Shiga/Verocytotoxins and Shiga/verotoxigenic *Escherichia coli* in animals

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**Abstract** — Vero/Shiga toxins (VT/Stx) have an A-B structure: the A subunit carries the enzymatic activity and the B subunit binds the toxin to the membrane receptor (Gb3 or Gb4). The VT/Stx inhibit protein synthesis in the target eucaryotic cells, mainly the endothelial cells of blood vessels. The VT/Stx are subdivided into two families. VT1/Stx1 is a homogeneous family of toxins identical to the Stx of *Shigella dysenteriae*. VT2/Stx2 is a more heterogeneous family of toxins more distantly related to this Stx toxin. The VT2/Stx2 variants can be distinguished by the polymerase chain reaction (PCR) and/or the reaction with monoclonal antibodies. The VT/Stx-producing *Escherichia coli* are also subdivided into two main groups on the basis of the presence or absence of additional properties: the enterohaemorrhagic *E. coli* (EHEC) induce the formation of attaching/effacing lesions and carry a 60 MD plasmid encoding a specific haemolysin (the enterohaemolysin); the vero/shigatoxigenic *E. coli* (VTEC/STEC) do not show these properties. The EHEC are isolated from humans and ruminants, especially young calves. They are associated with haemorrhagic enterocolitis and its sequelae in humans; the haemolytic-uraemic syndrome (HUS). The VT/Stx play a role in the occurrence of blood in the faeces and in the HUS by their action on the endothelial cells of blood vessels in the intestinal submucosa and in the renal glomeruli, after resorption through the intestinal walls. The VTEC/STEC are isolated from piglets, calves and humans. In recently weaned piglets, they cause the oedema disease, an enterotoxaemia characterized by subcutaneous, mesenteric and cerebral oedemas, with nervous disorders as main clinical signs. The oedema disease is the consequence of the action of the VT/Stx on the endothelial cells of blood vessels in various organs. In calves and humans, the role in disease of VTEC/STEC is controversial, but they could be associated with some cases of diarrhoea and HUS. The case of the O157:H7 EHEC which are present in healthy cattle of various ages, but are highly virulent for humans is of special interest. The potential zoonotic aspect of VT/Stx-producing *E. coli* infections in animals is detailed chapter by chapter. Prophylaxis of these infections by vaccination is the subject of the discussion on the future of the research studies on these pathogenic bacteria. © Inra/Elsevier, Paris.

*E. coli* / shigatoxins / verotoxins

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Résumé – Cytotoxines Shiga/Vero et *Escherichia coli* Shiga/Vérotoxinoğènes chez les animaux.


**E. coli / toxines Shiga / toxines Véro**

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1. INTRODUCTION

1.1. Historical background

In 1977, Konowalchuk et al. [69] reported a toxin active on cultured Vero cells, [hence the name verocytotoxin (VT)] produced by a dozen human and porcine diarrhoea- and edema disease-associated Escherichia coli. The cytopathic effect of VT is quite different from the non-cytopathic effect of the classical heat-labile enterotoxin of enterotoxigenic E. coli (ETEC). The same year, O'Brien et al. [92] reported that several E. coli, including the human H30 strain also studied by Konowalchuk et al. [69], are cytotoxic for cultured HeLa cells. This cytotoxicity is neutralized by an immune serum specific for the Shiga toxin (Stx) from Shigella dysenteriae type 1, hence the name Shiga-like toxin (SLT) [93].

These groups of cytotoxins are heterogeneous because the toxicity of several human (H.I.8. E32511, 933, etc.) and porcine (E57, P104) strains is not, or only partially, neutralized by immune sera directed to the toxin of the human strain H30 and to the Stx of Shigella dysenteriae type 1. This is in contrast to the toxicity of other strains [61, 69, 117, 128]. The latter cytotoxins are identical to the Stx of Shigella dysenteriae and were renamed VT1 or SLT1, whereas the former toxins received the name VT2 or SLT2.

1.2. Nomenclature

The discovery of these cytotoxins by different research teams has resulted in a parallel nomenclature system. The terms ‘verotoxins (VTs)’ or ‘Shiga-like toxins (SLTs)’ on the one hand and ‘verotoxigenic E. coli (VTEC)’ or ‘Shiga-like toxin-producing E. coli (SLTEC)’ on the other have been used for many years by Canadian/British workers and American workers, respectively, although they are synonyms. More recently, the proposal to use the names ‘Shiga toxins (Stx)’ for all these cytotoxins was made [19], but did not receive unanimous approval [62].

The main arguments in favour of the newly proposed nomenclature are that VT/SLT are identical or related to the Stx of Shigella dysenteriae and that the description of the Stx precedes the description of VT/SLT. The main argument in favour of maintaining the original nomenclature of VT is based upon the fact that not all VTs are identical to the Stx of Shigella dysenteriae (VT1 are, but the various VT2 are not) and therefore distinct names should be used. VT/Stx must thus be considered as synonyms and throughout the text, the newly proposed nomenclature will be used.
Another confusing area concerns the nomenclature of the various Stx-producing E. coli. Basically three groups are described:

- those associated with haemorrhagic colitis (HC) and its sequelae, the haemolytic-uraemic syndrome (HUS) in human beings (which, in addition to the production of Stx, are also able to induce the ‘attaching-effacing’ (AE) lesions). They belong to a few main serotypes (O157:H7, O157:H-, O26:H11, O26:H-, O111:H-, O113:H21, O103:H2, etc.), and possess a ca. 60 MDa plasmid encoding for a specific enterohaemolysin [89, 95, 139];

- those showing basically the same properties, but isolated from animals, mainly calves, and causing enteritis and bloody diarrhoea [18, 75, 143];

- those not showing these properties, isolated from human beings, sometimes in association with HUS [89, 95, 139], from pigs as a cause of edema disease [4, 40, 139, 143], and from other animal species with or without clinical signs [16, 75, 96, 139].

