Chapter 4
Microbial Toxins in Foods: The Importance of *Escherichia coli*, a Versatile Enemy

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Abstract When speaking of food microbiology, analysts and hygiene professionals are accustomed to starting their discussions with a relatively less number of food pathogen bacteria, in spite of the great variety of microbiological risks in the food and beverage sector. A few micro-organisms are well known with reference to the possibility of showing diversified strains. In other words, one specific micro-organism can spread into the environment and in selected 'culture media' like foods with the possible 'permutation' of the original strain in several sub-strains with different properties and features. This is the situation of *Escherichia coli*, the most important micro-organism in the group of so-called 'Coliform' bacteria. *E. coli* is a typical commensal of the intestinal tract of animals and humans. For its abundant presence in the colonic microflora, it is used as one of the most important indicators of faecal contamination in food and water. However, some subsets within this species have acquired specific virulence genes. Enteric strains have been divided into different pathotypes, depending on virulence factors and pathogenic features. In the present chapter, various *E. coli* pathotypes are described; particular relevance is given to toxin-producing strains. Additionally, this work intends to provide useful information about related toxins, their known chemical properties and the most used analytical methods in the food sector.

Keywords *Escherichia coli* · Faecal contamination · Food-borne outbreak · Heat-labile toxin · Heat-stable toxin · Shiga toxin

Abbreviations

AAF	Aggregative Adherence Fimbrium
A/E	Attaching-and-Effacing
CT	Cholera Toxin
CF	Colonisation Factor
CFU	Colony Forming Unit
DNA	Deoxyribonucleic Acid
DAEC	Diffusely Adherent *E. coli*
EAEC	Enteroaggregative *E. coli*
EAST-1	Enteroaggregative Heat-Stable Toxin 1

EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
LT	Heat-Labile
ST	Heat-Stable
STa	Heat-Stable a
STb	Heat-Stable b
IMS	Immunomagnetic Separation
LEE	Locus Enterocyte Effacement
LPS	Lipopolysaccharide
PCR	Polymerase Chain Reaction
RTE	Ready-to-Eat
RKI	Robert Koch Institute
SPATE	Serine Protease Auto Transporter of *Enterobacteriaceae*
Stx	Shiga Toxin
STEC	Shiga Toxin-Producing *E. coli*
ShET-1	*Shigella* Enterotoxin 1
SMAC	Sorbitol-MacConkey
VTEC	Verotoxigenic *E. coli*

4.1   Introduction

Escherichia coli is a member of the family *Enterobacteriaceae*: it is represented mostly by motile Gram-negative bacilli. It occurs in diverse forms in nature, displaying an impressively versatile behaviour, which reflects the immense diversity within the species (Bergthorsson and Ochman 1998). Far from being only an enteric species, *E. coli* has recently been also recognised as a free-living bacterium; environmental reservoirs of this organism are known (Cox et al. 1988). In fact, evidences from a number of studies suggest that *E. coli* may not only persist, but also multiply in natural environments such as warm, sub-tropical soils and waters (Byappanahalli and Fujioka 1998, Solo-Gabriele et al. 2000), even if survivorship in open environments is strain -dependent (Topp et al. 2003). Nevertheless, the intestinal system of humans and other mammals is considered to be more favourable for its growth, as resource availability and the constancy of the abiotic features represent the ideal conditions for *E. coli* (van Elsas et al. 2011). Among the human colonic flora, this microorganism is the predominant facultative anaerobe. *E. coli* can colonise the gastrointestinal tract of infants within hours after birth and afterwards, establishing an enduring relationship with mutual benefits for the rest of the

host's life (Kaper et al. 2004). *E. coli* commensal strains thrive in the mucous layer of the mammalian colon, allowing the host to break down particular carbon compounds. These commensal strains remain confined harmlessly in their niche and are rarely pathogenic, except when gastrointestinal barriers are broken.

