in neither the reference nor the tested strains. When segments could not be amplified, additional LR-PCR using other combinations of primers were used considering three possible explanations: (1) polymorphism or deletion in the primer sequences, (2) large insertion between primers that inhibit sequences

amplification, (3) genomic rearrangement (f.i. translocations and inversions) that results in improper primers pairs.

The Table 2 shows the results of the amplifications of the segments by the WGPS for each tested strain. Amplification of the same size products occurs in a range of 82% to 94% depending on the strain. One to seven percent of the segments were not amplified. And 5 to 11% of the segments presented a difference in size. Consistently with the PFGE data, the rate of amplification of the same size segments was not correlated with the host.

As we can observe in Figure 2, many of the segments different in size or not amplified are located in integrative elements (IE) or prophages regions. The Table 3 shows the repartition of these segments in relation to these regions (IE or prophages). These results indicated that the genomic diversity of O26 strains is largely due to variations in these genetic elements, especially for segments that we could not amplified (in the average, 86% of these segments were located in IE or prophages regions).

Divergence between human and bovine strains.

The results of the WGPS were analyzed with the aim to find host-specific regions of strains isolated either from bovines or humans. Segments that seemed to present differences (absence of amplicon or difference in size) mainly in one host and not in the other one were chosen for further characterizations. Seven genomic regions were then selected based on these observations. The genes content of these regions are described in Supplemental Table 1.

Distribution of divergent segments in a collection of O26 strains.

In order to confirm the potential host-specific regions revealed by the WGPS, 55 EHEC and EPEC strains of serogroup O26 were screened by LR-PCR for the seven regions underline by the

143 WGPS analysis. Statistical analyses were performed to assess differences according to the host or
144 the pathotype. The results in Table 4 shows that in the seven tested regions, five were statistically
145 associated with EPEC strains ($p<0.01$). Moreover, two of the seven segments were statistically
146 associated to strains isolated from bovines ($p<0.05$). Nevertheless, the other regions, despite the
147 fact that they were not statistically associated to the bovine strains, were more often associated to
148 these strains than to the strains isolated from humans. Finally, when comparing their distribution
149 in strains isolated from healthy or diarrheic bovines, three of the segments were statistically
150 associated to diarrheic bovines ($p<0.05$).
151
152 4. Discussion
153
154 The situation regarding host specificity of EHEC strains is for the moment not clear.
155 Serogroups of EHEC strains, such as O26, can infect both humans and calves, and can also be
156 found in healthy cattle. Therefore, we wanted to explore the genomic differences between ten
157 O26 EHEC strains isolated from bovines and from humans by the WGPS with the aim to identify
158 host-specific genomic regions.
159 The WGPS results revealed a structural diversity in the genome of O26 EHEC strains of
160 12% in the average. A majority of the variations (difference in size or absence of amplicons)
161 were located in Integrative Elements (EI) or prophages regions. These findings are not surprising
162 considering that these regions are known to be unstable and mobile chromosomal regions. A
163 horizontal transfer from other bacteria of these genomic regions is probably the basis of these
164 variations. These genomic deletions and insertions via a horizontal transfer are probably a way to
165 better survive and be better adapted to certain environmental condition. In comparison to the
166 results obtained by Onhishi *et al.* on O157 EHEC strains, we observed a higher variation rate

among the O26 strains (12% in the average for O26 strains and 8,8% in the average for O157 strains).

When analyzing the distribution results of the potentially host-specific segments in the collection of strains, several segments were specific to strains isolated from bovine, especially bovine with diarrhea. They were isolated from diverse countries (United Kingdom, Ireland, The Netherlands, Belgium and USA) and two-third of the strains were isolated in 1991. Therefore, we could suspect that these strains represent a sub-group of strains specifically adapted to the production of diarrhea in calves. Nevertheless, more strains, especially strains isolated from diarrheic calves, have to be studied before concluding such existence of a sub-group. This one could be a clonal group instead of being a host/pathotype-associated sub-group.

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Table 1: strains compared by the Whole Genome PCR Scanning (WGPS) (n.i. : no information).

	Serogroup	Origin	Disease	Country	Shiga toxin	Intimin
EH193	O26:H11	Human	Diarrhea	Belgium	2	beta
EH182	O26:H11	Human	Diarrhea	Belgium	1	beta
EH296	O26:H11	Human	Diarrhea	Belgium	2	beta
03/151	O26:H11	Human	n.i.	France	1	beta
122	O26:H11	Bovine	n.i.	Ireland	1	beta
379S89	O26:H11	Bovine	Diarrhea	Belgium	1	beta
357S89	O26:H11	Bovine	Diarrhea	Belgium	1	beta
4276	O26:H11	Bovine	Diarrhea	Ireland	1	beta
11368	O26:H11	Human	Diarrhea	Japan	1	beta
13247	O26:H11	Human	Diarrhea	Japan	1	beta
ED80	O26:H11	Bovine	n.i.	Italy	1 and 2	beta

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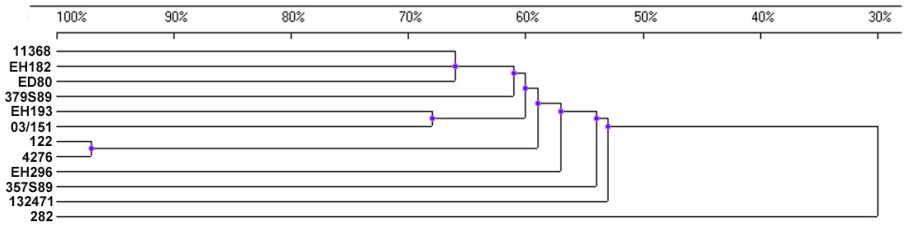
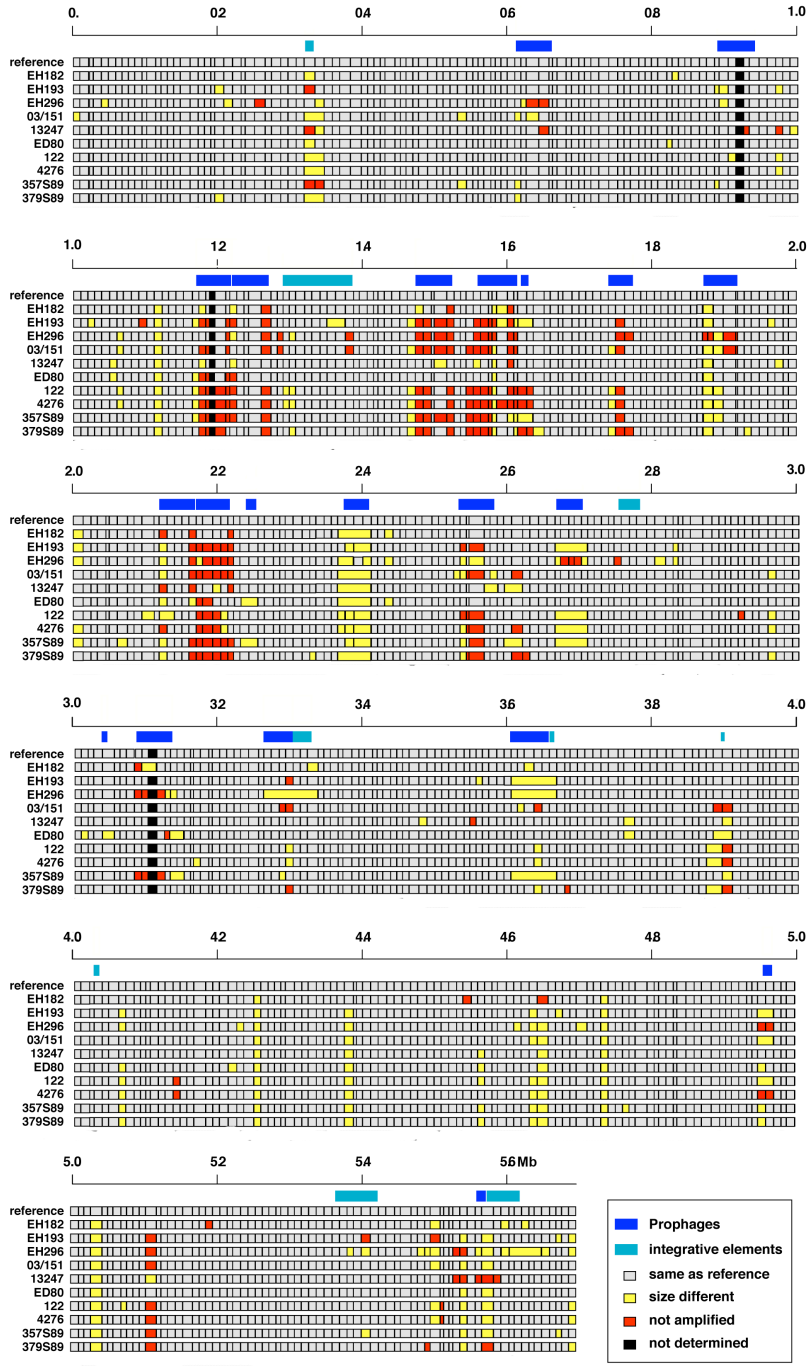


261

262 **Figure 1**: dendrogram of tested strains constructed by PFGE data (UPGMA, Dice coefficient, 1%

263 position tolerance).

264


0.
02
04
06
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1.0
1.2
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1.6
1.8
2.0
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3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6 Mb
reference
EH182
EH193
EH296
03/151
13247
ED80
122
4276
357S89
379S89
Prophages
integrative elements
same as reference
size different
not amplified
not determined

265

12

Figure 2: WGPScanning results of O26 strains. Each rectangle symbolizes one LR-PCR performed on the 11 strains (10 tested strains and 1 reference strain). Segments presenting products of identical sizes from those of the reference strain (11368) are represented by gray rectangle; segments presenting products of different sizes from those of the reference strain (11368) are represented by yellow rectangle; segments that were not amplified are represented by red rectangle. LR-PCR that could not work nether in the reference and the tested strains are represented by black rectangle. Regions in the genome of prophages and integrative elements (IE) are respectively depicted as dark and light blue.

Table 2: diversity of the ten O26 strains tested by the WGPS.

	No. (%) of segments										
Host	Human	Human	Human	Human	Human	Bovine	Bovine	Bovine	Bovine	Bovine	
Strain No.	**EH182**	**EH193**	**EH296**	**03/151**	**13247**	**ED80**	**122**	**4276**	**357S89**	**379S89**	**AVERAGE**
Absent segments	10 (1.8%)	29 (5.1%)	40 (7%)	34 (6%)	15 (2.7%)	7 (1.2%)	29 (5.1%)	32 (5.7%)	28 (5%)	31 (5.5%)	25 (4.5%)
Segments different in size	26 (4.6%)	48 (8.5%)	63 (11.2%)	35 (6.2%)	30 (5.3%)	35 (6.2%)	48 (8.5%)	43 (7.6%)	54 (9.5%)	36 (6.4%)	42 (7.4%)
Segments with the same size	529 (93.6%)	488 (86.4%)	462 (81.8%)	496 (87.8%)	520 (92%)	523 (92.6%)	488 (86.4%)	490 (86.7%)	483 (85.5%)	498 (88.1%)	498 (88.1%)

Table 3: distribution of the segments different in size or not amplified in relation to the integrative elements or prophages genomic regions.

		No. (%) of segments										
	Host	Human	Human	Human	Human	Human	Bovine	Bovine	Bovine	Bovine	Bovine	
	Strain No.	**EH182**	**EH193**	**EH296**	**03/151**	**13247**	**ED80**	**122**	**4276**	**357S89**	**379S89**	**AVERAGE**
Segments different in size	**Out of integrative elements or prophages**	10 (1.8%)	19 (3.4%)	26 (4.6%)	17 (3%)	14 (2.5%)	20 (3.5%)	20 (3.5%)	19 (3.4%)	20 (3.5%)	21 (3.7%)	19 (3,3%)
	In integrative elements or prophages	16 (2.8%)	29 (5.1%)	37 (6.6%)	18 (3.2%)	16 (2.8%)	15 (2.7%)	28 (5%)	24 (4.2%)	34 (6%)	15 (2.7%)	23 (4.1%)
Absent segments	**Out of integrative elements or prophages**	3 (0.5%)	2 (0.4%)	4 (0.7%)	3 (0.5%)	4 (0.7%)	0 (0%)	5 (0.9%)	5 (0.9%)	1 (0.2%)	5 (0.9%)	3 (0.5%)
	In integrative elements or prophages	7 (1.3%)	27 (4.7%)	36 (6.3%)	31 (5.5%)	11 (2%)	7 (1.2%)	24 (4.2%)	27 (4.8%)	27 (4.8%)	26 (4.6%)	22 (4%)

281 **Table 4**: distribution of the seven selected segments in a collection of EPEC and EHEC strains
282 isolated from bovines and humans (segments that are statistically associated are presented in
283 bold).

		Type of changing in the WGPS results						
		Deletion	Deletion	Deletion	Deletion	Insertion	Insertion	Insertion
		EC106.6	**EC112.1**	**EC112.2**	**EC220**	**EC297**	**EC311**	**EC359**
	No. of strains tested	No. of strains giving an amplicon different from the reference strain						
EPEC	17	**9**	**13**	**13**	**13**	**9**	11	5
EHEC	38	3	0	5	7	0	25	5
Bovine	31	8	**11**	13	**15**	8	23	8
Human	24	4	2	5	5	1	13	2
Diarrheic bovine	12	**5**	5	5	**8**	**5**	10	3
Healthy bovine	13	0	3	5	3	0	7	0

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6.3. Article 8: "Typing of O26 enterohaemorrhagic and enteropathogenic *Escherichia coli* isolated from humans and cattle with IS621 multiplex PCR-based fingerprinting"

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ORIGINAL ARTICLE

Typing of O26 enterohaemorrhagic and enteropathogenic *Escherichia coli* isolated from humans and cattle with IS*621* multiplex PCR-based fingerprinting

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Keywords
cattle, EHEC, EPEC, human, IS*621* fingerprint, O26, PFGE.

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Abstract

Aims: This study evaluated a typing method of O26:H11 enterohaemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC) based on the variation in genomic location and copy numbers of IS*621*.
Methods and Results: Two multiplex PCRs, targeting either the left (5′) or right (3′) IS/chromosome junction of 12 IS*621* insertion sites and one PCR specific of another truncated copy, were developed. Thirty-eight amplification profiles were observed amongst a collection of 69 human and bovine O26:H11 EHEC and EPEC. Seventy-one per cent of the 45 EHEC and EPEC with identical IS*621* fingerprints within groups of two, three or four isolates had >85% pulsed field gel electrophoresis (PFGE) profile similarity, including four groups of epidemiologically related EHEC or EPEC, while most of the groups had <85% similarity between each others. Epidemiologically related EHEC from each of three independent outbreaks in Japan and Belgium also exhibited identical IS*621* fingerprints and PFGE profiles.
Conclusions: The IS*621* fingerprinting and the PFGE are complementary typing assays of EHEC and EPEC; though, the former is less discriminatory.
Significance and Impact of the Study: The IS*621* printing method represents a rapid (24 h) first-line surveillance and typing assay, to compare and trace back O26:H11 EHEC and EPEC during surveys in farms, multiple human cases and outbreaks.