Strains from the first group are referred to as ‘enterohaemorrhagic E. coli’ or EHEC, although bloody diarrhoea is not systematically observed. The name EHEC was at one time restricted to the strains belonging to the O157 serogroup. Similar strains from animals are referred to as EHEC or EHEC-like isolates, even if they are not especially associated with haemorrhagic colitis. If the author agrees with these two definitions and names, he does not, however, agree with the most often proposed name for the strains from the third group, i.e. EHEC also or ‘atypical EHEC’ [89]. Indeed it is his feeling that this name does not suit strains from this third group, especially porcine strains which are not basically associated with intestinal disorders [4]. The author will use the name STEC (Shigatoxigenic E. coli) for them, which best describes their main property, i.e. the production of Stx, until other virulence factors or virulence-associated properties are described.

1.3. Structure of the manuscript

After a presentation of the most recent molecular data on Shiga toxins, the clinical, bacteriological and epidemiological data on Stx-producing E. coli will be extensively reviewed in ruminants and in pigs. After a short note on Stx-producing E. coli in other animal species, the past and future history of Shiga toxins will be discussed. A review on human Stx-producing E. coli would have been justified because of the public health problems and because the Stx-producing E. coli from animals are almost always studied in comparison with their human counterparts. However, a lot of current literature focuses on them and the role of Stx-producing E. coli in human infections and diseases (diarrhoea, haemorrhagic colitis and systemic complications, such as the haemolytic-uraemic syndrome) has thus been reviewed recently by several authors [89, 95, 97, 139]. Moreover, the most recent epidemiological and clinical data on O157 strains are covered in a recently published book [59]. There is thus no need to repeat what has already been presented and discussed several times.

2. THE SHIGATOXINS OF ESCHERICHIA COLI

Extensive and numerous reviews on the Shiga toxin (Stx) families have been published since their description. This chapter is based on the most recent ones [83, 89, 95, 114]. Moreover, the recently published book mentioned above presents the most recent cellular and molecular data on Stx [59]. A few specific and/or even more recent references have been added in the text.

2.1. The Shiga toxin family

As already mentioned, the Stx produced by E. coli consist of two families: Stx1, a homogeneous group of toxins virtually identical to the Stx of Shigella dysenteriae type
1 and Stx2, a heterogeneous group of toxins more distantly related to the Stx. The prototype Stx1 is produced by the *E. coli* strains H19, H30 and 933 [69, 93, 117, 128]. Variants have been described which only differ by one amino acid with no consequence on toxicity or on antigenicity.

The prototype Stx2 is produced by *E. coli*-type strains 933 and E32511 [61, 114, 128]. The first Stx2 variants were actually described by Konowalchuk et al. [69], but were rediscovered a few years later [117], and characterized even later. The Stx2e toxin is produced by *E. coli*-type strains E57, S1191 and 412 associated with the edema disease in recently weaned piglets [50, 81, 135] and occasionally by strains of other origins [44, 98, 105, 129, 136]. Other names such as Stx2v (after variant) or Stx2vp (after variant pig) exist. The other Stx2 variant described by Konowalchuk et al. [69] is produced by the human *E. coli* strain H.1.8. It is more closely related to Stx2e than to Stx2 and is named Stx2ev, after edema disease variant (or Stx2va, or Stx2vh after variant human) [47]. The Stx2ev has not been described for other strains.

Several other Stx2 variants produced by human and animal *E. coli* have been and are still being described [2, 99]. Amongst them, the Stx2c variant produced by the *E. coli* E32511 strain [114] appears to be the most epidemiologically and clinically important in humans. These variants were recognized on the basis of partial neutralization by anti-Stx2 immune serum and/or the absence of a positive PCR reaction. *E. coli* can produce Stx1 or Stx2 only, or both (strain 933 f.i.), or Stx2 and a Stx2 variant, mainly the c variant (strain E32511 f.i.), or two Stx2 variants.

### 2.2. Structure and biological activities

Stx have an A-B subunit structure and consist of one A subunit of approximately 33 kDa and five B subunits of approximately 7.5 kDa each. The A subunit is the biologically active portion of the toxin and the B subunits bind the toxin to the glycolipid membrane receptor, globotriaosylceramide or Gb3 (Galα1-4Gal) for Stx1 and Stx2, but globotetraosylceramide or Gb4 (GalNacβ1-3Galα1-4Gal) for Stx2e and Stx2ev. Other variants have intermediate affinity for these receptors. Receptor specificity is based on a few amino acids in the B subunit.

Internalization of the toxins is achieved by receptor-mediated endocytosis followed by transport to the Golgi apparatus and then to the endoplasmic reticulum. The A subunit is translocated into the cytoplasm and cleaved to yield a 28 kDa N-terminal peptide (A1) with the enzymatic activity and a 4 kDa C-terminal peptide (A2). The A1 peptide acts as an N-glycosidase by removing an adenine residue from the 28S rRNA of the 60S subunit of eucaryotic ribosomes, therefore preventing elongation-factor-1-dependent binding of aminoacyl-tRNA to ribosomes. The end result is the inhibition of protein synthesis leading to cell death.

The range of biological activities of Stx include cytotoxicity, enterotoxicity and neurotoxicity.

An observation already reported by Konowalchuk and Speirs [68] and confirmed a few years later is that all Stx are highly toxic in vitro for Vero cells but not necessarily for other cells [14, 64]. Stx1 and Stx2 are also toxic for HeLa cells. Stx2e is 10 000 times less toxic and other Stx2 variants are 10–100 times less toxic for HeLa cells than for Vero cells. The situation is the opposite for MDBK cells. These observations are related to the amount of specific receptors present on the cells: mainly Gb3 (receptor for Stx1 and Stx2) on HeLa cells, mainly Gb4 (receptor for Stx2e) on MDBK cells, similar levels of Gb3 and Gb4 on Vero cells. In vivo, the target cells are mainly the endothelial cells in the kidneys, brain and/or stomachal and intestinal submucosa according to the infected species. However, in theory, any cell which possesses the appropriate receptor(s) at its surface may be a target cell.
Stx1 and Stx2 are also considered to be enterotoxic, i.e. to cause fluid accumulation in ligated intestinal segments in rabbits, such as the heat-stable and heat-labile enterotoxins of ETEC. Although this effect has not been observed by all authors, it may be related to the various Stx produced. In contrast the Stx2e toxin is poorly (100 times less) enterotoxic. The mechanism of this enterotoxicity is still not completely understood and the following explanations are considered:

- selective destruction of the absorptive villus tip enterocytes which present high levels of Gb3 receptor in their membrane in contrast to the secretary crypt enterocytes;
- inhibition in the absorption of NaCl, without any effect in ion secretion, especially Cl⁻;
- damage to the underlying vessels by a direct effect on endothelial cells with loss of fluids and cells into the gut lumen;
- production of another toxin which is enterotoxic, such as the EAST1 toxin, or enteroaggregative heat stable toxin, produced by enteroaggregative E. coli [11].