However, the versatile behaviour of *E. coli* ranges also to pathogenic forms. In fact, several strains have acquired specific virulence factors that affect a wide array of cellular processes; these strains possess genomic islands (i.e. regions) which are full of genes that encode key traits for adherence, colonisation, invasion and production of toxic compounds (Touchon et al. 2009). These virulence determinants have been acquired by means of bacteriophages or plasmid deoxyribonucleic acid (DNA); these determinants have been frequently spread by horizontal gene transfer, leading to the creation of novel combinations of pathogenic strains. The various commensal and pathogenic forms of *E. coli* are known to have genomes that may diverge by up to 20 % (Ochman and Jones 2000). Generally, infections due to pathogenic *E. coli* result in three general clinic syndromes: urinary tract infections, in which *E. coli* is by far the most common causing agent; sepsis/meningitis; enteric/diarrhoeal diseases. Among the diarrheagenic strains, there are (Kaper et al. 2004) six well-known pathotypes (i.e. group of strains of a single species that cause a disease using a common set of virulence factors): enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC).

Several other potential *E. coli* pathotypes have been reported but have not been as well established as the ones listed above. Among the six pathotypes, some include strains of worldwide public health importance, which are recently emerging. In fact, certain groups can cause life-threatening diarrhoea, which in addition may lead to severe sequelae.

4.2 Diarrhoea-Associated *E. coli*

All the six pathotypes possess specific proteins, which enhance their colonising and adherence ability, overwhelming competition with the indigenous flora. On the other hand, various pathotypes are classified on the basis of virulence factors, as they differ in the pathogenetic strategy, which may consist in:

- Intracellular invasion
- Intimate adherence to epithelial cell membranes
- Production of toxins able to disrupt fundamental eukaryotic processes.

In addition to the so-called virotyping, serology is also used. In fact, *E. coli* strains are classified on the basis of antigenic differences. Serotyping is performed on the primary components of cell surface: the lipopolysaccharide (LPS) which is termed O antigen, the flagella (H antigen) and some strains possess a capsular (K) antigen. Each O antigen defines a serogroup, while the combination of O and H

antigens identifies the serotype (Kaper et al. 2004). More than 180 O, 60 H and 80 K different antigens have been proposed (Stenutz et al. 2006). Nevertheless, there is not always a definite correlation between the serogroup and the pathotypes. One pathotype may comprise several serogroups, which conversely may belong to various pathotypes on the basis of virulence factors. Furthermore, virulence factors usually reside in mobile genetic elements (as plasmids and phages), which thus may be transferred from one strain of *E. coli* to another.

Therefore, even if these categorisations may become useful in approaching classification, they should not be considered absolute.

4.2.1 *Enteropathogenic* **E. coli** *(EPEC)*

EPEC has been the first identified pathotype. The characteristic of infections due to EPEC is the 'attaching-and-effacing' (A/E) histopathology. Specifically, bacteria intimately attach to the intestinal epithelial cells causing marked cytoskeletal changes, including accumulation of polymerised actin and the effacement of intestinal microvilli. EPEC virulence factors are encoded in a plasmid of 70–100 kb, called EPEC Adherence Factor (EAF) plasmid, and in genes on a 35 kb pathogenicity island, named Locus Enterocyte Effacement (LEE). EAF is important when speaking of the adherence to epithelial cells and the recruitment of bacteria in the environment through the formation of three-dimensional networks (Giron et al. 1991); intimate adherence is also mediated by a protein, intimin, whose gene is present in LEE.

LEE also encodes a specialised secretion system, which assists in the translocation of relevant proteins to the extracellular environment, and associated proteins. Some strains also produce the EspC enterotoxin which is homologous to members of the autotransporter family of proteins (Mellies et al. 2001), which will be addressed in Sect. 4.2.4.

Among the EPEC, some of the most common serogroups are O20, O25, O26, O44, O55, O86, O91, O111, O114, O119, O125ac, O126, O127, O128, O142 and O158 (Nataro and Kaper 1998).