Introduction

In developed countries (USA, Canada, UK, France, Japan, etc.), enterohaemorrhagic *Escherichia coli* (EHEC) are responsible for small- or large-scale outbreaks of uncomplicated diarrhoea, haemorrhagic colitis and/or haemolytic–uraemic syndrome (HUS). In many cases, infection occurs via consumption of vegetal and animal foodstuffs contaminated by the faeces of ruminants (mainly cattle), which can be asymptomatic carriers. Typical EHEC can also be defined based on microbiological criteria; that is, the presence of two major virulence properties or of their encoding genes: the production of Shiga toxins (Stx, also called verocytotoxins) and of the histological and ultramicroscopic 'attachment and effacement' (AE) lesions on the enteric epithelial cells. Based on these microbiological criteria, EHEC belong to scores of O:H serotypes whose virulence in humans and host range can differ. The reference EHEC serotype is O157:H7. Other O serogroups of importance nowadays are O26 (the second in importance

worldwide), O111, O103 and O145, with O26 and O111 also being responsible for uncomplicated diarrhoea in young calves up to 3 months of age (Nataro and Kaper 1998; Campos *et al.* 2004; Mainil and Daube 2005; Naylor *et al.* 2005; Beutin 2006; Hussein 2007; Karmali 2009; Bolton 2011).

Some Stx-producing *E. coli* do not cause AE lesions on eukaryotic cells (verocytotoxigenic or Shiga toxin-producing *E. coli sensu stricto* or VTEC/STEC), while others produce AE lesions but no Stx (enteropathogenic *E. coli sensu stricto* or EPEC). EHEC and EPEC are sometimes grouped together under the name 'attaching–effacing *E. coli*'. EPEC are further subdivided into typical (tEPEC) and atypical (aEPEC), basically on the production, or absence of, of bundle-forming pili, or presence/absence of encoding genes: tEPEC are isolated from humans predominantly and, though more rarely, from dogs and cats, while aEPEC are isolated on similar scale from humans and animals. Many aEPEC form a distinct group of pathogenic *E. coli* with specific properties, but some that belong to EHEC-specific serogroups, including O26, can derive from EHEC after loss of phage-located *stx* genes (Karch *et al.* 1992; Nataro and Kaper 1998; Campos *et al.* 2004; Bielaszewska *et al.* 2007; Hernandes *et al.* 2009; Gyles and Fairbrother 2010; Moxley and Smith 2010; Bolton 2011).

To perform epidemiological studies and surveillance of EHEC infections, suitable, rapid and efficient molecular methods for isolate typing are needed. Although highly discriminatory, pulsed field gel electrophoresis (PFGE) (Olive and Bean 1999; Davis *et al.* 2003; Terajima *et al.* 2006) requires special equipment and is both time- and labour-consuming, taking up to one working week to perform. Moreover, it can be difficult to obtain reproducible results between laboratories, despite the fact that an official and standard protocol exists (CDC. http://www.cdc.gov/pulsenet/); this lack of consistency can hinder data comparison.

Complete genome sequences of three EHEC of the O157:H7 serotype (Sakai, EDL933 and TW1435 strains) have been published (Hayashi *et al.* 2001; Perna *et al.* 2001; Kulasekara *et al.* 2009). Chromosomes and plasmids of several O157:H7 EHEC have been compared to the Sakai genome sequence by the whole-genome PCR scanning (WGPS) method, a long-range PCR-based method, which systematically identifies regions of closely related genomes with structural polymorphisms: most of the large-size structural polymorphisms were found to be generated by alteration of prophage regions, whereas most of the small-size structural polymorphisms (SSSPs) were found to be generated by genetic events associated with IS elements, especially with IS*629* (Ohnishi *et al.* 2002; Iguchi *et al.* 2006; Ogura *et al.* 2006; Ooka *et al.* 2009a). A rapid multiplex PCR typing method, called the O157 IS*629* printing method, was subsequently developed based on the differences in genomic locations and copy numbers of IS*629* between O157 *E. coli* (Ooka *et al.* 2009b).

Recent genome sequencing of the human O26:H11 EHEC (hEHEC) strain 11368 (accession numbers AP010953–AP010956) has revealed the presence of a total of 119 copies of 29 different IS elements. IS*621* is the most frequent, with 14 copies, all located on the chromosome. IS*629* is present in 13 copies, but only eight are located on the chromosome and only six are complete copies (Ogura *et al.* 2009). Very recently, we compared the chromosomes and plasmids of five O26:H11 hEHEC and five O26:H11 bovine EHEC (bEHEC) by the WGPS method using a primer set designed according to the genome sequence of strain 11368: IS*621*-containing genomic regions exhibit important levels of SSSPs among the O26 EHEC, but IS*629*-containing genomic regions do so at a very low level (Mainil *et al.* 2009; Bardiau *et al.* 2010b).

The purpose of this study was therefore: (i) to develop an IS*621*-based multiplex PCR printing method, similar to the O157 IS*629* printing method, of O26 EHEC and EPEC based on the variation in genomic location and copy numbers of IS*621*, (ii) to analyse a collection of O26 EHEC and EPEC isolated from humans and from cattle using the O26 IS*621* printing method and to compare the results with those obtained by PFGE and with the epidemiological data of the isolates and finally, (iii) to examine the applicability of the IS*621* printing method to the identification and tracing of O26:H11 EHEC during outbreaks.

Materials and methods

Bacterial strains and isolates

The collection of 69 O26:H11 *E. coli* comprises 29 bEHEC, 21 hEHEC, 15 bEPEC and four hEPEC, including the sequenced hEHEC strain 11368 and the other ten bEHEC and hEHEC that were previously studied by WGPS analysis (Bardiau *et al.* 2010b; hereafter referred to as WGPSed EHEC) (Table 1). Most have already been reported in other publications (Szalo *et al.* 2002, 2004; Bardiau *et al.* 2009). The pair of Belgian hEHEC was isolated from the same patient at a 1-month interval (epidemiological group 1: EG1; D. Piérard, unpublished data), the two pairs of Belgian bEHEC were isolated from two different veal calves (EG2 and EG3; Bardiau *et al.* 2010a) and the quartet of Dutch bEPEC was isolated from two different calves in the same farm (EG4; H. Imberechts, personal communication), each comprised epidemiologically related isolates (Table 1). Some American

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bEHEC and bEPEC from 1991 (Cray *et al.* 1996) and the two Irish bEHEC (Kerr *et al.* 1999) may also be epidemiologically related (Table 1), but their case history data could unfortunately not be recovered (H.J. Ball, T.O. Bunn, personal communications). The other EHEC and EPEC were not related (T.A. Casey, H. Imberechts, I.

Table 1 Origin, pathotypes and serotypes of the collection *Escherichia coli* (blank cells in the 3rd, 4th and 6th columns mean 'no data')

Pathotype	Host	Age*	Status	Country†	Year	Isolate‡	*eae*	*stx*	*wzx-wzy*$_{O26}$	*fliC*$_{H11}$	WGPS§
EHEC	Calf		Diarrhoeic	USA	1961	TC193	+	1	+	+	
	Calf		Diarrhoeic	USA	1963	TC659	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3108	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3109	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3117	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3180	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3269	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3273	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3302	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3305	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3375	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3380	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3629	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3630	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3631	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3632	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3656	+	1	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3657	+	1	+	+	
	Calf		Diarrhoeic	USA	1989	DEC10E	+	1	+	+	
	Cattle			GB	1991	A39	+	2	+	+	
	Cattle			IRL	<1999	122	+	1	+	+	+
	Calf		Diarrhoeic	IRL	<1999	4276	+	1	+	+	+
	Calf		Diarrhoeic	B	1987	357S89	+	1	+	+	+
	Calf		Diarrhoeic	B	1989	379S89	+	1	+	+	+
	Calf			I	1992	ED80	+	1	+	+	+
	Calf	<0·5	Diarrhoeic	B	2008	11·2‡	+	1	+	+	
	Calf	<0·5	Diarrhoeic	B	2008	11·4‡	+	1	+	+	
	Calf	<0·5	Diarrhoeic	B	2008	20·1‡	+	1	+	+	
	Calf	<0·5	Diarrhoeic	B	2008	20·2‡	+	1	+	+	
	Human		Diarrhoeic	JP	2001	11368	+	1	+	+	+
	Human		Diarrhoeic	JP	2001	13247	+	1	+	+	+
	Human			GB	1967	H19	+	1	+	+	
	Human		HUS	GB	1967	H30	+	1	+	+	
	Human		Diarrhoeic	USA	1961	DEC9A	+	1	+	+	
	Human		Diarrhoeic	USA	1966	DEC9B	+	1	+	+	
	Human	Infant	Diarrhoeic	USA	1977	DEC10C	+	1	+	+	
	Human		Diarrhoeic	B	1989	EH031	+	1	+	+	
	Human		Diarrhoeic	B	1994	EH182	+	1	+	+	+
	Human		Diarrhoeic	B	1994	EH193‡	+	2	+	+	+
	Human		Diarrhoeic	B	1994	EH196‡	+	2	+	+	
	Human		Diarrhoeic	B	1996	EH296	+	2	+	+	+
	Human		Diarrhoeic	B	1996	EH298	+	2	+	+	
	Human		Diarrhoeic	B	1995	EH284	+	1	+	+	
	Human		Diarrhoeic	B	1996	EH322	+	1	+	+	
	Human		Diarrhoeic	B	1996	EH324	+	1	+	+	
	Human	<1		F	1999	99/109	+	2	+	+	
	Human	<1		F	1999	99/147	+	1	+	+	
	Human	1–5		F	2002	02/113	+	1	+	+	
	Human	1–5		F	2003	03/139	+	1	+	+	
	Human	1–5		F	2003	03/151	+	1	+	+	+

Table 1 (*Continued*)

Pathotype	Host	Age*	Status	Country†	Year	Isolate‡	*eae*	*stx*	*wzx-wzy*$_{O26}$	*fliC*$_{H11}$	WGPS§
EPEC	Cattle	<0·5	Healthy	USA	1991	TC3145	+	−	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3486	+	−	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3748	+	−	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC3848	+	−	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC4004	+	−	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC4219	+	−	+	+	
	Cattle	<0·5	Healthy	USA	1991	TC4221	+	−	+	+	
	Cattle			IRL	<2003	C15333	+	−	+	+	
	Cattle			GB	1991	A14	+	−	+	+	
	Calf		Diarrhoeic	NL	1991	333KH91‡	+	−	+	+	
	Calf		Diarrhoeic	NL	1991	351KH91‡	+	−	+	+	
	Calf		Diarrhoeic	NL	1991	352KH91‡	+	−	+	+	
	Calf		Diarrhoeic	NL	1991	354KH91‡	+	−	+	+	
	Calf	0·5	Diarrhoeic	B	1991	631KH91	+	−	+	+	
	Cattle			IRL	<1999	63	+	−	+	+	
	Human		Healthy	CH	1952	C240-52	+	−	+	+	
	Human			BR	1981	7-81 HSJ	+	−	+	+	
	Human	1–5		F	2000	00/106	+	−	+	+	
	Human	1–5		F	2000	00/113	+	−	+	+	
non-H11	Cattle		Diarrhoeic	USA	1962	TC282	−	−	+	−	
O26	Human	1–5		F	1999	99/145	−	−	+	−	
E. coli	Human			F	2002	02/057	−	−	+	−	
	Human	<1		F	2002	02/145	−	−	+	−	
	Human	6–14		F	2003	03/023	−	−	+	−	
	Human			F	2003	03/178	−	−	+	−	
	Human			IRL		C4071	−	−	+	−	
	Piglet	0·03	Diarrhoeic	USA	1963	TC652	−	−	+	−	
	Avian			F	1999	739	−	−	+	−	

HUS, haemolytic–uraemic syndrome; WGPS, whole-genome PCR scanning; EHEC, enterohaemorrhagic *Escherichia coli*; bEHEC, bovine EHEC; hEHEC, human EHEC; EPEC, enteropathogenic *Escherichia coli*; bEPEC, bovine EPEC.

*In years.

†B, Belgium; BR, Brazil; F, France; GB, Great Britain; IRL, Ireland; I, Italy; JP, Japan; CH, Switzerland; NL, the Netherlands; USA, United States of America.

‡Epidemiologically related isolates are in bold (see Materials and Methods): EH193 and EH196 hEHEC form epidemiological group (EG) 1; 11·2 and 11·4 bEHEC, EG2; 20·1 and 20·2 bEHEC, EG3; 333KH91, 351KH91, 352KH91 and 354KH91 bEPEC, EG4.

§+: isolates studied by the WGPS method (Bardiau *et al.* 2010b).

Filliol, H.J. Ball, personal communications; D. Piérard and T. Hayashi unpublished data). Nine *eae*− and *stx*− negative avian, bovine, human and porcine non-H11 O26 *E. coli* were also included in the study.

The O26:H11 outbreak EHEC isolates (Table 2) were two groups of hEHEC isolated from human faeces during two independent outbreaks in Fukuoka, Japan in 1997 (Horikawa *et al.* 1998) and 2003 and one group of EHEC isolated in the north of Belgium (province of Antwerp) in 2007 during an outbreak associated with the consumption of ice cream produced at a dairy farm, from a human with HUS, from the home-made ice cream and from the farm environment (overshoes) (De Schrijver *et al.* 2008).

Prior to carrying out any other test, all isolates were tested for confirmation with PCRs specific to the *wzx-wzy* and *fliC* genes coding for the O26 and H11 antigens, respectively, to the *eae* gene coding for the intimin adhesin, and to the *stx1* and *stx2* genes coding for Stx 1 and 2, respectively (Tables 1, 2 and 3a) (Oswald *et al.* 2000; Bardiau *et al.* 2009).

DNA sequencing and sequence analysis

Genome sequence analysis of the O26:H11 hEHEC strain 11368 identified 13 complete (1277 bp long) and one truncated (72 bp long at the left (5′)-end) chromosomal copies of IS*621* (Ogura *et al.* 2009). Sequencing analysis of one additional hEHEC from Belgium (EH296) and two bEHEC from Ireland (4276) and from Belgium (379S89) using Sequencing by Oligonucleotide Ligation and Detection (SOLiD, Applied Biosystems Inc., Tokyo, Japan) identified four additional IS*621* insertion sites (T. Ooka,

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Table 2 Origin, pathotypes and serotypes of the outbreak EHEC

Outbreak (reference)	No. of isolates	Origin	Status (no. of isolates)	*eae*	*stx*	*wzx-wzy*$_{O26}$	*fliC*$_{H11}$	IS*621* fingerprint	PFGE profile
Japan 1: 1997 (Horikawa *et al.* 1998)	10	Human	Diarrhoeic (9) Asymptomatic (1)	+	1	+	+	6753–	Profile 1
Japan 2: 2003	11	Human	Diarrhoeic (2) Asymptomatic (9)	+	1	+	+	4753–	Profile 2
Belgium: 2007 (De Schrijver *et al.* 2008)	One	Human	HUS	+	1	+	+	6153–	Profile 3 (De Schrijver *et al.* 2008)
	Three	Farm environment (overshoes)	–	+	1	+	+	6153–	
	Five	Ice cream produced at the farm	–	+	1	+	+	6153–	

HUS, haemolytic–uraemic syndrome; PFGE, pulsed field gel electrophoresis; EHEC, enterohaemorrhagic *Escherichia coli*.