Neurotoxicity of Stx has been observed repeatedly in mice and rabbits parentally inoculated with extracts of Sh. dysenteriae and of Stx-producing E. coli. It is illustrated by an ascendant paralysis and by lethality. This neurotoxicity is most probably a consequence of the damage caused by the toxins to the vascular endothelial cells of the central nervous system. Neurotoxicity has also been reproduced in piglets during studies on edema disease and is thus demonstrable for all types of Stx.

2.3. Genetics and production

Stx are encoded by an operon (stxAB) with two open-reading frames for the A and B subunits. The stxA and stxB genes form one transcriptional unit. The Stx-producing E. coli can possess one stx operon (stxl or stx2) or two stx operons [stxl and stx2 (strain 933 [57]), or stx2 and one stx2 variant (strain E32511 [114]), or two stx2 variants (strain B2F1 [56]).

The stxl operons are identical or very closely related to the stx operon of Shigella dysenteriae type 1. As already said the Stx2 are more distantly related to the Stx toxin of Shigella dysenteriae type 1, so that the stxlAB and stx2AB prototype operons only share 57 % nucleotide sequence identity in their stxA and 60 % in their stxB genes. Although Stx1 and Stx2 differ in their genes encoding the B subunits, they have the same receptor specificity, Gb3.

Phylogenetic trees based on the published nucleotide sequence of the A and B subunit-encoding genes of the stx2 operon variants have been constructed showing the existence of clusters [3, 99, 115]. The stx2 operon variants are closely related to each other within each cluster (>95 % overall identity) and more closely related to the stx2 operon (74-96 % overall identity) than to the stxl operon (55-60 % overall identity). The stx2e and stx2ev operons are the most distantly related to the stx2 operon with 94 and 70 % identity, respectively, in the genes encoding the A subunit and only 78/79 % in the genes encoding the B subunit. This explains their different receptor specificities (Gb4).

Originally O'Brien et al. [93] described two groups of Stx-producing E. coli: those producing high levels of toxins detectable in the culture supernatants and those producing low levels of toxins detectable only in bacterial extracts. Other bacterial species could also produce high or low levels of toxins [11, 93, 113, 120]. The stx genes have been detected by DNA hybridization and/or PCR only in the E. coli and in other bacterial species (Citrobacter freundii and Enterobacter spp.) producing high levels of toxins. They have, however, never been detected in the E. coli nor in the other bacterial species producing low levels of toxins.
The *stxl*, *stx2* and the *stx2c* operons are located on lambdoid phages in *E. coli* belonging to various serotypes, especially O157, whereas the *stx* operon in *Shigella dysenteriae*, the *stx2e* and possibly the other *stx2* variant operons in *E. coli* are not. The transfer of *stxl* and *stx2* operons to recipient strains can be performed in vitro by phage conversion and is suspected in vivo [2, 116, 122, 125].

The production of the *Stx1* and *Stx* of *E. coli* and *Sh. dysenteriae*, but not of *Stx2* and of the *Stx2* variants of *E. coli*, is regulated at the transcriptional level and is repressed by a high concentration of iron and by a low temperature. The production of the *Stx2e* by porcine strains is repressed in the wild-type bacteria by another still unknown mechanism.

After their synthesis the *Stx* are exported to the periplasm by a type II signal sequence-mediated secretion system. Their main localization seems to be the periplasmic space, especially for *Stx1*, whereas *Stx2* and the *Stx2* variants appear to be more easily excreted into the culture medium.

### 2.4. Detection and diagnosis

The *Stx* can be detected in the supernatants of bacterial cultures and in biological samples such as faeces. In bacterial culture, even higher titres can be obtained after treatment to lyse the cells [93] or by polymyxin B [37] to release the cell-bound toxins present in the periplasmic space.

Three types of assays are basically used: cell toxicity assays, immunological assays and genetic assays.

The cell toxicity assays are of course well known [69, 93]. They are still considered as the most sensitive, especially when Vero cells are used, and can be applied on culture supernatants, on cell extracts and also directly on faecal samples after the adaptation of the protocols. The use of Vero cells is recommended to detect all variants. Otherwise the use of other cells is to be considered (HeLa, MDBK f.i.). Primary cell cultures, such as endothelial cells, are also sensitive to the *Stx* but are not used for routine detection assays. Cells are observed for any cytopathic effect for 3 days and the identity to one of the so far described Stx is performed by neutralization with specific immune sera. There is no cross-reaction between *Stx1* and *Stx2* and only partially between *Stx2* and most variants.

Several immunoassays have been developed to detect *Stx* in culture supernatants, in bacterial extracts, or directly in faecal samples [89, 95]: sandwich and other ELISA assays with immune sera, monoclonal antibodies or a Gb3 receptor as a capture system and the reverse passive latex agglutination assay. Only the use of some specific monoclonal antibodies allows us to fully distinguish between the different *Stx2* variants. Interestingly enough, *Stx*-producing *Pseudomonas aeruginosa* strains can cause false positive reactions in a commercial enzyme immunoassay for *Stx* [11]. Other *Stx*-producing bacteria may thus represent a cause of false positive reactions.

The genetic assays are subdivided into the colony hybridization assays with polynucleotidic or oligonucleotidic probes and the polymerase chain reaction (PCR) assays. The colony hybridization assay with polynucleotidic probes was the first one to be used [90]. The distinction between *stxl* and *stx2* and variants is easy to perform with any of the probe systems. Most of the probes derived from one *stx2*-related operon cross-hybridize with all *stx2* operon variants. It is, however, possible to use oligonucleotide probes and especially PCR assays to achieve a differential detection of the *Stx2* variants. It is also possible that other bacterial species harbouring *stx*-related genes represent a cause of false positive reactions in hybridization assays, as they can in enzyme immunoassays.