Usually, infection due to EPEC results in severe watery diarrhoea, together with vomiting and low-grade fever. Diarrhoea likely results from multiple mechanisms, as malabsorption due to loss of microvilli, local inflammation, augmented intestinal permeability and active ion secretion. At present, EPEC strains are no longer as relevant as in the middle of 20th century. Sporadic outbreaks are still observed both in Europe and in the United States (Nataro and Kaper 1998), caused mostly by the so-called atypical EPEC, that contain LEE (but EAF plasmid is absent). However, in contrast to their modest importance in industrialised countries, typical EPEC (i.e. containing the EAF plasmid) continue to be a major cause of diarrhoea in developing countries. In particular, EPEC are responsible for a high mortality in infants younger than 2 years. EPEC strains are often the most frequently isolated bacterial diarrhoeal pathogens in the 0–6-month group (Nataro and Kaper 1998). Adults and

older children are relatively resistant and probably serve as the reservoir being asymptomatic carriers. Various foodborne outbreaks have been reported: prawn mayonnaise, salads and infant formula are described as vehicles, even if in the majority of cases the food sources have been rarely identified (Meng et al. 2001).

4.2.2   *Enterotoxigenic* E. coli *(ETEC)*

The importance of ETEC is correlated with the secretion of at least one group of enterotoxins: heat-stable (ST) and heat-labile (LT) toxins. In fact, ETEC strains may express either one of the two toxins only, or both a ST and a LT. Anyway, both the toxins are under the genetic control of transmissible plasmids, as also colonization factors (CF). CF mediate the colonisation of the small intestine, thus allowing the elaboration of toxins (fimbrial or fibrillar proteins). More than 22 CF have been detected among human ETEC (Qadri et al. 2005), but nonetheless epidemiological studies report that about 75 % of human ETEC express either colonization factor antigens (CFA) I, II or IV. It has also been reported that CF have been detected in less than 10 % of ETEC strains expressing only LT, in comparison to much higher percentages in strains expressing ST or both (over 60 %). These data, together with the more frequent isolation of LT-producing strains from healthy people (in respect to the other two combinations), suggest CF may have a role in pathogenesis. However, the main role is surely due to the production of LT and ST enterotoxins.

LT are closely related to Cholera toxin (CT), an enterotoxin produced by *Vibrio cholerae*, with which they share approximately 80 % amino acid identity (Sixma et al. 1993a). Besides protein sequence, LT and CT have the same enzymatic activity, holotoxin structure, primary receptor and activity in animal and cell culture assays, while they differ in toxin processing and in the immune system response (Nataro and Kaper 1998). LT have an AB_5 multimer structure, where the single A subunit is involved in catalytic activity and the B pentamer is necessary for membrane binding. LT is of ca. 86 kDa; in particular, the A subunit is 28 kDa and each B subunit is 11.5 kDa (Streatfield et al. 1992). In relation to LT, proteins are produced as monomers and the A subunit stimulates the assembly of the five B subunits. The B monomer is small, compact and highly structured, as it consists of a small *N*-terminal helix (α_1), two three-stranded anti-parallel sheets (sheet I: β_2, β_3, β_4, and sheet II: β_1, β_5, β_6) and a long helix (α_2). The two sheets interact with each other through three hydrogen bonds (Sixma et al. 1991). The α_2 helix shows amphipathic behaviour, as on one side it has eight charged residues. On the other side, it is mostly hydrophobic except on the last turn, which has hydrophobic residues on both sides. The loops can be divided in two classes, depending on the end of the sheets: a 'narrow' end of the subunit, where the loops are shorter, involving the connections β_1–β_2, β_3–β_4, α_2–β_5 and the *C*-terminus, and a wider end, with longer loops connecting secondary structure α_1–β_1, β_2–β_3, β_4–α_2, β_5–β_6 and the *N*-terminus nearby (Sixma et al. 1993a).