T. Itoh and T. Hayashi, unpublished data); each of the four EHEC harboured eight to 14 copies of IS*621*. The 18 IS*621* insertion sites were assigned numbers 1–18 according to their genomic locations (Fig. 1).

The sequences located left and right of each of the 14 IS*621* insertion sites in the hEHEC strain 11368 were available from its published sequence (Ogura *et al.* 2009). The sequences located left and right of each of the four additional copies of IS*621* were determined by PCR amplification of these genomic regions from the appropriate EHEC with two IS*621* internal outward primers designed from the highly conserved left and right end regions of IS*621* (OW_L and OW_R primers; Table 3b) and PCR product–directed sequencing using the ABI 3710 auto-sequencer (Applied Biosystems Inc.), as previously described (Ogura *et al.* 2008) (DDBJ/GenBank/EMBL accession numbers AB583558, AB583559, AB583560, AB583561, AB583562, AB583563, AB583564 and AB583565).

IS*621* detection PCRs

The presence and copy numbers of IS*621* in the 11 WGPSed EHEC were determined by PCRs using external primers located outside the left (5′) and right (3′) IS/chromosome junctions of each IS*621* copy (OS_L and OS_R primers; Table 3b). As far as possible, OS primers were located at different distances from the ends of each IS*621* copy, within a range of *c.* 1 kbp, as described for the IS*629* in O157 *E. coli* (Ooka *et al.* 2009b). The final concentration of each primer was 0·4 μmol l^{-1}.

For the IS*621* detection PCRs, each pair of OS_L and OS_R primers was individually tested on the 11 O26 WGPSed EHEC after overnight growth on Luria–Bertani agar at 37°C and DNA extraction from one colony by the alkaline-boiling method, as previously described (Ooka *et al.* 2009a). The DNA template was 1 μl of the supernatant that was obtained after the alkaline-boiling extraction in a total reaction volume of 15 μl using the Blend-Taq PCR kit (Toyobo, Osaka, Japan). The PCR conditions were as follows: initial denaturation at 96°C for 2 min; 30 cycles of denaturation for 30 s at 96°C, annealing for 30 s at 60°C, elongation for 2 min at 72°C; and final elongation at 72°C for 5 min. The amplicons were detected by electrophoresis in 2% agarose S gels (Nippon Gene, Tokyo, Japan). Lambda DNA digested by *Sty*1 (Marker 6; Nippon Gene) and the 100-bp ladder (Sigma Genosys, Ishikari, Japan) were used as size markers.

IS*621* printing PCRs

Four initial multiplex printing PCRs were set up by combining two sets of nine OS_L primers with the OW_L primer and two sets of nine OS_R primers with the OW_R primer and tested on the 11 O26 WGPSed EHEC. After comparing the results of the detection and initial printing PCRs, two final multiplex printing PCRs were set up targeting either the left or right IS/chromosome junction. The final concentration of each OS primer was 0·1 μmol l^{-1}, and the final concentration of the OW primer was '×' times 0·1 μmol l^{-1} (× being the number of OS primers in the primer mix).

The DNA template was 5 μl of the supernatant that was obtained after alkaline-boiling extraction in a total reaction volume of 25 μl using the KOD Dash kit (Toyobo, Asaka, Japan), and the amplification parameters were optimized as follows: an initial denaturation step at 94°C for 2 min; 20 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 64°C, elongation for 30 s at 68°C; and no final elongation step. The amplicons were detected by electrophoresis in 3·2% agarose S gels (Nippon Gene). The 100-bp ladder (Sigma Genosys) was used as a size marker.

Table 3 Primers used in this study

Primers		Sequence (5′–3′)	Target gene/sequence	Size in bp of the amplified fragment (reference)
(a) Typing primers				
wzx-wzy$_{O26}$	F	AAATTAGAAGCGCGTTCATC	*wzx-wzy*$_{O26}$	596 (Bardiau *et al.* 2009)
	R	CCCAGCAAGCCAATTATGACT		
fliC$_{H11}$	F	ACTGTTAACGTAGATAGC	*fliC*$_{H11}$	224 (Bardiau *et al.* 2009)
	R	TCAATTTCTGCAGAATATAC		
eae	F	CCCGAATTCGGCACAAGCATAAGC	*eae*	881(Oswald *et al.* 2000)
	R	CCCGGATCCGTCTCGCCAGTATTCG		
stx1	F	GTCATTCGCTCTGCAATAGGTAC	*stx1*	151 (Ooka *et al.* 2009b)
	R	GCCGTAGATTATTAAACCGCCCT		
stx2	F	CCATGACAACGGACAGCAGTT	*stx2*	181 (Ooka *et al.* 2009b)
	R	CTGCTGTGACAGTGACAAAACG		

				Detection PCR		
				With IS*	Without IS	Printing PCR
(b) IS*621* primers						
IS*621*_OS_1	L†	CTCTGGCGATGCAGCGATG	C_006_007‡	2132	853	328
	R†	AGTAATCGGCGGTATACGGG				659
IS*621*_OS_2	L	CTTAAGCGTCTGTAGGTCGGA	C_013_014	1647	368	162
	R	ACGGACAGGTACCGGAACG				340
IS*621*_OS_3	L	TAGCGCCCGATACGGCGG	C_025_026	1528	249	189
	R	AGGCGGGTCGCTTTGTTGG				194
IS*621*_OS_4	L	AACAGTTTAGGCGCGACGCC	C_029.2_029.3	711§	639	510
	R	CGCCGTGACGTTAAGCTCC				–§
IS*621*_OS_5	L	CTGGAAGAAAATCAGATCCCAG	C_051_052	1630	351	255
	R	AAGGCGATGACCCGACGCC				230
IS*621*_OS_6	L	GGTTTCTGCCGATGGCGCA	C_087_088	1734	455	292
	R	GGTGGTAATGGTAAGGGCAAG				297
IS*621*_OS_7	L	CGCGTGATCGACTTTAGCGG	C_089_090	1979	700	216
	R	GTACTGTTACGTCCGCTGC				618
IS*621*_OS_8	L	GGTATCGACCCTACGCTGG	C_101_102	2652	1373	792
	R	AGCGGCCTGACACTGATGC				715
IS*621*_OS_9	L	GGTGATCGTTCGCTGGAGGT	C_182_183	1901	622	369
	R	ACACCTTAAACCGATTGCGCT				387
IS*621*_OS_10	L	CGATGAACTGGCTGTTCTGG	C_311_312	1926	647	617
	R	TGCCATGAGCATCATCGACG				164
IS*621*_OS_11	L	CTGGCAGATCTGTTTGGTTTC	C_327_328	2075	796	457
	R	CATACGCGATGGTGTGCATCA				473
IS*621*_OS_12	L	CTCGTGACCTACAATCTGTCA	C_366_411	2273	994	561
	R	TCTTCGCCATATCTTCGTGCA				567
IS*621*_OS_13	L	TCAGCCCATACGCTGAGCAC	C_411_410	2126	847	418
	R	TCGTCGCTCCATGAGAAGC				563
IS*621*_OS_14	L	CTGAATACGGCGAGCAACGT	C_406_405	2482	1203	666
	R	GTGTAAGGAGCATAAGGAATGA				671

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Table 3 (*Continued*)

				Detection PCR		
				With IS*	Without IS	Printing PCR
IS*621*_OS_15	L	ACGCGCCTGTTCCCGTAGC	C_367.1_413	2272	993	709
	R	AGATGCGCCCCGCATTCGG				418
IS*621*_OS_16	L	ACCCGTTCTGCTCTGCAGTAC	C_435_436	2688	1409	771
	R	CTGGAACTTACCGATAATCAAG				772
IS*621*_OS_17	L	GATCAGGTAGATCAACTGGCA	C_444_445	2871	1592	871
	R	GCCTGATGGTGCTGGAAGAG				855
IS*621*_OS_18	L	TCCGTTGCCGTCGAGAGC	C_457_458	1854	575	570
	R	ACAGCCTGAAAGCCGCGCC				139
IS*621*_OW	L¶	TACGGCATGCGGACTGCAG	IS*621*	–	–	–
	R¶	TGTCTGGCATGCCTGCAATC				–
IS*621*_IN	L	CTGCAGTCCGCATGCCGTA	IS*621*	–	–	–
	R	GATTGCAGGCATGCCAGACA				
(c) Redesigned IS*621* primers						
IS*621*_OS_1	R_new	AGTACTGCCCCATACGCAGC	C_006_007	2252	973	779
IS*621*_OS_5	L_new	TGCTGCTGGAAGAAAATCAGATC	C_051_052	1635	356	260
IS*621*_OS_13	L_new	CATACGCTGAGCACGGCAAG	C_411_410	2120	841	412
IS*621*_OS_14	R_new	TTCCTGGCATCTGGCAAGGG	C_406_405	2068	789	257
IS*621*_OS_16	L_new	TGCGTGGTCAGAACG	C_435_436	2458	1179	983
	R_new	TCAGGTCTATGTTCGCACCG				330

*IS*621* is 1277-bp long and its insertion is accompanied by a 2-bp duplication.
†Combined with the OW_L primer to target the left (5′) IS/chromosome junction and with the OW_R primer to target the right (3′) IS/chromosome junction, respectively.
‡Coordinates of the IS*621* insertion site according to the coordinates of the corresponding whole-genome PCR scanning primers
§Truncated copy of IS*621*: only 72 bp are present at the left (5′) end.
¶IS*621*_OW_L begins 69 bp far from the left (5′) end of the IS; IS*621*_OW_R begins 65 bp far from the right (3′) end of the IS.

Pulsed Field Gel Electrophoresis

PFGE analyses were performed as already described (Cobbaut *et al.* 2009; Ooka *et al.* 2009b). In brief, bacterial cells were embedded in 0·9% Certified Low-Melt Agarose (Bio-Rad Laboratories Inc., Tokyo, Japan), lysed with a buffer containing 0·2% sodium deoxycholate, 0·5% N-lauroylsarcosine and 0·5% Brij-58, and treated with 100 μg ml^{-1} proteinase K. Restriction fragments of *Xba*I-digested genomic DNA were separated using CHEF MAPPER (Bio-Rad) with 1% pulsed field certified agarose (Bio-Rad) at 6·0 V cm^{-1} for 22 h and 18 min, with pulsed times ranging from 47 to 44·69 s. The molecular weight marker was the lambda ladder starting from 48·5 kbp with 48·5 kbp increments (Bio-Rad). The size of each DNA band was estimated by lane analyzer (Atto Corp., Tokyo, Japan). The banding patterns were analysed using the Dice coefficient, with an optimization and position tolerance of 1%. Dendrograms were prepared by the Unweighted-Pair Group Method using arithmetic average Algorithm.

Results

Identification of informative IS*621* insertion sites

To identify informative IS*621* insertion sites, we first determined the presence or absence of IS*621* in the 18 insertion sites for each of the 11 WGPSed EHEC (including the sequenced strain and the three strains analysed by SOLiD sequencing) by the detection PCRs using the 18 OS_L/OS_R pairs of primers: the predicted sizes of amplified fragments were between 249 and 1592 bp when IS*621* was absent and between 711 and 2871 bp when the IS*621* copy was present (Table 3b). We then performed the four initial multiplex printing PCR analyses of the 11 WGPSed EHEC: the predicted sizes of the amplified fragments ranged from *c.* 139 to 983 bp depending on which IS*621* copy was present; in the case of absence of IS*621*, no amplified fragment was expected (Table 3b). Six primers were redesigned because they failed to work properly in the multiplex printing PCRs (Table 3c).

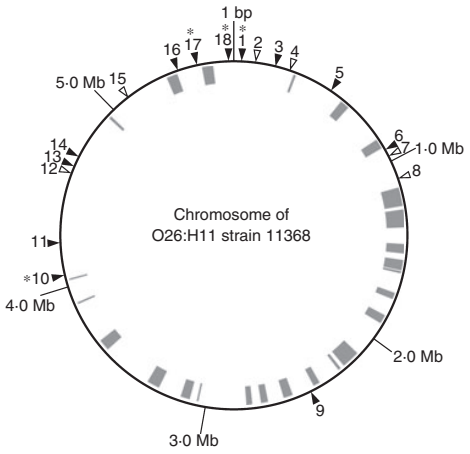

Figure 1 Location of the 18 copies of IS*621* on the chromosome of the human enterohaemorrhagic *Escherichia coli* (hEHEC) sequenced strain 11368 (Ogura *et al.* 2009). (▶) The 12 IS*621* insertion sites targeted in the final multiplex printing PCRs; (▷) Additional IS*621* insertion sites; (*) The 4 IS*621* insertion sites not present on the chromosome of the hEHEC strain 11368 and (▥) Prophages and integrative elements.

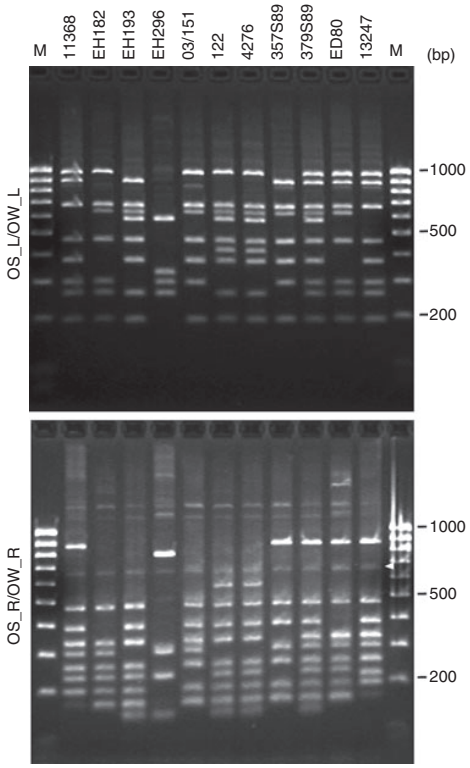

Figure 2 IS*621* final multiplex printing PCR_L targeting the left and PCR_R targeting the right IS/chromosome junction of the 11 O26:H11 WGPSed EHEC (Table 4) with 12 OS_L and the OW_L primers and with 12 OS_R and the OW_R primers, respectively. The molecular weight marker (M) is the 100-bp ladder (Sigma Genosys). The white arrow points out an unspecific amplification band.

After comparison of all the data, we identified 13 IS*621* informative insertion sites (1, 3, 4, 5, 6, 9, 10, 11, 13, 14, 16, 17 and 18; Fig. 1) with variations between the 11 EHEC that could be used as target sites in the IS*621* printing method. Five insertion sites were not informative: three (2, 7 and 8) because no variation was observed between the 11 WGPSed EHEC and two (12 and 15) because the detection and initial printing PCRs yielded multiple contradictory results and/or products with different sizes from the expected ones.

Development of the O26 IS*621* printing method

Two final multiplex printing PCRs were constructed targeting either the left or right IS*621*/chromosome junctions of 12 of the informative insertion sites with complete copies of IS*621* (PCR_L and PCR_R). The result of the detection PCR for insertion site 4 (truncated copy of IS*621*; Table 3b) was added as an additional marker. Taking into account those 13 insertions sites, the 11 O26 WGPSed EHEC tested positive for four to ten different insertion sites and yielded nine different amplification profiles (Fig. 2; Table 4). Nonagreement between PCR_L and PCR_R was observed at the height of insertion sites 16 and 17 in hEHEC EH193 (Table 4: shaded values). Two pairs of isolates yielded identical profiles: respectively, two Japanese hEHEC (11368 and 13247) positive for nine IS*621* insertion sites and two Irish bEHEC (122 and 4276), isolated from diarrhoeic calves, also positive for nine IS*621* insertion sites.