PCR systems have been developed to detect the presence of *stx* operons, either on their own or along with the detection of
other genes in multiplex PCR reactions [3, 25, 43, 99, 115]. Of the many primers designed some detect all stx (stxl and the various stx2) operons, others are specific for the stxl operons, for all the stx2 variants, or for only one of them. The PCR techniques are very sensitive and specific for the characterization of bacterial isolates, but are not as good for the characterization of faecal samples, because of the presence of non-specific inhibiting factors in the faeces. For faeces they are regarded as less sensitive than cell toxicity assays.

3. STX-PRODUCING ESCHERICHIA COLI AND RUMINANTS

Stx-producing E. coli present two kinds of interactions with cattle: i) intestinal diseases in newborn to 4-month-old calves; ii) carriage by healthy animals, young calves and adults. In addition to cattle, Stx-producing E. coli are also detected in the faeces of other domestic and wild ruminants either in association with disease or in healthy animals. The pathology of Stx-producing E. coli in cattle has been the object of reviews for several years [18, 75, 100, 136, 139, 143]. In contrast, the carrier state has been intensively studied only for the last 3 or 4 years and recent reviews are referred to [2, 31, 53, 89].

3.1. Clinical conditions in young calves

The first reports of production of Stx by E. coli isolated from diarrhoeic calves were published in 1980 [63, 140]. The cytotoxin of the strain isolated by Kashiwazaki et al. [63] was antigenically similar to the cytotoxin of strain H30 [64] and was thus a member of the Stxl family. The association between diarrhea in calves and the presence of Stx toxin-producing E. coli was confirmed in 1985 [51, 85, 120]. During the following years, a number of reports were published which in addition brought information about the general and specific properties and characteristics of these bovine Stx-producing E. coli.

Stx-producing E. coli are associated with diarrhoea in 1–8-week-old calves with a peak between 4 and 5 weeks of age, although newborn and older calves can also be affected [27]. The diarrhoea observed is mucoid, sometimes haemorrhagic and differs from the aqueous diarrhoea caused by ETEC. Diarrhoea does not cause the death of the calf very often (the case fatality rate does not exceed a few per cent), but it is recurrent even after treatments, leading to dehydration, weakening and a depressed growth rate. Necropsy lesions of enteritis are mainly localized in the large intestine with the possibility of extension to the small intestine in severe cases. Localized and diffuse haemorrhages can be observed. In severe cases, other diarrhoeagenic infectious agents are detected: cryptosporidia, rotavirus, coronavirus, coccidia and ETEC in very young calves.

Systemic complications, such as HUS, have never been observed in calves. The most probable reason for this is the absence of specific receptors on kidney glomerular endothelial cells (see section 2.2).

3.2. Serotypes and pathotypes of Stx-producing E. coli in young calves

Most of the Stx-producing E. coli associated with diarrhoea in young calves belong to the following serotypes: O5:H–, O8:H8, O20:H19, O26:H11, O103:H2, O111:H–, O111:H8, O111:H11, O118:H16 and O145:H+. Some of these serotypes are also recovered from humans (see section 3.5). Other serotypes are detected much less frequently, but many strains are either nontypable with the immune sera used or rough variants. The O157 strains have only exceptionally been associated with clinical diseases [15, 94].
The bovine Stx-producing *E. coli* can be subdivided into EHEC and STEC (see definitions in section 1.2). The former mainly belong to the O5, O26, O103, O111, O118 and O145 serogroups and are associated with clinical diarrhoea and enteritis. Most of the latter belong to the O8 and O20 serogroups. The situation of STEC is equivocal as some authors regard them as non-pathogenic because they are frequently isolated from healthy as well as from diarrhoeic calves. In some studies STEC are, however, clearly isolated in cases of diarrhoea. Bovine STEC may thus represent a heterogeneous population of bacteria [104, 107], only some of which are pathogens because they possess additional still uncharacterized virulence-associated properties.

3.3. Virulence properties and pathogenesis in young calves

In addition to the production of Stx bovine, EHEC and STEC indeed possess other properties which may be related to their pathogenicity. The data on bovine EHEC and STEC are reviewed in comparison with the properties of the human strains.

3.3.1. The Stx toxins

The majority of bovine EHEC associated with diarrhoea in young calves produce (or hybridize with a specific gene probe) Stx1 only and only a few Stx2, or both toxins. However, bovine STEC from young calves are positive for Stx1 and/or Stx2 [79, 137]. The Stx2 produced are either the prototype toxin or a variant, especially Stx2c, but along still untyped variants [77, 123, 137]. In human EHEC the Stx1, Stx2 and Stx2c can be phage-encoded [115]. Localization of the stx operons on phages has also been reported or suspected in bovine EHEC and STEC [2, 109].

Since the first publication associating Stx-producing *E. coli* with HC and HUS in humans, there have been numerous studies and debates over the exact role and mechanism of action of the toxins. Studies using different animal models have sometimes further confused the debate because the significance of Stx can vary according to the model used. After comparison of the wild-type EHEC with their isogenic mutants in the *stx* genes, a general agreement is reached: the Stx play no role in the development of the enteritis and in the occurrence of diarrhoea but are important in the haemorrhagic aspect of HC and in the development of HUS. A comparison of the wild-type and isogenic mutant strains in a calf infectious model would most probably confirm that in calves the Stx are not involved in the occurrence of diarrhoea but rather in the haemorrhagic aspect of the enteritis when present. Other properties must thus be expressed by the bovine EHEC and STEC to explain the diarrhoea.