The B subunits-pentamer is arranged in a 'doughnut' shape, with a pore in the middle. The pore is very polar, being formed by the long α_2 helices, and shows a net positive environment, due to the prevalence of positive residues in comparison to negative ones. The pentamer is stabilised by various interactions, including at least 26 hydrogen bonds between a subunit and the adjacent molecules, and at least four salt bridges between a subunit and each neighbour, with 10 being in the central pore. There is a flat surface on one side of the pentamer, due to the combination of the short loops on the narrow end of the subunits, where A subunit is found. The long loops, instead, form a more convoluted surface, which has been reported to be part of the membrane receptor-binding site (Sixma et al. 1992). Lastly, six-stranded antiparallel β sheets are formed by the combination of the three-stranded sheets with the opposite sheet of the adjacent monomers. The A subunit is responsible for the activity of the toxin and is proteolytically cleaved, generating two peptides: A_1 and A_2. A_1 consists of a wedge-shaped domain with numerous hydrogen bonds, while A_2 has a long helix, that in the complete A subunit is packed with one side against the A_1 fragment (Sixma et al. 1993a). A disulphide bridge joins the two fragments; besides, there are also four hydrogen bonds and one salt bridge. Although there are not so many hydrophilic contacts, the packing is not exceedingly hydrophobic with the A subunit surface being 52.9 %-hydrophobic. The N-terminal A_1 is the real catalytic moiety, and the C-terminal A_2 is inserted non-covalently into the central pore of the B pentamer. Finally, there are also various interactions between A and B subunits. A_2 is involved mainly in the formation of hydrogen bonds and five salt bridges with one monomer of the B subunit. In addition, there are two regions of hydrophobic interactions between A_2 and the B pentamer: one is located at the narrow end of the central pore and the second is at the wide end of the pore. There are also indirect contacts, mediated by a number of water molecules. Moreover, A_1 forms interactions with the B subunit (Sixma et al. 1993a).

The AB structure of the toxin is important in protecting the A subunit from degradation in a protease-rich environment as the lumen. In fact, it keeps trypsin-sensitive residues in a hidden structural conformation, preventing proteolysis and subsequent degradation of the A subunit. Instead, the arginine 192 residue, whose cleavage is required for LT activation, is located in a surface-exposed loop, allowing the biological function of the toxin.

Purified LT preparations proved stable over pH adjustments and storage at −70 and −20 °C for months; the biological activity of the holotoxin, and in particular of the A subunit, has not been removed completely after heating at 65 °C for 30 min. In addition, the biological activity has been enhanced by incubation with trypsin and has been cancelled by pronase and proteinase K (Kunkel and Robertson 1979).

LT are divided in two serogroups since they do not cross-react immunologically: LT-I and LT-II. LT-I is common as a pathogenic agent both in humans and in animals, while LT-II is found mostly in animals. LT-II has not been previously associated with disease, but recent isolations from diarrheic patients lead to hypothesise its toxicity (Nardi et al. 2005). LT-I and LT-II have highly homologous amino acid sequences for the A subunit, which reflects the evolutionary need to preserve the structure for toxigenic activity. In contrast, the two B subunits display

high divergence (Connell 2007). In fact, the differences in the B subunits reflect the fact that it has to bind gangliosides, which have an interspecific and intraspecific (in different cell types) heterogeneity. From the structural point of view, gangliosides are oligoglycosylceramides, that contain one or more *N*-acetylneuraminic acid or *N*-glycolylneuraminic acid forms of sialic acid, conjugated to the ceramide core by glycosidic linkages (Hajishengallis and Connell 2013). The gangliosides-binding activity of LT-I and LT-II is peculiar. However, different variants exist among both LT-I and LT-II. LTp-I and LTh-I have been discovered in strains isolated from pigs and humans, respectively, and show partial antigenic cross-reactivity. LT-II also shows subclasses; in detail, three variants have been described: LT-IIa, b and c. These members are antigenically distinguishable and have different toxic doses. LT-IIb has a lower isoelectric point (5.2–5.6 if compared with 6.8), a lower toxic dose and its behaviour during purification diverged significantly in comparison to LT-IIa (Guth et al. 1986). The last of these members, LT-IIc, has been only recently detected in an avian host, and it has been shown to be less cytotoxic than the other two variants using mouse bioassay (Nawar et al. 2010). The differences among the variants depend on the multiple amino acid substitutions in the B subunit, which enable the three LT to bind distinct gangliosides, reflecting their capacity to intoxicate several animals.