PFGE analysis revealed that the 11 O26 WGPSed EHEC had different PFGE patterns (Fig. 3). However, the two Irish bEHEC 122 and 4276 had very similar PFGE profiles with only a three-band difference suggesting that they are very closely related clones. In contrast, the two Japanese hEHEC 11368 and 13247 had distinct PFGE patterns.

To simplify the analysis and comparison of IS*621* fingerprints, the amplification profiles of the final multiplex printing PCR_L and PCR_R were presented according to the API20 numerical method originally developed in the

Table 4 IS*621* final multiplex printing PCR results on the 11 O26:H11 WGPSed EHEC

		IS insertion site	1	3	5	6	9	10	11	13	14	1 6	17	18	4*
		Size PCR_L†	328	189	260	292	369	617	457	412	666	983	871	570	
		Size PCR_R†	779	194	230	297	387	164	473	563	257	330	855	139	NR
EHEC isolates		Value‡	1	2	4	1	2	4	1	2	4	1	2	4	+/−
03/151	*eae*	PCR_L	−	+	−	+	+	+	+	−	+	+	−	−	
hEHEC	*stx1*	PCR_R	−	+	−	+	+	+	+	−	+	+	−	−	
France		Total value	2			7			5			1			−
357S89	*eae*	PCR_L	−	+	−	+	+	+	+	−	+	−	+	−	
bEHEC	*stx1*	PCR_R	−	+	−	+	+	+	+	−	+	−	+	−	
Belgium		Total value	2			7			5			2			−
EH296	*eae*	PCR_L	+	−	+	+	−	−	−	−	−	−	−	+	
hEHEC	*stx2*	PCR_R	+	−	+	+	−	−	−	−	−	−	−	+	
Belgium		Total value	5			1			0			4			−
11368	*eae*	PCR_L	−	+	+	+	+	−	+	−	+	+	+	−	
hEHEC	*stx1*	PCR_R	−	+	+	+	+	−	+	−	+	+	+	−	
Japan		Total value	6			3			5			3			+
13247	*eae*	PCR_L	−	+	+	+	+	−	+	−	+	+	+	−	
hEHEC	*stx1*	PCR_R	−	+	+	+	+	−	+	−	+	+	+	−	
Japan		Total value	6			3			5			3			+
EH182	*eae*	PCR_L	−	+	+	+	−	+	+	−	+	+	−	−	
hEHEC	*stx1*	PCR_R	−	+	+	+	−	+	+	−	+	+	−	−	
Belgium		Total value	6			5			5			1			+
ED80	*eae*	PCR_L	−	+	+	+	−	+	+	−	+	+	+	−	
bEHEC	*stx1*	PCR_R	−	+	+	+	−	+	+	−	+	+	+	−	
Italy		Total value	6			5			5			3			+
EH193	*eae*	PCR_L	−	+	+	−	+	+	+	−	+	−	+	+	
hEHEC	*stx2*	PCR_R	−	+	+	−	+	+	+	−	+	+	−	+	
Belgium		Total value	6			6			5			6/5§			−
122	*eae*	PCR_L	−	+	+	−	+	+	+	+	+	+	−	+	
bEHEC	*stx1*	PCR_R	−	+	+	−	+	+	+	+	+	+	−	+	
Ireland		Total value	6			6			7			5			−
4276	*eae*	PCR_L	−	+	+	−	+	+	+	+	+	+	−	+	
bEHEC	*stx1*	PCR_R	−	+	+	−	+	+	+	+	+	+	−	+	
Ireland		Total value	6			6			7			5			−
379S89	*eae*	PCR_L	−	+	+	+	+	+	+	−	+	+	+	+	
bEHEC	*stx1*	PCR_R	−	+	+	+	+	+	+	−	+	+	+	+	
Belgium		Total value	6			7			5			7			−

EHEC, enterohaemorrhagic *Escherichia coli*; bEHEC, bovine EHEC; hEHEC, human EHEC.
*The detection PCR of the IS*621* truncated copy 4 (72 bp long at the left (5′) end) with the OS_L and OS_R primers gave one amplified fragment of 711 bp long if the IS fragment is present (+) or of 639 bp long if the IS fragment is absent (–). NR, Not relevant.
†Size of the amplified fragments with the printing PCR with 12 OS_L and the OW_L primers and with 12 OS_R and the OW_R primers, respectively (see also Table 3b,c).
‡See Results for explanation (section: Development of the O26 IS*621* printing method).
§Non-agreement between PCR_L and PCR_R results.

late 1970s for the identification of *Enterobacteriaceae* (Smith *et al.* 1972; Willcox *et al.* 1980). The results were combined into sets of three, with each member of one triplet receiving the value of 1, 2 or 4, respectively, when the result is positive (Table 4). The value of each triplet varies between 0 and 7 and corresponds to only one possible combination of positive results. The results for the truncated copy were added as a '+' or a '–'.

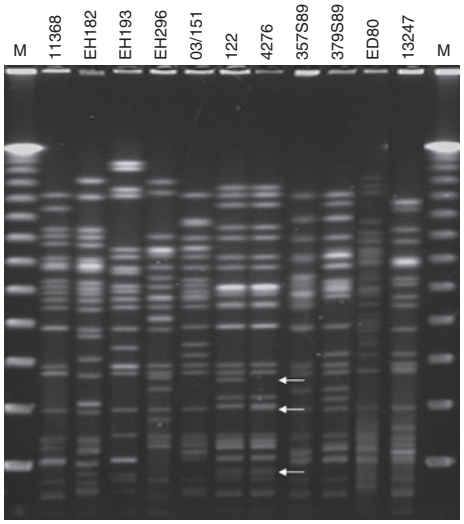

Figure 3 Pulsed field gel electrophoresis profile comparison of the 11 O26:H11 WGPSed EHEC. The molecular weight marker (M) is the lambda ladder starting from 48.5 kbp with 48.5 kbp increments (Bio-Rad Laboratories). The white arrows highlight the three-band difference between the two Irish bEHEC 122 and 4276 isolates.

IS*621* printing method of a collection of O26 bovine and human EHEC and EPEC

The two final multiplex printing PCR_L and PCR_R and the detection PCR for the truncated copy were subsequently applied on a collection of 29 bEHEC, 21 hEHEC (including the 11 WGPSed isolates), 15 bEPEC, four hEPEC and nine non-H11 O26 *E. coli* (Table 1). The bEHEC 4276 and the hEHEC 11368 were present in all gels as internal controls.

Amplified fragments were obtained from all 69 O26:H11 EHEC and EPEC with two to ten copies of IS*621* detected in each isolate: four insertion sites (1, 4, 14 and 18) yielded PCR fragments (IS*621* positive) from a minority (1–20) of isolates, while the remaining nine insertion sites (3, 5, 6, 9, 10, 11, 13, 16 and 17) yielded PCR fragments from a majority (40–62) of isolates. A total of 38 amplification profiles were obtained: 20 profiles were observed in only one isolate each, nine profiles in two isolates each, six profiles in three isolates each, two profiles in four isolates either and one profile in five isolates.

Nonagreement between PCR_L and PCR_R was observed 25 times at the height of seven different insertion sites in 17 isolates: insertion site 5 in three isolates, insertion site 6 in one isolate, insertion site 10 in four isolates, insertion site 13 in two isolates, insertion site 16 in three isolates, insertion site 17 in three isolates (including hEHEC EH193 as observed previously) and insertion site 18 in nine isolates, with eight isolates showing nonagreement at two different insertion sites: 6 and 13 (one isolate), 10 and 18 (four isolates), 16 and 17 (three isolates).

No fragment was amplified from any non-H11 O26 *E. coli*. Absence of IS*621* was confirmed by PCR with two IS*621* internal inward primers complementary to the OW_L and OW_R primers (IN_L and IN_R primers; Table 3b).

Comparison with PFGE profiles and epidemiological data

PFGE restriction profiles were obtained from 29 bEHEC, 20 hEHEC, 13 bEPEC and three hEPEC. One hEHEC (with unique IS*621* fingerprint), one hEPEC (grouping with IS printing group 2·1, but not epidemiologically related to) and two bEPEC (one forming a pair with another bEPEC and one grouping with IS printing group 4·3) gave no PFGE profile. A cut-off value of 85% similarity of PFGE profiles was chosen to assess the IS*621* multiplex PCR-based printing method of the O26:H11 EHEC and EPEC comparing their IS*621* fingerprints and PFGE profiles.

Forty-five (69%) of the 65 EHEC and EPEC with PFGE profiles had identical IS*621* fingerprints by groups of two, three or four isolates, respectively (Table 5). Thirty-two (71%) isolates within these pairs, triplets and quartets had >85% similarity of their PFGE profiles, including the EG1 to EG4 groups (IS printing groups 2·1, 2·2, 3·1 and 4·1), while most of the groups had <85% PFGE profile similarity between each others, with two noticeable exceptions (footnotes * and † of Table 5). Also noticeable was one quartet of EHEC isolates (IS printing group 4·3) made of one pair of American bEHEC of unknown epidemiological relation and of a second pair of epidemiologically unrelated hEHEC (one American, one British) with 100 and 94% PFGE similarity in either pair, respectively, but <50% between the two pairs (Table 5). The remaining 13 (29%) isolates within the pairs, triplets and quartets had <85% similarity of their PFGE profiles between each others (IS printing groups 2·8, 2·9, 3·4 and 3·5) or with other isolates sharing identical IS*621* fingerprints (within IS printing groups 3·1, 3·3 and 4·2) (Table 5). But when the results were based on the history data (country of origin, host, pathotype and year of isolation), only two of them, the Japanese hEHEC 11368 and 13247 (Table 5), still grouped together, sharing identical history data and IS*621* fingerprints while having <85% PFGE profile similarity (Fig. 3) and no confirmed epidemiological relation.

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Table 5 Comparison of the collection *Escherichia coli* isolates with identical IS*621* profiles: pathotype (EHEC, EPEC), host of origin (Bovine, Human), country of origin (B, Belgium; F, France; GB, Great Britain; IRL, Ireland; I, Italy; JP, Japan; CH, Switzerland; NL, the Netherlands; USA, United States of America), year of isolation (1952–2008) and PFGE percentage similarity. Epidemiologically related isolates are in bold (EG1–EG4: see also Materials and Methods)

IS printing group	IS fingerprint	Isolate	Pathotype	Host	Country	Year	>85% PFGE similarity	Epidemiological link
2·1	6656/5–	EH193	EHEC	H	B	1994	+	EG1
		EH196						
2·2	2711–	20·1	EHEC	B	B	2008	+	EG2
		20·2						
2·3	6675–	122	EHEC	B	IRL	<1999	+	Unknown
		4276						
2·4	6751+	TC3108	EHEC	B	USA	1991	+	Unknown
		TC3109						
2·5	6753–	TC3180	EHEC	B	USA	1991	+	Unknown
		TC3273						
2·6	2353–	TC3302	EHEC	B	USA	1991	+	Unknown
		TC3305						
2·7	4551+	TC3656	EHEC	B	USA	1991	+*	Unknown
		TC3657						
2·8	2753–	A39	EHEC	B	GB	1991		None
		99/109		H	F	1999	–	
2·9	6353+	11368†	EHEC	H	JP	2001		None
		13247					–	
3·1	6373–	03/139	EHEC	H	F	2003	NA‡ –	NA None
		11·2		B	B	2008	+	EG3
		11·4						
3·2	6743+	TC3630	EHEC	B	USA	1991	+*	Unknown
		TC3631						
		TC3632						
3·3	4553+	DEC10E	EHEC	B	USA	1989	NA –	None
		TC659				1963	+	
		DEC9B		H		1966		
3·4	6553+	ED80	EHEC	B	I	1992		None
							–	
		TC3117			USA	1991		
		TC193§				1961		
3·5	6753+	EH284†	EHEC	B	B	1995		None
		DEC10C			USA	1997	–	
		EH324			B	1996		
4·1	67/345/1–	333KH91	EPEC	B	NL	1991	+¶	EG4
		351KH91						
		352KH91						
		354KH91						
4·2	6745/1–	C240-52	EPEC	H	CH	1952	NA –	None
		A14		B	GB	1991	+	
		63			IRL	<1999		
		C15333				<2003		
4·3	2553–	TC3375	EHEC	B	USA	1991	+ –	Unknown or none
		TC3380						
		DEC9A		H		1961	+	
		H30			GB	1967		

PFGE, pulsed field gel electrophoresis; EPEC, enteropathogenic *Escherichia coli*; bEPEC, bovine EPEC; EHEC, enterohaemorrhagic *Escherichia coli*; bEHEC, bovine EHEC; hEHEC, human EHEC; EG, epidemiological group.

*90–94% PFGE profile similarity was observed between American bEHEC of groups 2·7 and 3·2, and TC3629 (IS*621* fingerprint 6543 +).

†89% PFGE profile similarity was observed between Japanese hEHEC 11368 (group 2·9) and Belgian hEHEC EH284 (group 3·5).

‡NA, not applicable.

§85% PFGE profile similarity between American bEHEC TC193 (group 3·4) and Belgian hEHEC EH031 (IS*621* fingerprint 6573–).

¶88–95% PFGE profile similarity was observed between Dutch bEPEC of group 4·1 and Belgian bEPEC 631KH91 (IS*621* fingerprint 6345/1–).

Of the 20 O26:H11 EHEC and EPEC (31%) with unique IS*621* fingerprints, 17 (85%) had <85% similarity of their PFGE profiles with all other isolates, but three (TC3629, EH031 and 631KH91; 15%) had >85% PFGE profile similarity with other isolates (footnotes *, § and ¶ of Table 5). Of these, only the American bEHEC isolate TC3629 is worth mentioning for sharing history data and high percentage PFGE profile similarity (90–94%) with five other American bEHEC (footnote * of Table 5).

Finally, no bEPEC or hEPEC shared IS*621* fingerprints or >85% PFGE profile similarity with any EHEC isolate. Four bEPEC formed the EG4 (or IS printing group 4·1), and one hEPEC and three bEPEC formed the IS printing group 4·2 (Table 5). The remaining six bEPEC and two hEPEC had unique combinations of IS*621* fingerprint and PFGE profile similarity.

Outbreak EHEC isolates

As the IS*621* fingerprint analysis must clearly take into account the origin of the isolates, three groups of EHEC from independent outbreaks in Japan and Belgium (Table 2) were compared. The two groups of Japanese hEHEC isolated during two outbreaks gave two different IS*621* fingerprints: 6753− for the isolates from outbreak 1 (Horikawa *et al.* 1998) and 4753− for the isolates from outbreak 2. Isolates of either group also gave similar but not identical PFGE profiles with a difference of a few bands (not shown). All Belgian EHEC isolated from one human with HUS, from the dairy farm environment (overshoes) and from the home-made ice cream (De Schrijver *et al.* 2008) gave identical IS*621* fingerprints (6153−) and PFGE profiles.