3.3.2. The attaching/effacing (AE) phenotype

Bovine EHEC are capable of causing AE lesions and this phenotype is considered to represent the primary cause of the diarrhoea observed [51, 106, 107]. Indeed *E. coli* producing AE lesions but not Stx (enteropathogenic *E. coli* or EPEC) are by themselves a cause of diarrhoea in humans and in different animal species. Production of AE lesions is a multistep event characterized by: i) initial adherence to the microvilli of the enterocytes; ii) transduction of a signal into the enterocytes; iii) cytoskeleton rearrangement with effacement of the microvilli of the enterocytes; and iv) intimate attachment to the enterocyte cytoplasmic membrane [84, 89].

The initial adhesin of bovine EHEC, if any, still awaits identification, although candidates [144] and positive adherence properties to appropriate cells of bovine origin in culture [138] have been described. The genetic determinants for the last three steps are, however, better known. These genes are grouped together on the chromosome
forming one pathogenicity island called LEE, for the locus of enterocyte effacement. The LEE of bovine EHEC (and EPEC) show similarities and differences in comparison to the LEE of human EHEC and/or EPEC. The genes differing between human and bovine EHEC could serve as epidemiological markers in public health studies (see section 3.5) [26, 48, 136].

STEC strains which can be isolated from clinical cases are AE phenotype-negative. If STEC are diarrhoeagenic E. coli, they must possess other properties which cause the occurrence of diarrhoea. These properties have not yet been characterized but some are suspected (see section 3.3.4).

3.3.3. The enterohaemolysin and the ‘EHEC virulence plasmid’

Human O157 and many non-O157 EHEC harbour a large plasmid called the ‘EHEC virulence plasmid’ [60]. It carries genes encoding one enterohaemolysin, i.e. a haemolysin active only on washed red blood cells (ehx genes), for a type II secretion system (etp genes), for a catalase-peroxydase (katP gene), and for a secreted serine protease (espP and pssA genes). Its involvement in the initial attachment of EHEC is, however, still controversial. The role of these properties in the survival of the bacteria and/or in the development of the disease is purely speculative.

Different enterohaemolysins (Ehly) have been described in E. coli, but this one is relatively specific for EHEC, from which the name EHEC Ehly comes [73]. The EHEC Ehly belongs to the RTX (repeat in toxins) family of toxins and ca. 60% identity were observed between the ehx and hly (encoding the α-haemolysin of E. coli) genes. The presence of the ‘EHEC virulence plasmid’ can be detected using gene probes and PCRs for the various systems described [60]. The ehx and etp genes are present in all O157 EHEC, whereas the katP and espP genes are detected in two-thirds of them. In non-O157 EHEC, the ehx genes are present in almost all strains tested, the etp in half of them, and the others in only a third of them.

The majority of bovine EHEC and STEC produce the enterohaemolysin phenotype on washed sheep blood agar plates or test positive with the pCVD419 probe which corresponds to the ehx genes. The EHEC enterohaemolysin phenotype or genotype is correlated with the production of Stx1 alone or with Stx2 (at least 75% of the strains tested), but less often with the production of Stx2 alone (50% of the strains tested) [103, 118, 136, 137]. The production of enterohaemolysin by bovine EHEC and STEC is also mediated by a plasmid [136, 137]. Whether this plasmid carries the other genetic determinants present on the ‘EHEC virulence plasmid’ of human EHEC is yet unknown.

3.3.4. Others

Human O157 and non-O157 EHEC and STEC can produce the heat-stable toxin of enteroaggregative E. coli (EAST1) [112] whose role in pathogenesis is speculative, but which may be responsible in part for the occurrence of non-bloody diarrhoea. Bovine EHEC and STEC have not been tested for the EAST1, to our knowledge.

Bovine EHEC and STEC are generally resistant to tellurite (Pohl and Mainil, unpublished data) as are human O157 EHEC and Shigella spp. [145]. The relevance to the pathogenesis of this resistance is questionable. This resistance might play some role in the pathology of those strains and/or in their ecology and survival in the cattle rumen and gut and in the environment. It is most useful for their selection by incorporating tellurite compounds in growth media [95].

The strains belonging to the O5:H-serotype possess very rare properties for E. coli, i.e. they produce no gas during carbohydrate fermentation and are capable of fermenting urea [22, 102]. The genetic determination of these properties has, to our knowledge, never been studied.
3.4. Carrier state in healthy cattle

Most cases of HC and HUS in humans are caused by ingestion of foods and drinks contaminated with faeces from cattle, especially ground beef, undercooked hamburgers, salami or other foods sometimes of cattle or small ruminant origin (raw milk or home-made cheese from raw milk), but also non-pasteurized apple cider and juice, uncooked vegetables and water from well or municipal systems. Less frequent modes of transmission of the infection are cattle-to-person and person-to-person direct contacts [89, 95, 97, 139]. These observations have prompted large scale surveys, over the presence of EHEC and STEC, especially of the 0157 serogroup, in beef and dairy herds of healthy adult cattle, in healthy young calves, and at slaughter in young bulls, culled cows and veal calves. The surveys were performed in European countries (Belgium, France, Germany, Holland, Italy, Spain, UK, etc.), in North America (Canada and USA), in South America (Chile, etc.), and in Asia (Thailand, Sri Lanka, etc.) [2, 27, 53, 89, 105].

Stx-producing E. coli were detected in all surveys in most if not in all herds, but the proportion of positive animals in herds varied greatly from study to study and sometimes reached 100 %. Many of the positive strains detected belong to serotypes common among human EHEC and STEC. Geographical, management and seasonal fluctuations are possible explanations for the variable prevalence of EHEC and STEC of the different serotypes, but most probably the population sampling and the techniques of detection account for most of the variations. O157:H7 and O157:H– EHEC have a much lower, but also highly fluctuable, prevalence on farms and at slaughter, as compared to other serotypes: from less than 0.5 % up to 10 % of positive animals.

The Stx-producing E. coli from healthy animals may represent non-pathogenic strains, or strains pathogenic for humans but not for cattle, or strains pathogenic for calves (although the conditions for the development of the disease were not present at the time of sampling). One way of answering this question is to develop challenge experiments in calves. Experimental infections have been conducted with O157 human EHEC. They confirmed that O157 EHEC are not pathogenic for calves aged 1 week and more [17, 28], but also demonstrated pathogenicity for newborn calves [30, 31]. In older animals O157:H7 EHEC behave much as commensal E. coli more than pathogenic E. coli with a short-term colonization (<2 months) of the rumen and/or the intestine and shedding [53].