In contrast to large LT, ST are low-molecular-weight peptides and contain several cysteine residues, which are responsible for their heat-stability. In fact, ST are still active after 30 min at 100 °C. ST include two unrelated classes that differ both in structure and in mechanism of action: heat-stable a (STa) and b (STb). STa are cysteine-rich peptides, highly homologous to proteins found in other enteric bacteria, as *Yersinia enterocolitica*, *Klebsiella pneumoniae* and *Citrobacter freundii* (Guarino et al. 1987; Klipstein et al. 1983; Takao et al. 1984). STa toxic activity is not destroyed after treatment with pronase, trypsin, proteinase K, periodic acid oxidation and several organic solvents (acetone, phenol, chloroform and methanol). In addition, STa is stable at acidic pH (up to pH 1.0), while a pH value greater than 9.0 destroys biological activity (Alderete and Robertson 1978). The isoelectric points of STa range from 3.88 to 4.08 (Dreyfus et al. 1983). Two variants are found among STa. However, they are nearly identical and can be both observed in humans: STp and STh, after their initial discovery in pigs and humans, respectively.

STa is initially synthesised as a 72-amino acid precursor peptide, while the mature protein contains 18 (STp) or 19 (STh) amino acids, with a molecular weight of 2 kDa. The 18–19 amino acid peptide contains the toxin, which reaches the final sequence after cleavage during translocation to the periplasm. In fact, the highly conserved region (residue 29–38) in the precursor protein increases the translocation of STa across the membrane (Yamanaka et al. 1993). The toxic domain (14 residues) is located at the *C*-terminus, while the last residues at *N*-terminus are not required for biological activity. The mature toxin contains six cysteines that form three disulphide bridges. Disulphide linkages are fundamental to enterotoxicity, as they play a crucial role in determining the strength of the bond with the receptor; consequently, biological activity is completely abolished by treatment with reducing and oxidising agents. Moreover, they contribute to stability of the

molecule, together with hydrogen bonds. In the crystal structure, the Cysteine 5–Cysteine 10 disulphide bridge has quite a rare geometry, adopting a right-handed conformation, whereas the other two bridges have a more common left-handed spiral conformation (Sato et al. 1994). The crystal structure of STp has shown good agreement with the structure of STh, determined by NMR (Matecko et al. 2008). A ring-shaped self-associated hexamer is formed by STa (Sato and Shimonishi 2004).

STb is associated prevalently with diseases in cattle while rare cases have been reported for humans (Lortie et al. 1991; Okamoto et al. 1993), where STb had probably been acquired from animals (e.g. pigs). Its sequence shows no homology when compared with STa. Moreover, STb can induce the loss of villus epithelial cells and partial villus atrophy (Nataro and Kaper 1998). STb is a highly basic molecule, with a determined isoelectric point of 9.6: in fact, it contains nine basic amino acids. In particular, lysine residues seem to contribute in an important way to the toxicity of STb (Fujii et al. 1994).

ETEC produce enterotoxins in the early stages of growth. Although they are apparently secreted in all conditions that enable cell growth, the release of LT in enriched culture media seems to be favoured at neutral to alkaline pH values if compared to acid pH values (Gonzales et al. 2013), while a pH-dependent release of ST has not been observed (Johnson et al. 1978).

Among the ETEC, some of the most common serogroups are O6, O8, O11, O15, O20, O25, O27, O78, O128, O148, O159 and O173 (Stenutz et al. 2006).

Known infections result in watery diarrhoea, usually without blood, mucus or pus, whereas vomiting and fever are present rarely. Clinical records may vary from mild, brief and self-limiting diarrhoea, to severe and life-threatening cases, especially in weanling infants.

The majority of cases are thus associated with childhood diarrhoea in the developing world, but also people who travel in these areas also frequently experience the infection. In fact, available data suggest 20–40 % of traveller's diarrhoea cases may be caused by ETEC. The infection requires quite high infectious doses: up to 10^8–10^{10} colony forming units (CFU), mainly because of faecal contamination of food and water sources (Neill et al. 2001). Indeed, an inadequate sanitation of drinking water is common in the developing countries; sampling in the endemic areas has shown impressive rates of contamination, above all during warm months and in the rainy season, when growth and multiplication in food and water are more efficient (Qadri et al. 2005). Transmission of ETEC diarrhoea in developed countries is also possible, due to asymptomatic travellers importing these microorganisms. Food-borne outbreaks have been reported in the United States and Sweden, with ETEC strains present in processed meat and cheese products (Danielsson et al. 1979; Sack et al. 1977). In addition, other types of foods have been involved with ETEC outbreaks, including curried turkey mayonnaise, crabmeat, salads and tuna paste, maybe because of infected food handlers or contaminated water used during preparation (Meng et al. 2001).