Discussion

Like the O157 IS*629* printing method (Ooka *et al.* 2009b), the IS*621* printing method with the two final multiplex printing PCR_L and PCR_R targeting 12 IS*621* insertion sites and the additional detection PCR of the truncated copy represents a rapid (24 h) first-line surveillance typing assay of O26:H11 EHEC and EPEC, complementary to, though less discriminatory than the PFGE method. Nevertheless, the IS*621* printing results of the four groups of epidemiologically related hEHEC, bEHEC and bEPEC (EG1–EG4; Table 5) and of the Japanese and the Belgian outbreak EHEC (Table 2) confirm that this typing assay can be applied to compare and trace back isolates during surveys in farms, multiple human cases and outbreaks. The numerical method, which was introduced to transform the printing patterns into digital data (Table 4), makes it possible to perform prompt and accurate interlaboratory data comparison. Such a numerical system can also facilitate the construction of a worldwide database.

Still, 29% of the isolates with identical IS*621* fingerprints have <85% PFGE profile similarity, and 15% of the isolates with unique IS*621* fingerprints are related by their PFGE profiles to other isolates. Although the results are much more conclusive when isolates from the same country, host, pathotype and year of isolation are compared, more field studies are necessary to assess the actual influence of such results on the validity of the IS*621* printing method. At this stage of our knowledge, one purely speculative explanation of such results is that genetic rearrangements (deletions, inversions and insertions) may have influenced results of either or both tests depending on the involvement of restriction sites for PFGE and/or of IS*621* position and surrounding sequences. Besides single mutation at the height of one primer sequence, genetic rearrangements like deletion of either IS*621* flanking region, or inversion of a DNA fragment involving two IS*621* copies may also explain modifications of the IS*621* fingerprints and nonagreements between PCR_L and PCR_R results. However, far from complicating the comparison of EHEC and EPEC during outbreaks, these genetic rearrangements actually increase the discriminatory power of the IS*621* printing method. Although all copies of IS*621* are located on the chromosomal backbone (Fig. 1), and not on prophages or plasmids, avoiding the short-term data instability that can be induced by deletion of these genetic elements during outbreak periods, storage and/or repeated cultivation in laboratories (Iguchi *et al.* 2006; Ogura *et al.* 2009; Ooka *et al.* 2009b), additional research work is also clearly needed to follow the *in vitro* and *in vivo* stability of the IS*621* fingerprints of O26:H11 EHEC and EPEC and of their PFGE profiles, like already performed for O157:H7 EHEC (Iguchi *et al.* 2002).

Another difficulty is the comparative analysis of the results obtained with aEPEC belonging to the same serotypes as EHEC, like O26:H11, as the former can derive from the latter by loss of the Stx-encoding phages, and *vice versa* by phage transfer, either *in vivo* or *in vitro* (Karch *et al.* 1992; Bielaszewska *et al.* 2007). Nevertheless, this does not seem to be the case in this study because none of the O26:H11 bEPEC and hEPEC (from Belgium, Brazil, Great Britain, Ireland, Switzerland, the Netherlands and USA) group with any EHEC, either by their IS*621* fingerprints or by their PFGE profiles.

As a conclusion, the results of additional laboratory and field studies will help not only in refining the discriminatory power of the O26 IS*621* printing method as an epidemiological tool, but also in understanding the putative divergent evolution of the different pathogenic and nonpathogenic *E. coli* carrying the O26 somatic antigen. This is especially true for the non-H11 O26 *E. coli* yielding no

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amplification profile, which may belong to another evolutionary lineage than the O26:H11 EHEC and EPEC. In the future, it will also be most interesting to check whether similar IS printing methods could be developed for other EHEC serotypes that have been sequenced so far, such as O103 and O111 (Ogura *et al.* 2009).

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7. General discussion

7.1. What is the problem?

The public health concern of enterohaemorrhagic and enteropathogenic *Escherichia coli* is now well established in developed countries. Foodstuffs contaminated mainly with cattle faeces, which act as healthy carrier of EHEC, infect humans. Both EPEC and EHEC strains can cause food poisoning with diarrhoeas. Moreover, Haemorrhagic Colitis (HC) generally accompanies EHEC infections and, in a few cases, Haemolytic Uremic Syndrome (HUS) appears and can lead the patient to death. Besides, some EPEC and EHEC strains specific serogroups (such as O26) are directly associated with diarrhoea in calves.

For such strains (O26 EPEC and EHEC that can be carried in healthy ruminants, cause disease in humans and in calves), the host specificity remains unclear. To date, no one knows if these strains are homogeneous inside the same serogroup (the same strain that can lead to the three different situations) or if some strains are specific to humans or calves. If such host specificity exists, the initial adherence via specific adhesins could represent a good candidate to investigate, considering that this pathogenic step is the host specificity basis for many pathogens, including other pathogenic *E. coli* (such as REPEC).

Therefore, the principal objective of this work was to address the of O26 EHEC and EPEC strains host specificity question and to identify specific factors that could be involved in this host specificity, keeping in mind that initial adherence factors may be the right candidate.

We approached this thesis by two different ways:

(i) The identification of O26 EHEC factors implicated in initial attachment and/or in host specificity (human or cattle) by analysing already described adherence factors (chapter 4) and searching new virulence factors implicated in the host specificity or in the initial adherence by Suppressive Subtractive Hybridisation method (chapter 5);

(ii) The whole genome comparison of EHEC strains isolated from human and cattle by comparing bovine and human strains genomes with Whole Genome PCR Scanning and multiplex PCR-based IS fingerprint methods (chapter 6). In addition, this last method could be used as a new first-line epidemiological typing method in association with current ones (e.g. PFGE).

Moreover, in view to obtain new O26 EPEC and EHEC strains from ruminants, we performed a prevalence study in veal calves and wild ruminants in Belgium.

7.2. Are O26 EPEC and EHEC present in veal calves and wildlife in Belgium?

The collection of new EPEC, EHEC and VTEC for further analyses gave us the opportunity to study the prevalence of these strains in veal calves (Article 2) in the North of Belgium (Antwerp) and wild cervids (Article 3) in the Walloon region, the south part of Belgium. Unlike most prevalence studies, we did not focus the search of strains on the serogroup. Instead, we used a random pick up of the isolated strains. Using this method, we probably get false negative animals and the "real" prevalence is most likely higher. Nevertheless, we surely obtain here a better representation of the real transmission risk of such human pathogenic strains. Indeed, many serogroups other than the common ones (e.g. O157, O26, O111, O103, O145) are also responsible for individual cases and outbreaks in human worldwide. For example, in 2008, 66% of the cases in Germany were due to strains that do not belong to the five major serogroups. One other special feature of this study is that we performed it on veal calves (and not on beef or dairy cattle) and on free wild cervids (and not reared animals).

We showed in our study that the EPEC, EHEC and VTEC strains prevalence in Belgium is about 12% in veal calves and 15% in wild cervids. In addition, about 4% of the calves carried O26 or O111 serogroup strains, which are often associated with human infections. Concerning the other strains that do not belong to a common serogroup (i.e. all the cervids strains and two-third of the calves strains), they could be emerging pathogenic serogroups. The former may lead to human diseases and could thus represent a zoonotic risk that is worth to pay attention to, or animal-specific strains.

Finally, in parallel to our main goal, we decided to study the collected strains resistance for several commonly used antibiotics. Results for the veal calves are significantly different from those obtained for the wild cervids: 83% of strains isolated from calves are resistant to two or more antibiotics in comparison to only 21.6% of the strains isolated from wild animals. In terms of human treatment, the EHEC or VTEC strains antibiotic resistance results are not relevant. Indeed, the use of antibiotics are generally contra-indicated during Stx-producing *E. coli* infection, because they can induce the toxin production which, in turn, can lead to HUS. Nevertheless, these results can be interpreted as an evidence of antibiotics utilisation overuse in veterinary practice and animal food production fields.

7.3. Are the already described adherence factors host-specific?

Before tempting to search for new virulence factors that could be involved in initial adherence or host specificity, we decided to study the potential association between the pre-existing adhesins and their host. Indeed, in the last decade, many new adhesins have been described in EPEC, EHEC and VTEC strains without having been systematically studied for their prevalence in relation with the host. Moreover, some polymorphisms in Tir were recently associated with human infection in O157 EHEC strains (Bono *et al.*, 2007).

The first study that we performed is the search of 27 described adhesins in a O26 EPEC and EHEC strains collection (Article 4). This study revealed the adhesins tendency to be present or not in serogroup O26 strains: presence in most of the strains of *loc3, loc5, loc7, loc11, loc14, paa, efa1, iha, lpfA-O26* and *lpfA-O113* genes and absence of *loc1, loc2, loc6, loc12, loc13, saa* and *eibG* genes. Concerning a possible host association, none of these adhesins was statistically associated with strains isolated from human or bovine. Only the *ldaE* gene was more often found in EHEC and a-EPEC strains isolated from diarrhoeic bovine in comparison to healthy bovine.

tir, eae and *tccP2* genes polymorphisms results were disappointing concerning potential host specificity (Article 5). Firstly, polymorphisms in *tir* and *eae* genes were not frequent and none of them were associated with bovine or human isolates. Secondly, different variants of *tccP2* were detected and two of them were associated with a specific pathotype (AB253564 variant with EHEC strains and AB275131 variant with a-EPEC strains) but none with any specificity host.

Therefore, according to our results, none of the already described factors seems to be host-associated in the case of O26 strains.

7.4. What about the genomic plasticity of the O26 strains?

The diversity and plasticity in O26 EHEC and EPEC strains genome contents were supported by the results obtained by the Suppressive Subtractive Hybridisation (SSH) and by the Whole Genome PCR Scanning (WGPS).

Firstly, the SSH revealed the presence of DNA sequences with similarities to specific genes or pathogenicity islands (PAIs). These genes are present in other specific *E. coli* pathotypes (e.g. PAI I_{CL3}) or other genders (e.g. *Klebsiella spp., Nitromonas spp.*), and not known to be present in EHEC and EPEC strains of serogroup O26. So far, we suggest the existence of horizontal acquisition of genomic regions from other pathogenic bacteria, one hypothesis already propounded by other researchers (Brzuszkiewicz *et al.*, 2009, Hacker and Carniel, 2001, Juhas *et al.*, 2009, Kelly *et al.*, 2009). Secondly, the WGPS revealed a structural diversity in O26 EHEC genome of 12% of variation (in the average) in comparison to the reference strain. Two-third of the variations were located in Integrative Elements (IE) or prophages regions. These findings are not surprising considering that these regions are known to be mobile and/or unstable chromosomal regions.

The results that we obtained reflect the genomic plasticity of EHEC and EPEC strains in particular, or even of *E. coli* in general. They support the hypothesis that these variations are a microbial evolutionary strategy to create a mixed assortment of virulence factors coming from various pathogenic strains. This combination leads to unique sets of such factors in the different evolutionary clones, which increases the chances for the bacteria to adapt and to survive to changing environmental conditions (Brzuszkiewicz *et al.*, 2009, Hacker and Carniel, 2001, Mokady *et al.*, 2005). This phenomenon leads to the creation of emerging bacteria that could be highly virulent pathogens if they acquire strong virulence factors from different pathogenic strains. One dramatic illustration is the recent 2011 Shiga toxin-producing *E. coli* O104:H4 German outbreak (Denamur, 2011, Rasko *et al.*, 2011). Indeed, this bacterium results from the *stx* gene acquisition by an O104:H4 enteroaggregative *E. coli* by horizontal transfer (Brzuszkiewicz *et al.*, 2011).

7.5. Does a host specific group stand out?

When compiling all the results together, a sub-group of strains isolated from calves with diarrhoea appeared to share specific genomic characteristics. Firstly, with the study described in the article 4, one adhesin, LdaE, appeared to be statistically associated to strains isolated from bovine with diarrhoea. In 2005, LdaE, coding for the "locus for diffuse adherence" chaperone, was described by Scaletsky *et al.* in a O26 a-EPEC strain isolated from an infant with diarrhoea after having been identified by Szalo *et al.* in 2002 (Scaletsky *et al.*, 2005, Szalo *et al.*, 2002). This adhesin is part of a locus for diffuse adherence, which is responsible for a diffuse

pattern of adherence to the host cell. In addition, Torres *et al.* have shown that the expression of this locus is enhanced at 37° C and in presence of bile salt (Torres *et al.*, 2007). It was suggested that it might help to the adhesion of the bacteria to intestinal cells leading to colonisation. Secondly, the *tccP2* variant obtained in the article 5 showed that one variant (accession number AB275131) was also found to be statistically associated with a-EPEC strains, mostly isolated from bovine with diarrhoea. The majority of the strains carrying this *tccp2* variant were *ldaE*-positive. This AB275131 variant was originally described in O26 a-EPEC EC38/99 reference strain and could therefore be associated with this a-EPEC pathotype. Moreover, the regions highlighted by the WGPS scanning methods (article 7) and two of IS*621* regions tested in the article 8 were also associated with these same strains.

These strains, sharing several genomic features, could represent a sub-group of strains specifically adapted to diarrhoea production in calves. Their characteristics are listed in Table 4. These strains were isolated in diverse countries (United Kingdom, Ireland, The Netherlands, Belgium and USA) and two-third of the strains were isolated in 1991. They are grouped together in the genomic tree resulting from the PFGE. Nevertheless, these results should be interpreted with care and do not allow to draw any definitive conclusions, including about their zoonotic potential. Indeed, the number of strains is too low, especially strains isolated from diarrhoeic calves. Moreover, even if the strains of the sub-group come from different countries, they belong to the same group in the PFGE and were isolated at the same period. Therefore, they could be a clonal group instead of being a host/pathotype-associated sub-group.

7.6. Can we use a faster epidemiological method to link O26 strains?

In the path of the O157 IS*629* printing method developed by Ooka *et al.* (Ooka *et al.*, 2009) and based on the WGPS results we obtained, we could developed a new and rapid (<24h) first-line surveillance typing assay of O26 EPEC and EHEC strains (Article 8). This tool would be based on the PCR amplification of 13 IS*621* regions that could be transformed in digital data and than could be easily shared between laboratories for results comparison and make possible worldwide database construction. This method could be used for epidemiological investigations in case of outbreaks or multiple individual human cases of EHEC or EPEC infections to trace their sources (animals, foodstuffs or human), their geographical origin and their routes of contamination.

Other molecular epidemiology methods are currently used for the monitoring and the surveillance of EHEC and VTEC outbreaks and sporadic cases (Karama and Gyles, 2010). PFGE is currently the golden standard method because of its high discriminatory power and its worldwide standardisation. Nevertheless, this technique is time-consuming, requires specific equipments (electrophoresis and software) and good technical skills (experiment and data analyses). Comparison of results from different laboratories is moreover not easy. At the opposite, the method that we have developed here is rapid, easy to perform and do not require any specific technical skills. Notwithstanding, the IS typing method is less discriminatory than PFGE. That's why we recommend the use of this technique as a first-line approach in parallel with the PFGE method.

7.7. What do the results of this PhD thesis teach us?

In conclusion, we have tried to produce a comprehensive comparison of a collection of strains isolated from diverse hosts (bovines and humans), coming from diverse countries (USA, Ireland, UK, Belgium, France, The Netherlands, Italy, Japan, Switzerland and Brazil) and collected at diverse time (from 1952 to 2008), with the aim to elucidate O26 EHEC and EPEC host specificity mechanisms.