Another way of answering the above questions is to compare the general and specific properties of the Stx-producing E. coli from healthy cattle to those of Stx-producing E. coli associated with clinical disease in humans and in calves.

3.5. Epidemiology, sources of infection and public health hazard

The epidemiology of EHEC and STEC infection in young calves has not been fully studied but it seems reasonable to speculate that they are both carried by healthy individuals, calves and adults, and that the clinical disease is triggered by management problems such as overcrowding, transport, presence of other pathogens, etc. Diseased calves and healthy cattle can be infected with more than one strain based on serotype, pathotype, and the pulse field gel electrophoresis (PFGE) profile ([2, 12, 104, 107], China and Mainil, unpublished results).

The main problem is, however, the public health concern about the transmission of bovine EHEC and STEC to humans. To answer that question a comparison of human and bovine strains must be carried out using the same epidemiological tools.

Identical serotypes and pathotypes have been detected for human and bovine EHEC,
f.i. O26:H11, O103:H2, O111:H–, and O157:H7 [89, 95, 97, 136, 137, 139]. Some of these strains could be pathogenic for humans and calves (O26, O103, O111), whereas others would be pathogenic only for humans (O157). More precise molecular studies of the bacteria and of their virulence-associated factors and properties (stx genes and phages, EHEC virulence plasmids, LEE, tellure resistance, etc.) must be carried out to fully compare these bacterial strains and group them according to their pathogenic specificity and public health hazard potential. A recent study has detected differences in the LEE of the majority of EHEC (and EPEC) associated with diarrhoea in calves and of those isolated from healthy animals [27].

3.6. Diagnosis

The diagnosis and characterization of EHEC and STEC in cattle follow the same general approach as with human strains [89, 95]: cell cultures, ELISAs and/or PCRs are used for the Stx and/or the other properties either on bacterial isolates or directly on faeces. Specific ELISAs and uni- or multiplex PCRs on bovine faeces have been recently designed [1, 2, 27, 43]. Positive E. coli can be subsequently serotyped (see section 3.2). For detection of O157 EHEC carriers, special methods with enrichment and magnetic bead separation are being used much as the methods used in food microbiology [89, 95].

3.7. Infections in other ruminants

Small ruminants have been the subject of fewer surveys than cattle until recently when O157 EHEC were detected in sheep and goat in faeces or at slaughter [13, 24, 54, 70], thus showing that small ruminants may also represent a source of contamination for humans. Sheep positive for Stx-producing E. coli of other serotypes have also been detected with a wide variation from flock to flock [9, 10, 38, 58, 71]. In goat flocks, the percentage of positive animals also varied greatly, from 7 to 95 % [2, 9, 10]. Positive strains have various Stx profiles and many hybridize with the pCVD419 probe for the ehx genes. Transmission of O157 and other serotype EHEC to humans by raw goat milk or home-made raw-milk cheese have been demonstrated [13, 20]. O157 EHEC have also been isolated from wild and farmed deer in the UK and USA and were associated with a human outbreak in one case [23, 65, 108]. Stx-producing E. coli of various serogroups have also been detected in diarrhoeic and non-diarrhoeic buffalo calves in Sri Lanka [85].

4. STX-PRODUCING ESCHERICHIA COLI AND PIGS

Besides cattle and humans, Stx-producing E. coli are also associated with a well-documented disease in piglets, the ‘edema disease’ (ED), whose first published clinical description dates from 1938 in Ireland [119]. Although many clinical studies were carried out already in the 1950s, it was not before the 1980s that the most important bacteriological and molecular data were obtained.

The pathogenesis of ED and the virulence factors of the associated Stx-producing E. coli are the subjects of several reviews [5, 40, 49, 55, 139, 143]. Most pertinent data are summarized below along with a few additional references.

4.1. Clinical conditions

ED occurs most generally as sudden outbreaks in recently weaned piglets (1–2 weeks after weaning). Many affected piglets are found dead while others present nervous clinical signs (ataxia, convulsions) along with subcutaneous edema, especially on the head (eyelids, frontal area, groin). Diarrhoea is only rarely observed in true edema dis-
ease. Morbidity can reach 30–40% in some farms with a case fatality rate higher than 90%. Sporadic cases have also been described in younger suckling piglets and in older sows and boars.

At necropsy, the bodies are in good condition with a full stomach. Edema of the subcutaneous tissue, mesentery, stomach submucosa (cardiac region) and spiral colon submucosa are the most remarkable features. Enteritis is present only in the animals with clinical diarrhoea.

The typical histological lesions are extravasation and haemorrhages in various organs, mainly the central nervous system, the stomach, the large intestine and the subcutaneous tissue.

4.2. Serotypes and pathotypes

No more than four serotypes of *E. coli* are responsible for the great majority of the clinical outbreaks of ED throughout the world: O45:K+ (type strain E65), O138:K81 (type strain E57), O139:K82 (type strain E4), and O141:K–, formerly O141:K– (type strains E68II, E145) [126]. The O141:K85 serotype is also associated with neonatal and post-weaning diarrhoea.

The porcine Stx-producing *E. coli* belong to the STEC pathotype. EHEC strains have been observed only once and no clinical data were available [80]. In addition to Stx, the strains associated with diarrhoea (post-weaning diarrhoea or PWD) produce classical enterotoxin(s) and/or the fimbrial adhesin K88(F4) of enterotoxigenic *E. coli* (STEC/ETEC strains) [52, 143].

4.3. Virulence properties and pathogenesis

The main virulence factors of ED-associated *E. coli* are the Stx2e toxin and the F18 fimbrial adhesins acting as colonization factors. Some accessory virulence factors have also been described.

4.3.1. The Stx2e toxin

Speculation about the production of a toxic factor in the pathogenesis of ED came from early experimental reproduction of the clinical signs by intravenous injection of bacteria-free fluid from the intestinal contents of affected animals [130, 131]. This toxic factor received different names according to its properties in different experimental models: edema disease toxin, neurotoxin, angiotoxin, etc. [4].