4.2.3   *Enterohaemorrhagic* **E. coli** *(EHEC)*

EHEC are a frequent source of foodborne outbreaks and may cause life-threatening complications. However, EHEC denote a subset within verotoxigenic *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC). The term STEC is correlated with the production of a Shiga toxin (Stx) which is quite identical to the Stx produced by *Shigella dysenteriae* I, while VTEC indicates the toxicity of Stx for Vero cells (African green monkey kidney cells). Whereas not all STEC are considered pathogens, EHEC strains are all pathogens by definition, as they have other virulence factors besides Stx.

In fact, EHEC denote strains possessing also the so-called 'eae' gene, responsible for A/E lesions in the intestinal epithelial cells, and a 60-MDa plasmid (i.e. typical EHEC). A/E-positive STEC strains contain in their chromosomes the LEE pathogenicity island, as seen in EPEC (Sect. 4.2.1).

The key virulence determinant is Stx. Two types of Stx are produced by STEC: Stx1 and Stx2, immunologically non cross-reacting. Stx1 is identical to Shiga toxin from *Shigella dysenteriae;* there may be a difference in one residue (a serine instead of a threonine), and is highly conserved. Three Stx1 variants—Stx1a, Stx1c and Stx1d—have also been reported (Burk et al. 2003; Zhang et al. 2002). In particular, Stx1c has been related to mild or no disease in humans, and is more frequently isolated in ovine. Conversely, Stx2 includes numerous antigenic variants—Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, Stx2 g and Stx2d-activable—which vary in their biological activity, immunological reactivity and association with disease. Stx2e and Stx2f are the two most divergent variants from Stx2 at the amino acid level and are usually associated with animals (Stx2e and Stx2f are specifically linked with oedema disease of swine and feral pigeons respectively). Instead, Stx2a, Stx2b, Stx2c and Stx2d variants are more frequently associated with human illness. Stx2 g has been isolated from a bovine strain of *E. coli* and displays the highest homology with Stx2a and Stx2c.

Stx1 and Stx2 may be present in a single EHEC strain both together or singularly. One strain may produce more than one Stx as it may harbour more than one Stx-encoding bacteriophage. These phages are important for the spreading of Stx genes and other species expressing Stx have been described, as some strains of *Citrobacter freundii* and *Enterobacter cloacae*. Both Stx1 and 2 have an AB_5 structure, in which the A subunit (32 kDa) is bound to 5 B subunits (each 7.7 kDa). However, Stx1 and 2 share only 55 and 57 % amino acid homology, respectively, in the A and B subunits (Jackson et al. 1987).

Stx has a complex structure (as LT in ETEC) and the two toxins share some similarities, although they have almost no sequence identity (three identical residues in 52 superimposed residues, Sixma et al. 1993b). Stx, in fact, shows a pentameric ring formation for the B subunit (Stein et al. 1992), which is associated to the A subunit, that can be cleaved in two smaller fragments: A_1 (27.5 kDa) and A_2 (4.5 kDa). The two portions are covalently associated through a disulphide bond, with A_1 retaining the catalytic activity. The ring formed by the B subunits encircles

a helix at the C-terminus of the A subunit, while the rest of the A subunit lies on one side (Fraser et al. 1994). The A subunit interacts with the B pentamer via the A_2 fragment, that is inserted in the central pore. The B monomer is formed by two three-stranded antiparallel β-sheets and the α-helix. Therefore, unlike LT B-monomer, Stx lack an additional a-helix at its N-terminus. The β-sheet interaction between adjacent monomers is similar to LT but it is exposed due to the shorter N-terminus, forming six-stranded antiparallel β-sheets around the outer surface of the pentamer. Moreover, the interface between monomers is not as large as in LT, making Stx less stable (Sixma et al. 1993b). In Stx, the central pore is delineated by the five helices and shows no charges (on the other hand, LT pore is highly charged).