We did not obtain any specific factor associated with the isolate original host. Nevertheless, a sub-group of a-EPEC strains isolated from calves with diarrhoea appeared to share specific genomic characteristics. This sub-group could possess some specific properties to produce diarrhoea in young calves not including their zoonotic potential at this stage. Specific analyses on a larger population of strains have to be performed before concluding that this kind of sub-group really exists.

For the other strains, several hypotheses concerning potential host specificity can be put forward. First, other adhesins, not detected during this work, could be at the basis of the host-specific character. Secondly, variations in the expression of some host-specific adhesins could be related to growth environment factors (such as temperature, pH, presence of some substances). For example, LdaE adhesin expression is enhanced at 37° C and in presence of bile salt (Torres *et al.*, 2007) and two adhesins from UPEC strains production is regulated by environmental nutrient concentration (Crost *et al.*, 2004). Thirdly, the host specificity could be based on properties other than adherence, such as metabolism. Recently, Bertin *et al.* have reported ethanolamine use by O157 EHEC strain in bovine intestine as a nitrogen source,

conferring growth advantages and favouring persistence in bovine gut (Bertin *et al.*, 2011). Moreover, human and bovine microbiota could influence EHEC and EPEC growth or virulence factors (such as Stx or Intimin) secretion by producing specific inhibiting-factors (Chaucheyras-Durand *et al.*, 2006, de Sablet *et al.*, 2009). Finally, one last hypothesis is that there is no host specificity.

8. *Curriculum vitae* and publications

Curriculum vitae

Marjorie Bardiau was born in Charleroi in December 1982 and studied at the Seminary of Bonne-Espérance in Vellereille-les-Brayeux (an old abbey near Binche in Belgium). She moved to Liège to study biology and obtained her master degree in 2004 and a complementary master degree in Chemistry and Pharmacology of Natural Substances in 2005, both from the University of Liège in Belgium.

She began her PhD study in Veterinary Science in 2006 in Pr. Jacques Mainil's laboratory. The aim of her work is to identify some specific host virulence factors of enterohaemorrhagic Escherichia coli strains of serogroup O26 by suppressive subtractive hybridisation (SSH). As part of her PhD research, she spent twice two months (in 2007 and in 2009) in Pr. Tetsuya Hayashi's laboratory at the University of Miyazaki in Japan. She also collaborated with three other laboratories (the "institut scientifique de recherche agronomique" (INRA) in Toulouse, France (Dr. Eric Oswald), and the Veterinary Laboratories Agency (VLA) in Weybridge, UK (Dr. Anjum). Now she is currently working on bovine mastitis caused by *Staphylococcus aureus* in the Pr. Mainil's lab.

In parallel to the Science, Marjorie has a passion for photographing given by her grandfather since her early age (www.marjorieb.com). She also loves travelling and culinary art that she tries to practice as often as possible.

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2. **Bardiau M.**, Labrozzo S., Mainil J.G., Identification, par hybridation suppressive soustractive (SSH) et par microarray, de facteurs de virulence impliqués dans l'attachement initial et dans la spécificité d'hôte de souches entérohémorragiques d'Escherichia coli (EHEC) appartenant au sérogroupe O26. 7ème congrès National de la SFM, Nantes, France. Mai 2007.

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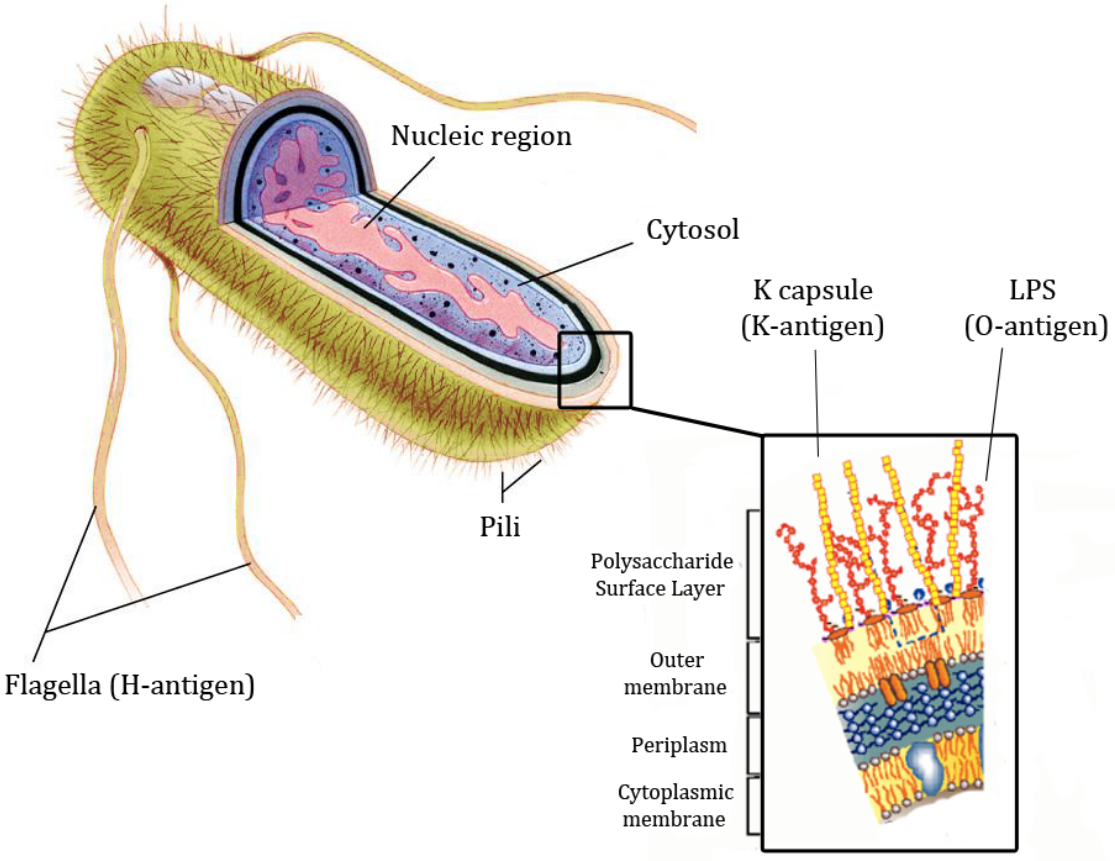

Figure 1: *Escherichia coli* strain (adapted from Alexander *et al.*, 2001).

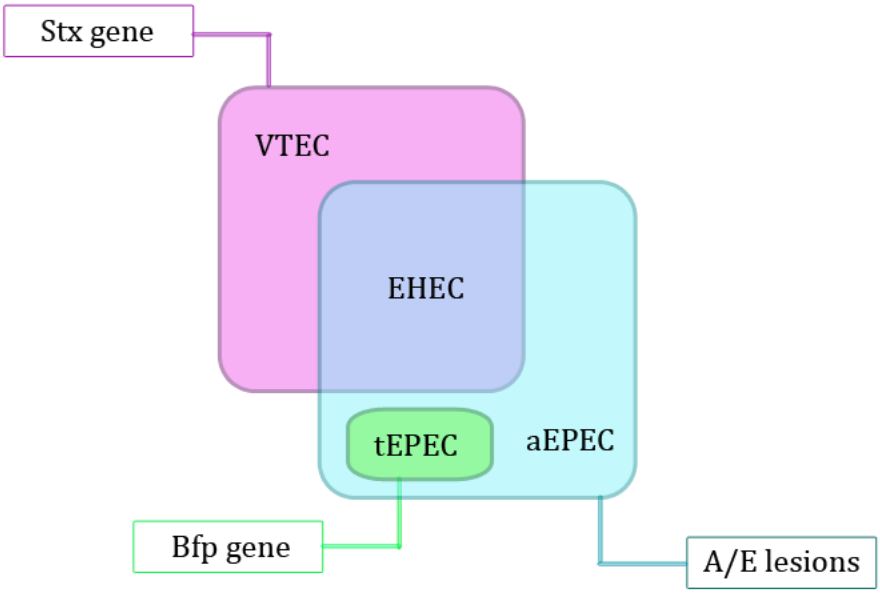

Figure 2: relationship between the three pathotypes (EPEC, EHEC and VTEC) and the Stx, the Bfp and the A/E lesions production.

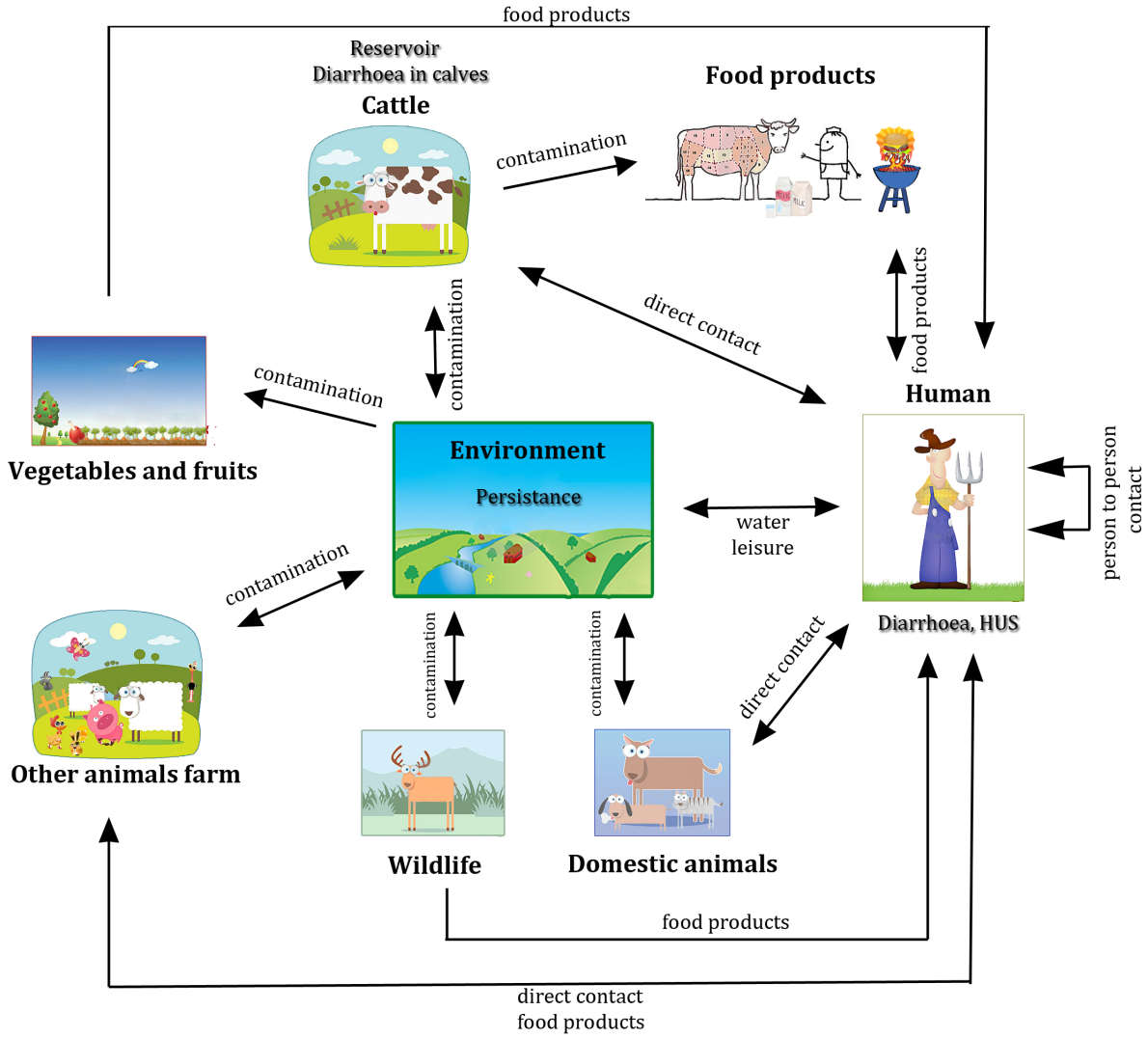

Figure 3: ecology and way of transmission of EHEC, VTEC and most likely EPEC strains.

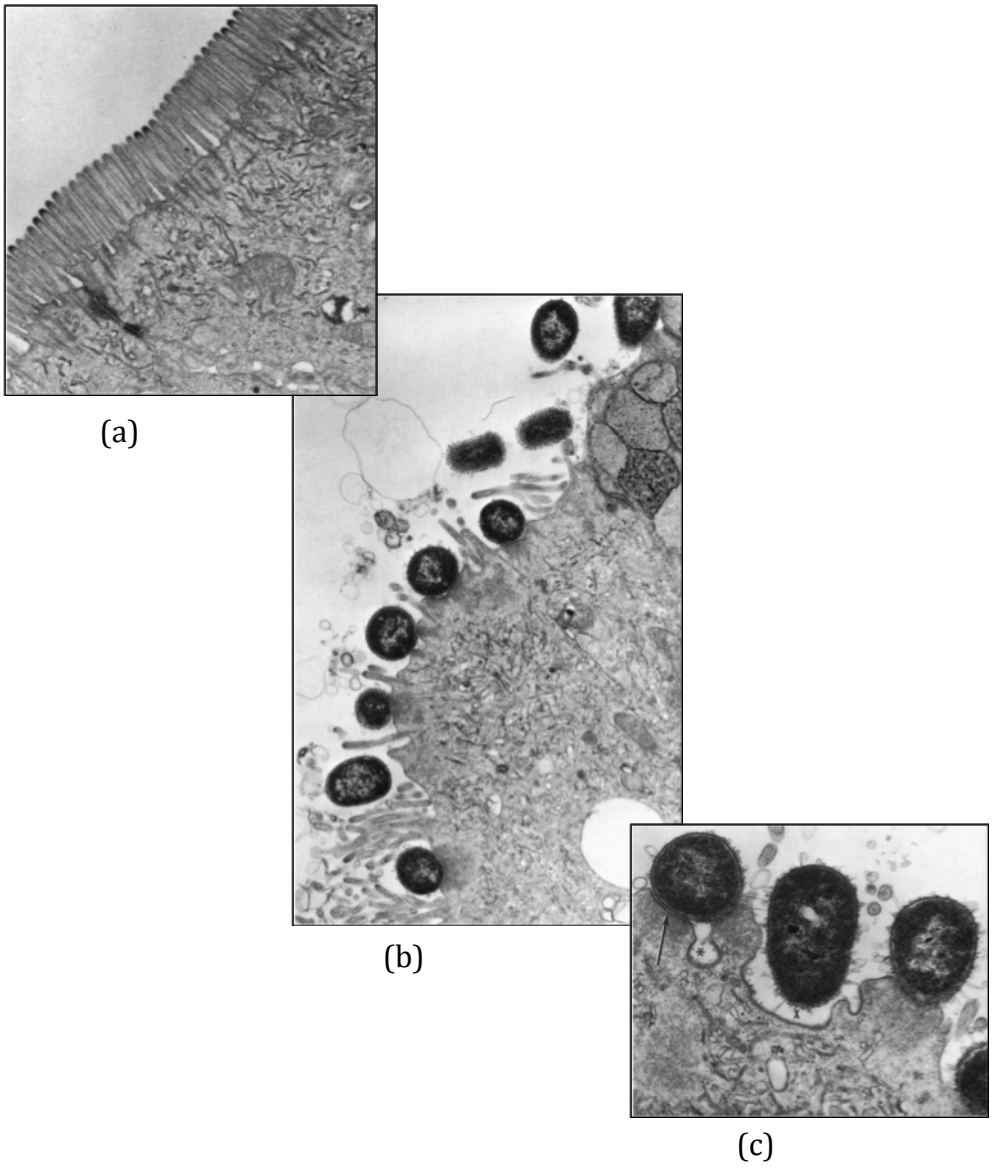

Figure 4: photography's of A/E lesion. (a) Healthy microvilli, (b) and (c) intimate attachment of the bacteria and effacement of the microvilli.