Only 20 years later Konowalchuk et al. [69] reported the production of a verocytotoxin by several *E. coli* including strain E57. The E57 verocytotoxin activity was not neutralized by an immune serum to the Stx1 of strain H30 [14, 64, 69]. Dobrescu [36] identified this verocytotoxin with the edema disease toxin. The pig verocytotoxin is actually a variant of the Stx2 family (STx2e, previously Stx2vp), with differences in its biological activity (no activity on HeLa cells, no enterotoxicity) and partial neutralization by immune serums to Stx2 (see sections 2.2 and 2.3).

The Stx2e described so far are homogeneous and with a very few exceptions are pig specific [44, 98, 105, 129, 136]. The only related variant is produced by the human strain H.1.8 and has been named Stx2ev (for edema disease variant) [47]. Stx other than Stx2e (Stx1, Stx2) have only exceptionally been detected in porcine *E. coli* not associated with ED [45, 46, 72, 76].

Stx2e plays a central role in the pathogenesis of ED and in the occurrence of clinical signs. After colonization of the gut, STEC produce Stx2e which crosses the intestinal epithelium and reaches the bloodstream. From there Stx2e reaches its target, i.e. the Gb4 receptor present on the endothelial cells of small arteries and arterioles in various tissues and organs causing the microscopic lesions which are the basis of typical macroscopic lesions and clinical signs. Clinical disease can be reproduced by intravenous administration of purified
Stx2e [74]. STEC are also associated with PWD, but Stx2e plays no role in the occurrence of the diarrhoea which is caused by classical enterotoxin(s) (STEC/ETEC strains) [52].

### 4.3.2. The colonization factors

ED-associated STEC produce no AE lesions but rather fimbrial adhesins adhere to the microvilli of the enterocytes of the small intestine. A specific fimbrial adhesin, called F107 at first, was not described before the 1990s because its production in vitro was very difficult to obtain [5]. Optimal expression is obtained by growing the bacteria on blood agar under microaerobic conditions [141]. F107 antigenic variants produced by strains associated with PWD were described: O141 fimbriae [66], 2134P [21, 88], F8813 [111], but the existence of only two variants was later suggested [141]: F107ab, corresponding to the variant produced by the ED-associated E. coli, and F107ac, corresponding to the variant produced by the PWD-associated E. coli. These fimbrial antigens have now been renamed F18ab and F18ac [110]. In vitro and in vivo expression of F18ab by ED-associated STEC is generally poorer as compared to the expression of F18ac by PWD-associated STEC [88a]. The F18 fimbrial adhesin-encoding genes are also present in non-toxigenic E. coli [80, 86].

The genes encoding the F18 fimbrial adhesins are located on plasmids, sometimes along with genes encoding the α-haemolysin and the classical enterotoxins of ETEC ([29, 141], Mainil and Remy, unpublished data).

The F18 fimbrial adhesin acts as a colonization factor of the piglets small intestine (midjejunum and ileum) by mediating adherence of the STEC to the enterocyte microvilli without any alteration of the eukaryotic cells. The STEC subsequently proliferate and produce high amounts of Stx2e. Clinical observations of the 1950s of genetic and age resistance to ED are in fact scientifically based upon the resistance of the piglets to STEC colonization. The genetic resistance is due to the lack of specific receptors to the F18 fimbrial adhesin on the enterocyte cytoplasmic membrane. The production of specific receptors is controlled by one genetic locus and the susceptible phenotype is dominant [6]. The susceptibility of the small intestine to colonization by F18+ E. coli actually increases with age [88]. This observation is most probably related to the absence of receptors on the enterocyte membrane of newborn and very young piglets. The receptors for the F18ab and F18ac variants are most likely to be the same, but differ from the receptors for the F4 fimbrial adhesin of ETEC [88a].

### 4.3.3. The α-haemolysin

ED-associated STEC produce an α-haemolysin [73, 127]. Although there is no evidence that the α-haemolysin contributes to the pathogenesis of STEC in ED its production is very convenient for diagnostic purposes even if many haemolytic E. coli isolated from piglets are not STEC. However, non-haemolytic porcine STEC have only rarely been described [46, 72, 101, 125, 134].

The genes encoding the α-haemolysin are located on a plasmid in porcine STEC. Very often, this plasmid also carries genes encoding the F18 fimbrial adhesin and/or classical enterotoxin(s) ([29, 141], Mainil and Remy, unpublished data).

### 4.3.4. Others

The production of Stx2e is not systematically related to any other property such as the production of a colicin or to any specific biotype [46, 101].

As already mentioned porcine STEC can also produce classical heat-stable (STa, STb) and heat-labile (LT) enterotoxins and the F4 fimbrial adhesin of ETEC. The F4 fimbrial adhesin can also act as a colonization factor of the small intestine in recently weaned piglets and the enterotoxins are
responsible for clinical diarrhoea observed in some outbreaks of ED [52, 142].

4.4. Epidemiology and sources of infection

ED occurs as unpredictable sudden outbreaks in groups of recently weaned piglets and is the consequence of the proliferation of haemolytic STEC with the production of high amounts of Stx2e in the intestinal lumen. Haemolytic E. coli can, however, be isolated from healthy piglets in almost all pig farms with and without clinical manifestations of ED. What are the differences between the various haemolytic E. coli? And what causes their proliferation at weaning time?

The main conclusions of a large field study carried out by Deprez et al. [33, 34] are:

- most haemolytic E. coli in farms with ED but only a few of those in farms without ED are STEC;
- the haemolytic STEC are much more competitive in vivo to suppress the commensal intestinal flora than are the other haemolytic E. coli;
- the proliferation of STEC in the intestines of recently weaned piglets is mainly the consequence of the sudden removal of the maternal lactogenic protection;
- the other changes at weaning time (diet, regrouping, etc.) can play an additional role.

These observations are potential explanations for the fact that ED is an endemic transmissible disease in some farms only, i.e. in those in which the STEC circulate.