As previously indicated, Stx1 is structurally different from Stx2, which is 400 times more lethal to mice (Tesh et al. 1993) and 1,000 times more toxic for human renal microvascular endothelial cells than Stx1 (Louise and Obrig 1995). Stx2 is associated with high virulence in humans, and it is a predictor of HUS if associated with the presence of the eae gene. Structural differences account for the greater pathogenicity of Stx2.

STEC strains have the potential to produce Stx in food. The Stx2 molecule is quite heat stable, as it resists pasteurisation processes (Rasooly and Do 2010). Stx2 retains almost full activity after heating at 60 °C for one hour, while a significant reduction occurs after heating at 80 °C for one hour (He et al. 2012). Consequently, it may account for those disease cases in which no live bacteria have been found after mild heat treatments.

In addition to Stx, EHEC contain numerous virulence factors, for instance a variety of fimbrial and non-fimbrial adhesins, all involved in various stages of adhesion, and other toxins that may contribute to EHEC pathogenesis (Dautin 2010; Kaper et al. 2004; Schmidt et al. 1995; Taneike et al. 2002).

Infection with STEC may result in no clinical symptoms, but in most cases it causes diarrhoea which can evolve in bloody diarrhoea (haemorrhagic colitis, HC), abdominal cramps, accompanied by vomiting and fever in some patients . The incubation period is on average 3–4 days, even if shorter (1 day) to longer (up to 8 days) period has been described (Nataro and Kaper 1998). In some cases, patients may develop haemolytic uremic syndrome (HUS) which is a potentially lethal complication, characterised by haemolytic anaemia, thrombocytopenia (decreased number of platelets) and acute kidney failure. The onset of HUS is more likely to occur (Gyles 2007) with O157 serotype—up to 10 % of individuals—and in patients affected by haemorrhagic colitis. In fact, production of enough Stx results in damage to blood vessels in the colon. Should a sufficient level of toxin be concentrated in the blood, Stx could reach various districts of the body causing HUS and/or impairment of other organs, as central nervous system and heart. In particular, children under five years of age and the elderly usually are more involved in these progressions.

The most famous EHEC serotype is O157:H7, because of its prevalence and importance in human disease, even if there are several other common serogroups: O4, O5, O16, O26, O46, O48, O55, O91, O98, O111ab, O113, O117, O118, O119,

O125, O126, O128, O145, O157 and O172. Recently, new EHEC serogroups—O176, O177, O178, O179, O180 and O181—have been isolated (Stenutz et al. 2006). On the basis of serogroups, STEC have been divided in classes—from A to E—depending on the virulence and the frequency of the outbreaks, where O157 belongs to seropathotype A while STEC serotypes that have not been implicated with outbreaks belong to seropathotype E (Karmali et al. 2003).

Escherichia coli O157:H7 is the leading cause of STEC infections worldwide: the United States Center for Disease Control and Prevention has estimated that it is responsible for more than 50 % of all EHEC infections in North America. Additionally, the O157:H7 serotype is also very common in Europe, Japan, Australia, Argentina, Chile and South Africa, even if non-O157 EHEC—O26, O103 and O111—are often causing outbreaks in these countries (Brooks et al. 2005). However, O157:H7 is statistically the serotype most often associated with haemorrhagic colitis and HUS. Interestingly, EHEC are less frequently isolated in developing countries if compared with other pathotypes as EPEC and ETEC (Nataro and Kaper 1998).