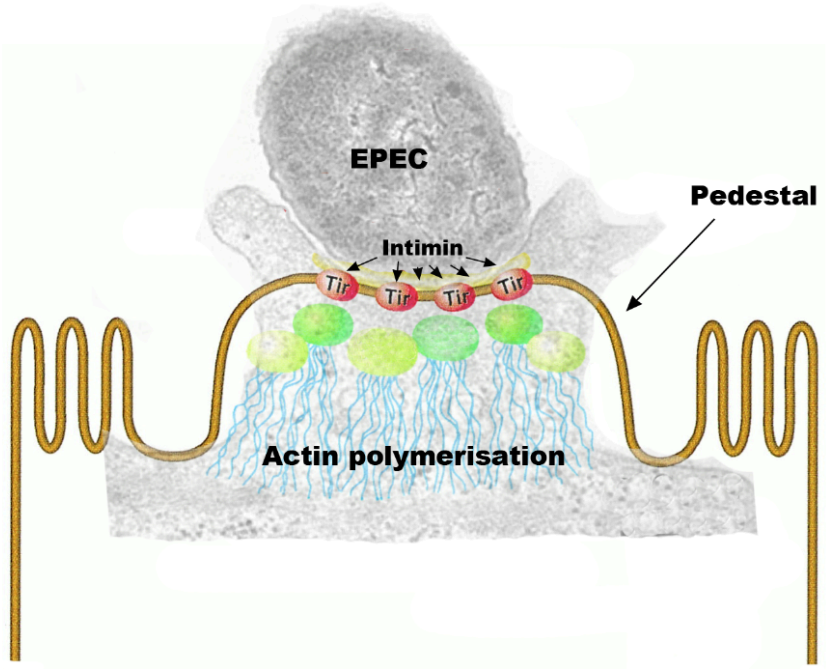

Figure 5: intimate attachment of the bacteria and formation of the pedestal.

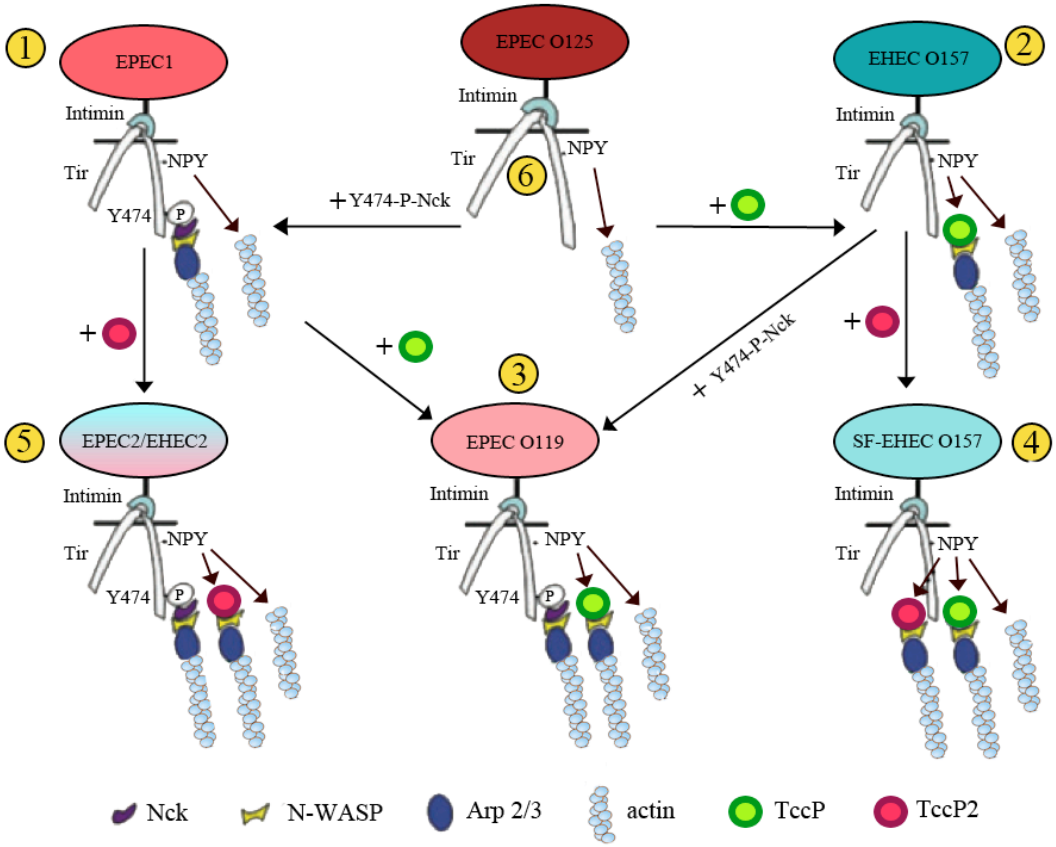

Figure 6: Different actin polymerisation pathways used by EPEC and EHEC strains: (1) EPEC1 strains utilise the host adaptor protein Nck; (2) typical EHEC O157 use the bacterial TccP adaptor; (3) some EPEC strains, represented by the serotype O119:H6, can activate N-WASP by Nck and TccP; (4) sorbitol-fermenting (SF) EHEC O157 strains trigger actin polymerisation via TccP and TccP2; (5) EPEC2 strains and the most of non-O157 EHEC strains can activate N-WASP by Nck and TccP2; (6) some EPEC strains, represented by serotype O125:H6, can trigger efficient A/E lesion formation with the help of the conserved NPY motif, present in all strains (adapted from Frankel and Phillips, 2008).

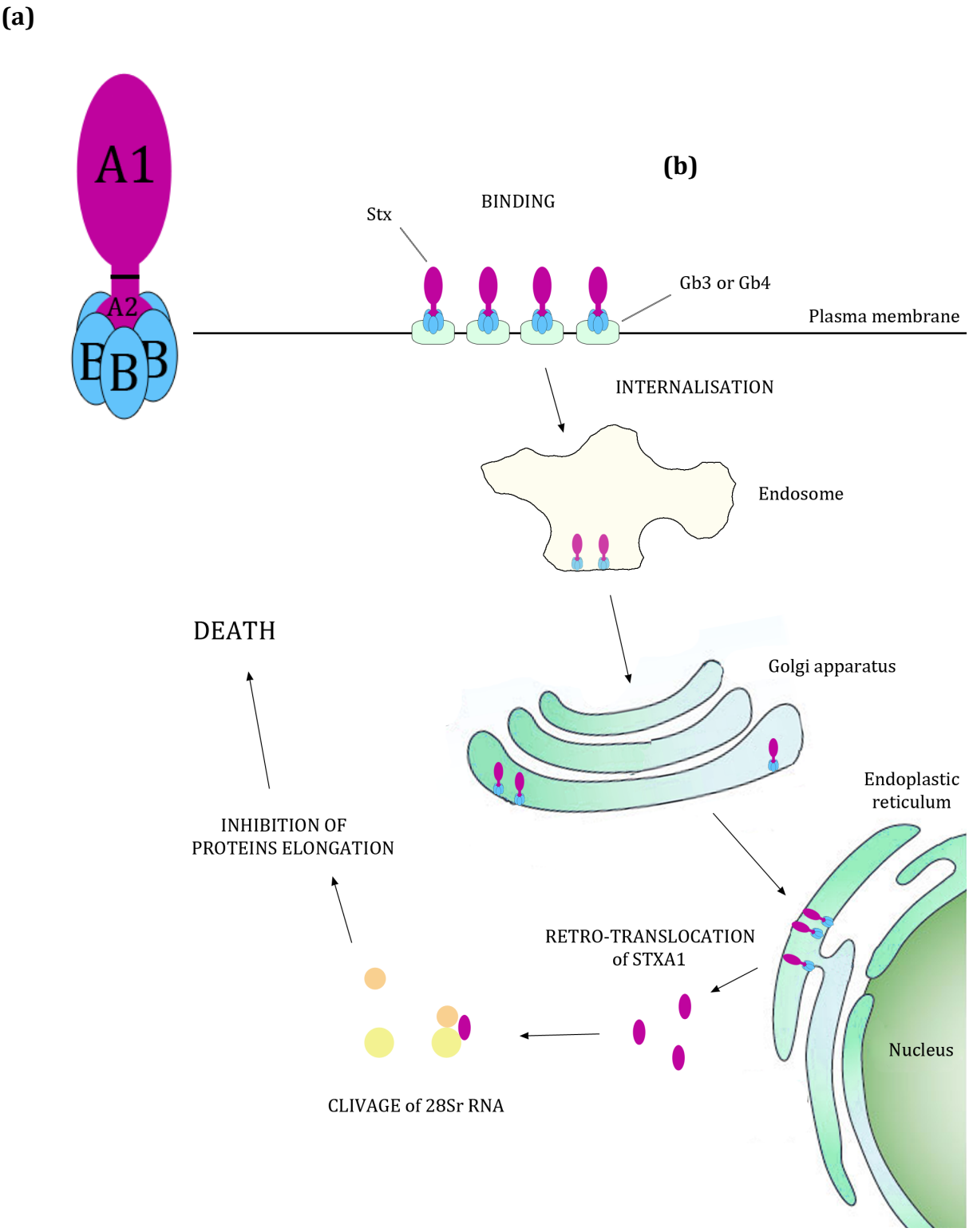

Figure 7: (a) structure of the Shiga toxin (Stx). The toxin has an A-B structure type composed of one A-subunit (divided in A1 and A2) and five B-subunits (adapted from Sandvig *et al.*, 2001). (b) Mechanism of action of the Shiga toxins on host cells.

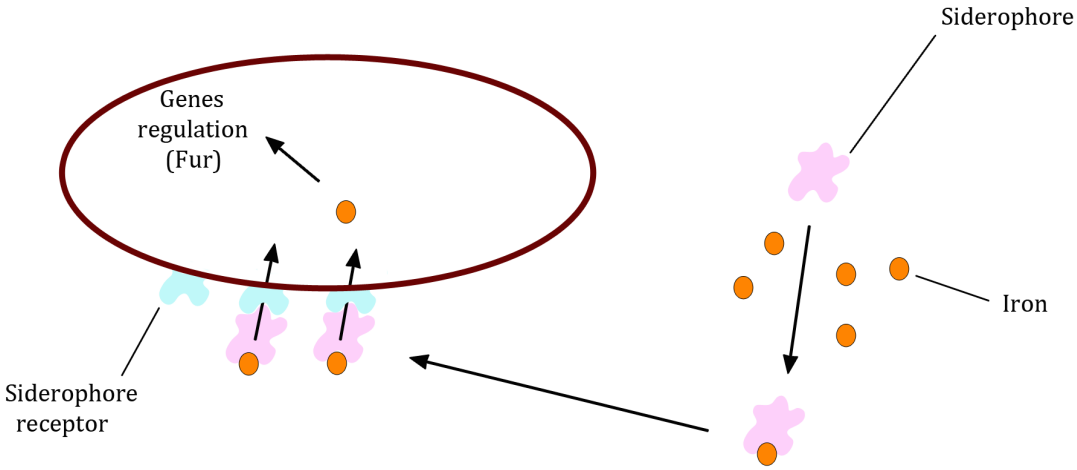

Figure 8: siderophore-mediated mechanism for iron uptake in *Escherichia coli*.

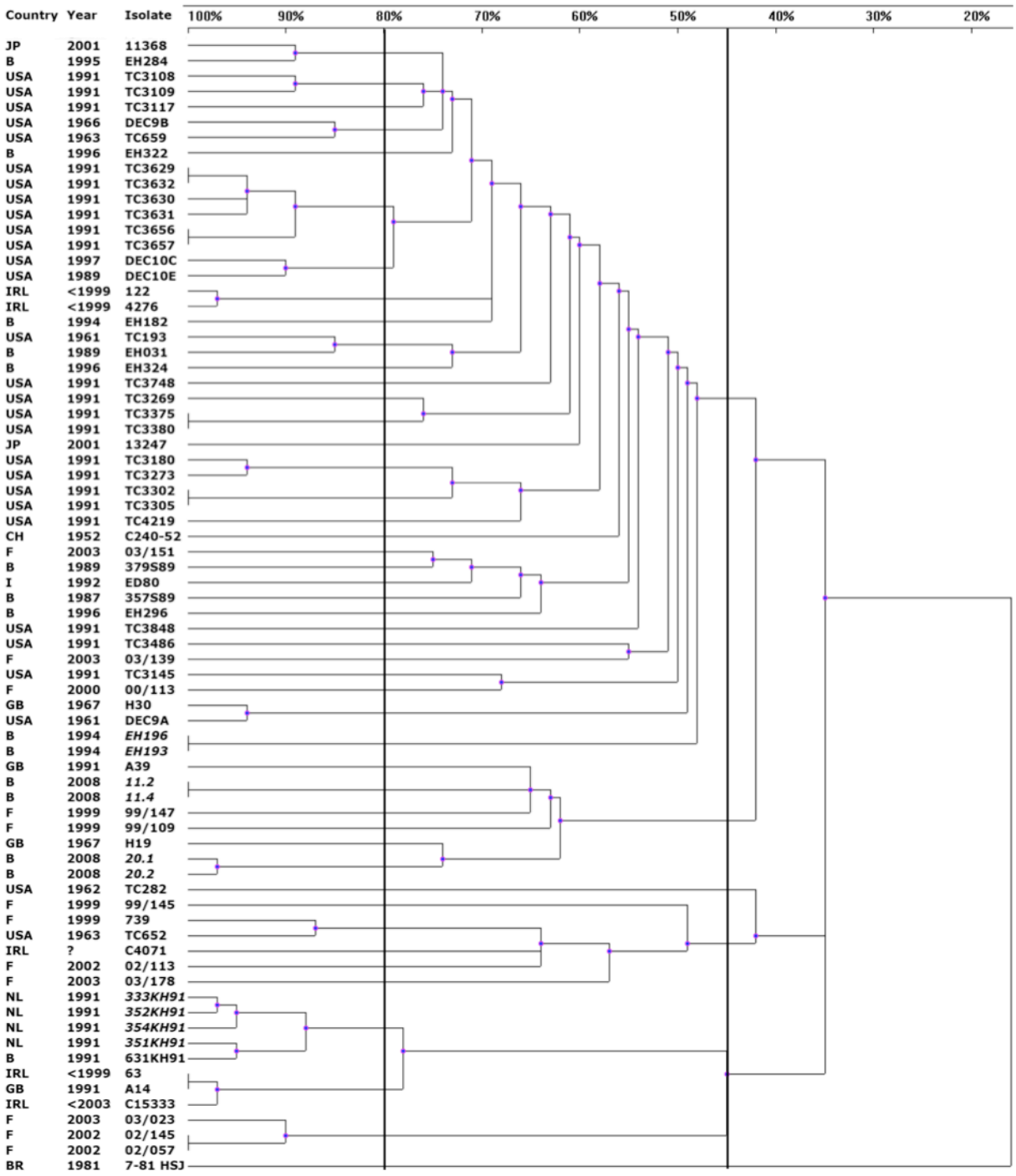

Figure 9: dendrogram of 74 *E. coli* strains used in this thesis constructed by PFGE data (Dice coefficient, with an optimisation and position tolerance of 1%, UPGMA).