4.5. Diagnosis

Diagnosis of ED is mostly clinical, based on the age of the piglets, on the circumstances of occurrence of the disease, on the clinical signs and on the lesions observed at necropsy. Bacteriological confirmation is, however, often requested. A pure culture of haemolytic E. coli is usually obtained from the small intestinal content. However, haemolytic E. coli are also isolated from healthy and PWD piglets in farms with or without ED outbreaks. Pohl et al. [101] detected 21 STEC out of 23 haemolytic E. coli isolated from piglets with ED, 15 STEC out of 25 haemolytic E. coli recovered from healthy piglets in farms with ED and only six STEC out of 35 haemolytic E. coli when they were recovered from healthy piglets in farms without ED.

Confirmation that the haemolytic E. coli recovered are STEC is thus necessary and can be obtained by cell culture assays (Stx2e is toxic for Vero cells and not for HeLa cells), by immunological and/or by genetic assays. Stx2e cross-reacts to some extent with other Stx2 variants in immunological assays (see section 2.4). Only monoclonal antibodies can distinguish Stx2e from Stx2 and the other Stx2 variants. Similarly, a gene probe derived from the stx2e gene cross-hybridizes with other stx2 gene variants [78], but specific PCR for the different stx2 gene variants have been developed [3, 96, 99].

4.6. Public health hazard

Similarities between edema and central nervous disorders in piglets with ED and in a few humans with HUS on the one hand, and the isolation of Stx2e-producing STEC from human beings [44, 98, 129] on the other, have raised the question of human contamination with porcine STEC strains. However, porcine STEC serogroups have not yet been observed in humans with HC and HUS. In addition, if other STEC serogroups have been isolated from healthy piglets and from pork products, only a very few belong to the O157 serogroup and none are true EHEC [24, 139]. In conclusion, piglets, pork and pork products are not considered as a source of contamination with STEC and EHEC for humans.
5. STX-PRODUCING ESCHERICHIA COLI AND OTHER ANIMAL SPECIES

5.1. Mammals

Stx-producing E. coli (EHEC and STEC) have also been isolated from pets such as dogs and cats, although these reports are rare and usually incompletely documented. A quite recent publication even presents evidence of dogs as vectors for human contamination with O157:H7 EHEC [132]. Data were reviewed by Broes [16] and Peeters [96] and are reviewed in this issue [7].

Horses may also act as vectors of O157:H7 EHEC [132]. Although an agreement on the involvement of E. coli in intestinal disorders in horses exists, their general and specific characteristics are still largely unknown [96].

5.2. Birds

In addition to mammals Stx-producing E. coli have also been isolated from birds (poultry [39, 41, 82], seagulls [133] and feral pigeons [32]). Their association with disease is uncertain but they may act as vectors for infection of domestic ruminants and humans. The most recent data on pathogenic E. coli for birds are also reviewed in this issue [35].

6. CONCLUSION: PAST AND FUTURE OF THE SHIGA TOXINS

The history of Shiga toxins encompasses one century considering that the description of the causal agent of bacterial dysentery, Shigella dysenteriae, was published in 1898 by Shiga [121] (cited in [91]). But the most intense period of research on Shiga toxins of E. coli began only 20 years ago with the works of Konowalchuk et al. and O’Brien et al. [69, 93]. Many subsequent research studies demonstrate that history (and research) is a perpetual (re)discovery of old observations.

In 1971, Smith and Linggood [124] referred to the production by the human E. coli H19 of a ‘heat-labile enterotoxin’ which was, however, not neutralized by an immune serum specific for the LT of ETEC. Moreover, if the capacity of producing this toxin was transferable in a conjugation experiment, as was the capacity of LT production which is plasmid-encoded, no plasmid was demonstrable in the recipient strain. Explanations came later: H19 produces Stxl (not LT) whose encoding genes are located on a phage [69, 116, 125].

Speculations that the HUS syndrome in humans was caused by an enteropathogenic E. coli after acquisition of a bacteriophage was raised in the late 1960s by Kibel and Barnard [67] (cited in [94]). Interestingly enough, retrospective studies have reported the existence of Stx-positive E. coli (EHEC and STEC) in collections of bovine strains of the 1960s [8, 77, 79, 103].

Another conclusion from Shiga toxin history is that an exception today can become a general rule tomorrow.

In their initial work, Konowalchuk et al. [69] reported the production of different verocytotoxins. Indeed two E. coli produced a verocytotoxin whose toxicity was not neutralized by an immune serum to the cytotoxins of the other strains: strain E57 from a piglet with ED and strain H.I.8 from an infant with diarrhoea. Moreover, their range of cytotoxicity differed from the other cyto-toxins [68]. It was later demonstrated that strain E57 produces the Stx2e toxin and strain H.I.8, the Stx2ev toxin.

Let us now take a prospective look into the future [91]. Much has been learned during the last 20 years, but there are still areas which escape our knowledge. Of the many questions to be addressed in the future research works on Stx, STEC and EHEC, the following two are very important (from the author’s point of view).
What is the ecology of EHEC, especially of the O157 serogroup, in cattle?

What is the vaccination potential against STEC and EHEC in humans and in animals?

These questions will not be answered easily, especially the former one which will need the use of time-consuming animal models to study colonization and survival [30, 53]. To answer the latter question in humans, one needs animal models to reproduce HUS [87]. Stx produce intestinal, cerebral and kidney lesions in rabbit and mice after parenteral injections, but not the typical HUS syndrome. Besides non-human primate models, another potential animal model for HUS is the greyhound in which a clinical condition similar to HUS (the cutaneous and renal glomerular vasculopathy, or CRGV) has been linked with 0157:H7 [42, 87].

The situation is quite different in piglets in which ED is reproducible by intravenous administration of purified Stx2e and which can be protected in experimental and field trials by vaccination with a purified toxoid or a mutant Stx2e [49].

The situation in cattle is two-fold. First, if EHEC are a cause of diarrhoea in young calves, the role of Stxl is still speculative and no clinical systemic complications such as HUS have been described. Vaccination against EHEC thus targets the AE phenotype more than the Stx [49]. There is also much excitement and speculation about preventing the carriage of the O157:H7 EHEC by vaccination. It is, however, too early to say that this will represent a powerful way of preventing carriage of O157:H7 EHEC in cattle [30, 49].

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