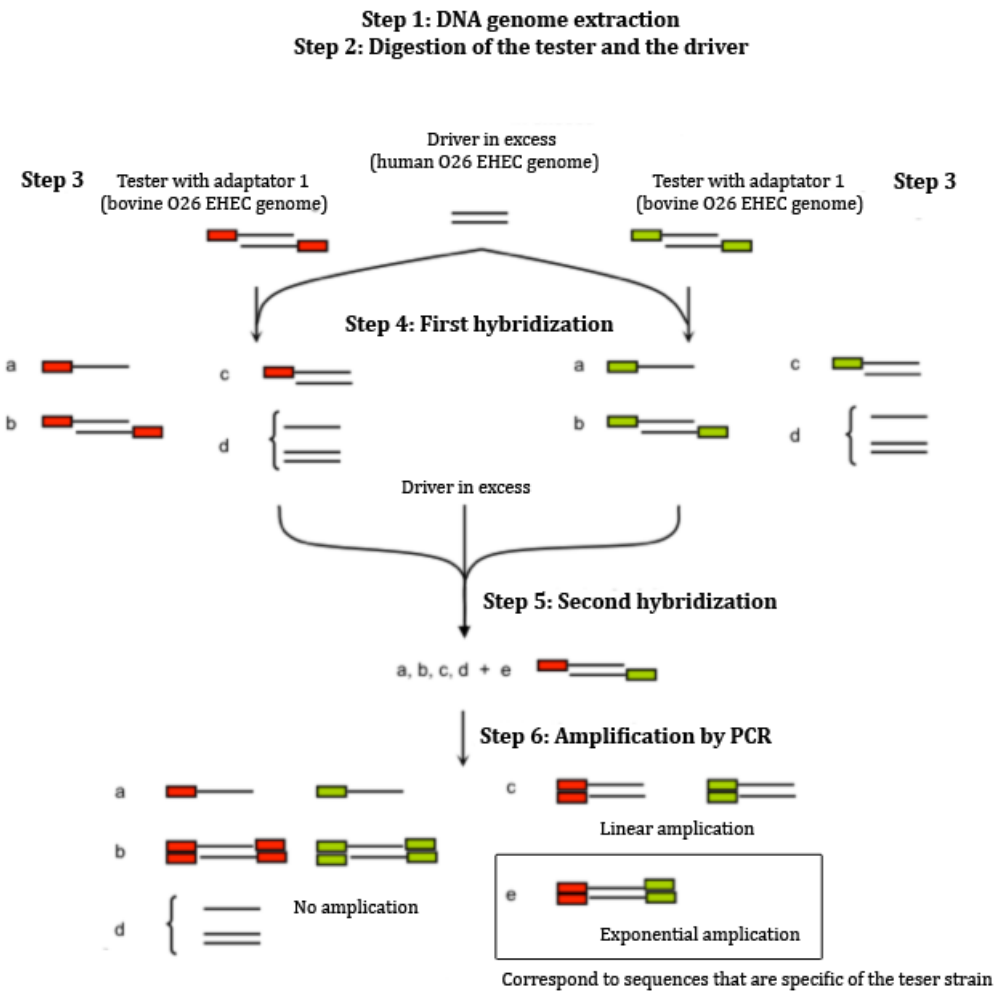

Figure 10: Suppressive Subtractive Hybridisation (SSH) method.

Table 4: characteristics of the strains that could form a sub-group.

EPEC/ EHEC	Host	Age	Status	Country	Year	Isolate	*eae*	*stx*	*wzx-wzyO26*	*fliCH11*	EHEC-*hlyA*	Phylogenetic groups
EHEC	Cattle	n.i.	n.i.	Great Britain	1991	A14	+	1	+	+	+	B1
EHEC	Cattle	n.i.	n.i.	Ireland	n.i.	63	+	1	+	+	+	B1
EPEC	Cattle	n.i.	n.i.	Ireland	<2003	C15333	+	-	+	+	-	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	333KH91	+	-	+	+	+	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	351KH91	+	-	+	+	-	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	352KH91	+	-	+	+	+	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	354KH91	+	-	+	+	-	B1
EPEC	Calf	6m	Diarrheic	Belgium	1991	631KH91	+	-	+	+	-	B1
EPEC	Human	n.i.	Healthy	USA	n.i.	TC6167	+	-	+	+	-	n.i.

Table 1: definition, host range and virulence properties of enteric and enterotoxaemic *E. coli* in animals and humans (adapted from Piérard et al., 2012 and Mainil 2003).

Pathotype	Host range	Diseases	Virulence
Enterotoxigenic *E. coli* (ETEC)	Pigs, ruminants, humans (more rarely dogs)	Traveller's diarrhoea Watery neonatal diarrhoea in babies, calves and piglets Post-weaning diarrhoea in piglets	Fimbrial adhesins; heat-stable (STb) and heat-labile (LT) enterotoxins
Enteropathogenic *E. coli* (EPEC)	Humans, all mammals	Diarrhoea	Attaching and effacing (A/E) lesion
Verotoxigenic *E. coli* (VTEC)	Humans, piglets	Haemolytic-uremic syndrome (HUS) in humans Oedema disease in piglets	Shiga toxins (Stx); afimbrial and fimbrial adhesins
Enterohaemorrhagic *E. coli* (EHEC)	Humans, calves	Watery and bloody diarrhoea and HUS in humans Diarrhoea in young calves	Stx and A/E lesion
Enteroinvasive *E. coli* (EIEC)	Humans, primates	Dysentery	Intracellularly proliferation in the enterocytes
Enteroaggregative *E. coli* (EAEC)	Humans (sporadically in animals)	Diarrhoea	Small fimbrial adhesins; toxins (Pet, EAST1, ShET1); transcriptional activator gene (*aggR*)
Diffuse adherent *E. coli* (DAEC)	Humans, animals	Diarrhoea Extra-intestinal infections (urinary tract infections, septicaemia)	Diffuse adherence (DA) on cell culture mediated by adhesins of the AFimbrial Adhesin (AFA) family, or by AIDA adhesin
Necrotoxigenic *E. coli* (NTEC)	Humans, animals	Diarrhoea Extra-intestinal infections (urinary tract infections, septicaemia)	Cytotoxic Necrotizing Factors (CNF) 1 or 2; different fimbrial and/or afimbrial adhesins

Table 3: origin, pathotypes and serotypes of the *E. coli* strains of serogroup O26 used in this thesis (n.i.: no information, w: week; m: month; y: year).

EPEC/ EHEC	Host	Age	Status	Country	Year	Isolate	*eae*	*stx*	*wzx-wzyO26*	*fliCH11*	EHEC-*hlyA*	Phylogenetic groups
EHEC	Calf	n.i.	Diarrheic	USA	1961	TC193	+	1	+	+	+	B1
EHEC	Cattle	n.i.	n.i.	Great Britain	1991	A14	+	1	+	+	+	B1
EHEC	Cattle	n.i.	n.i.	Ireland	n.i.	63	+	1	+	+	+	B1
EHEC	Calf	<6m	Diarrheic	Belgium	2008	11.2	+	1	+	+	+	B1
EHEC	Calf	<6m	Diarrheic	Belgium	2008	11.4	+	1	+	+	+	B1
EHEC	Calf	<6m	Diarrheic	Belgium	2008	20.1	+	1	+	+	+	B1
EHEC	Calf	<6m	Diarrheic	Belgium	2008	20.2	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3108	+	1	+	+	-	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3109	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3117	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3180	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3269	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3273	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3302	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3305	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3375	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3380	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3629	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3630	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3631	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3632	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3656	+	1	+	+	+	B1
EHEC	Cattle	<6m	Healthy	USA	1991	TC3657	+	1	+	+	+	B1
EHEC	Cattle	n.i.	Diarrheic	USA	1989	TC6169 (DEC10E)	+	1	+	+	+	B1
EHEC	Cattle	n.i.	n.i.	Great Britain	1991	A39	+	1	+	+	+	B1
EHEC	Cattle	n.i.	n.i.	Ireland	<1999	122	+	1	+	+	+	B1
EHEC	Calf	n.i.	Diarrheic	Ireland	<1999	4276	+	1	+	+	+	B1
EHEC	Calf	n.i.	n.i.	Italy	1992	ED80	+	1	+	+	n.i.	n.i.
EHEC	Calf	n.i.	Diarrheic	Belgium	1987	357S89	+	1	+	+	+	B1
EHEC	Calf	n.i.	Diarrheic	Belgium	1989	379S89	+	1	+	+	+	B1
EHEC	Cattle	n.i.	Diarrheic	Belgium	n.i.	331S89	+	1	+	+	+	B2
EHEC	Human	n.i.	Diarrheic	Japan	2001	11368	+	1	+	+	+	n.i.
EHEC	Human	n.i.	Diarrheic	Japan	2001	13247	+	1	+	+	n.i.	n.i.
EHEC	Human	n.i.	n.i.	Great Britain	1967	H19	+	1	+	+	n.i.	n.i.
EHEC	Human	n.i.	n.i.	Great Britain	1967	H30	+	1	+	+	n.i.	n.i.
EHEC	Human	n.i.	HUS	USA	n.i.	TC5710	+	1 and 2	+	+	-	B2
EHEC	Human	n.i.	HUS	USA	n.i.	TC5711	+	1	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	USA	1977	TC6168 (DEC10C)	+	1	+	+	-	B1
EHEC	Human	n.i.	Diarrheic	Belgium	1989	EH031	+	1	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	Belgium	1994	EH182	+	1	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	Belgium	1994	EH193	+	2	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	Belgium	1994	EH196	+	2	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	Belgium	1996	EH296	+	2	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	Belgium	n.i.	EH298	+	2	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	Belgium	1995	EH284	+	1	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	Belgium	1996	EH322	+	1	+	+	+	B1
EHEC	Human	n.i.	Diarrheic	Belgium	1996	EH324	+	1	+	+	+	B1
EHEC	Human	<1y	n.i.	France	1999	99/109	+	2	+	+	+	B1
EHEC	Human	<1y	n.i.	France	1999	99/147	+	1	+	+	+	B1
EHEC	Human	1-5y	n.i.	France	2002	02/113	+	1	+	+	+	B1
EHEC	Human	1-5y	n.i.	France	2003	03/139	+	1	+	+	+	B1
EHEC	Human	1-5y	n.i.	France	2003	03/151	+	1	+	+	+	B1
EHEC	Human	1-5y	n.i.	France	1999	99/145	-	1	+	+	+	B1

EPEC	Cattle	<6m	Healthy	USA	1991	TC3145	+	-	+	+	+	B1
EPEC	Cattle	<6m	Healthy	USA	1991	TC3486	+	-	+	+	+	B1
EPEC	Cattle	<6m	Healthy	USA	1991	TC3748	+	-	+	+	+	B1
EPEC	Cattle	<6m	Healthy	USA	1991	TC3848	+	-	+	+	+	B1
EPEC	Cattle	<6m	Healthy	USA	1991	TC4004	+	-	+	+	+	B1
EPEC	Cattle	<6m	Healthy	USA	1991	TC4219	+	-	+	+	+	n.i.
EPEC	Cattle	<6m	Healthy	USA	1991	TC4221	+	-	+	+	+	n.i.
EPEC	Cattle	n.i.	n.i.	Ireland	<2003	C15333	+	-	+	+	-	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	331KH91	+	-	+	+	-	B2
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	333KH91	+	-	+	+	+	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	334KH91	+	-	+	+	-	n.i.
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	335KH91	+	-	+	+	-	B2
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	351KH91	+	-	+	+	-	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	352KH91	+	-	+	+	+	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	354KH91	+	-	+	+	-	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	355KH91	+	-	+	+	-	B1
EPEC	Calf	6m	Diarrheic	Belgium	1991	631KH91	+	-	+	+	-	B1
EPEC	Calf	n.i.	Diarrheic	USA	1963	TC659	+	-	+	+	+	B1
EPEC	Human	n.i.	Diarrheic	USA	1961	TC6165 (DEC9A)	+	-	+	+	+	B2
EPEC	Human	n.i.	Diarrheic	USA	1966	TC6166 (DEC9B)	+	-	+	+	+	B2
EPEC	Human	n.i.	Healthy	USA	n.i.	TC6167	+	-	+	+	-	n.i.
EPEC	Human	1-5y	n.i.	France	2000	00/106	+	-	+	+	-	n.i.
EPEC	Human	1-5y	n.i.	France	2000	00/113	+	-	+	+	-	B1
EPEC	Human	n.i.	n.i.	France	n.i.	00/054	+	-	+	+	+	n.i.
EPEC	Human	n.i.	n.i.	France	2002	02/057	+	-	+	+	-	B1
EPEC	Human	<1y	n.i.	France	2002	02/145	+	-	+	+	-	B1
EPEC	Human	n.i.	n.i.	France	2003	03/178	+	-	+	+	+	n.i.
EPEC	Human	n.i.	Healthy	Switzerland	1952	C240-52	+	-	+	+	n.i.	n.i.
EPEC	Human	n.i.	n.i.	Brazil	1981	TC1988 (7 81 HSJ)	+	-	+	+	+	B1
-	Cattle	n.i.	Diarrheic	USA	1962	T282	-	-	+	+	n.i.	A
-	Human	n.i.	Diarrheic	France	n.i.	00/103	-	-	+	+	n.i.	A
-	Human	n.i.	Diarrheic	France	n.i.	00/130	-	-	+	+	n.i.	A
-	Human	n.i.	Diarrheic	France	n.i.	03/023	-	-	+	+	n.i.	A
-	Human	n.i.	Diarrheic	France	n.i.	C4071	-	-	+	+	n.i.	n.i.
-	Piglet	1-2 w	Diarrheic	USA	1963	TC652	-	-	+	-	n.i.	n.i.
-	Avian	n.i.	n.i.	France	1999	739	-	-	+	-	n.i.	n.i.

Table 4: characteristics of the strains that could form a sub-group.

EPEC/ EHEC	Host	Age	Status	Country	Year	Isolate	*eae*	*stx*	*wzx-wzyO26*	*fliCH11*	EHEC-*hlyA*	Phylogenetic groups
EHEC	Cattle	n.i.	n.i.	Great Britain	1991	A14	+	1	+	+	+	B1
EHEC	Cattle	n.i.	n.i.	Ireland	n.i.	63	+	1	+	+	+	B1
EPEC	Cattle	n.i.	n.i.	Ireland	<2003	C15333	+	-	+	+	-	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	333KH91	+	-	+	+	+	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	351KH91	+	-	+	+	-	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	352KH91	+	-	+	+	+	B1
EPEC	Calf	n.i.	Diarrheic	The Netherlands	1991	354KH91	+	-	+	+	-	B1
EPEC	Calf	6m	Diarrheic	Belgium	1991	631KH91	+	-	+	+	-	B1
EPEC	Human	n.i.	Healthy	USA	n.i.	TC6167	+	-	+	+	-	n.i.