Therefore, EHEC epidemiology is very complex, involving animal reservoirs, a long duration of shedding and a very low infectious dose. Specifically, STEC are shed in stools of infected patients for weeks. In fact, duration ranges from two to 62 days, with an average of 13; the range is far longer for HUS patients (5–124 days). The infective dose for *E. coli* O157:H7 is reported by the Food and Drug Administration (FDA) to be 10–100 cells, while the number is slightly higher for other EHEC. Transmission is by the faecal-oral route and occurs by direct contact with the infected animal, from person to person and by ingestion of contaminated food or water. In particular, edible materials are the most relevant way, as it has been estimated that up to 85 % of STEC infections are transmitted by food (Meng and Schroeder 2007). In fact, most EHEC outbreaks can be traced from food that had previously entered into contact with animal faeces. STEC strains have been isolated from a variety of animals, such as cattle, sheep, pigs, birds, goats, as well as horses, cats and dogs, though ruminants are thought to be the prevalent reservoir (Hussein 2007). They can host several strains simultaneously and the rate of colonisation is high, with reported prevalence of up to 70 % in bovine herds (Pradel et al. 2000), and concentrations of 10^7 CFU/g (Fegan et al. 2004). Furthermore, shedding seems to be related to age and period of the year with the highest frequency in young animals and in warmer months (Gyles 2007). Additionally, STEC are also commonly detected in cattle feedlots.

Thus, meat derived from these animals may be contaminated with STEC strains if there is any deficiency in hygiene practices. Food associated with STEC hence includes firstly undercooked beef and other types of meat, as porcine and sheep meat, but also fresh produce, salami, raw milk and cheeses. STEC outbreaks have been increasingly associated with consumption of fruits and vegetables, as lettuce, cantaloupes, alfalfa sprouts and radish sprouts, bagged salads and even potatoes in one case in Great Britain (Meng et al. 2001). In these situations, it can be speculated that contamination has occurred in the field due to irrigation or the use of soil that had been treated with farm effluent (Fremaux et al. 2008). Subsequently, the contamination has

reached the whole batch. Sources of infection include as well chicken, turkey and seafood (i.e. fish and shellfish), maybe due to cross-contamination.

STEC can also survive for long periods in water. As a result, almost every water source, as drinking water, streams, ponds, well water and even municipal water system, has been linked to outbreaks (WHO 2011). Notably, also food that has been regarded as safe and ready-to-eat for its acidity, as mayonnaise and unpasteurised apple cider and juice, has been found implicated. *E. coli* has a wide range of acid tolerance, being capable of growing at pH 4.4–10, but many STEC strains—in particular, O157:H7—have shown to survive in a really acidic environment, with a pH of 2.5–3 for more than 4 h (Molina et al. 2003). Leyer and co-workers have found that *E. coli* O157:H7 is able to survive for several days at pH 3.4, even if acid tolerance is dependent on the type of acid and is increased in microorganisms which had been previously exposed to weaker acids (Leyer et al. 1995). Furthermore, Stx production is also influenced by environmental factors. In fact, Stx amounts are more abundant in strains grown at 37 °C than at lower temperatures (Abdul-Raouf et al. 1995); higher concentrations have been obtained in minced meat in respect to milk.

4.2.4 *Enteroaggregative* E. coli *(EAEC)*

EAEC are the most recently described category of diarrheagenic *E. coli*. In contrast to the EPEC localised adherence pattern, EAEC show an aggregative adherence in Hep-2 cells (Nataro and Kaper 1998). The aggregative adherence pattern is mainly due to fimbrial structures, known as aggregative adherence fimbriae (AAF), related to the Dr family of adhesins (Nataro et al. 1992). Genes coding for AAF are located on a 60-MDa plasmid; four variants of AAF exist at least, each expressed in only a minority of EAEC strains. However, some EAEC strains with the characteristic adherence pattern have been found to lack AAF, suggesting the existence of other adhesion mechanisms. For instance, a novel adhesion pilin has recently been reported in EAEC strains lacking known AAF (Boisen et al. 2008). Another protein instead, dispersin, acts to disperse the bacteria, hence partly counteracting the aggregating effect mediated by AAF (Sheikh et al. 2002).

EAEC pathogenesis is also due to the elaboration of several toxins. The enteroaggregative heat-stable toxin 1 (EAST-1) is present in ca. 40 % of EAEC strains and seems to play a role in EAEC pathogenicity, even if its role is still questioned, given that it can be detected also in commensal strains. In addition, this toxin is associated with diarrhoea also in cattle and pigs. EAST-1 is a 38-amino-acid protease-sensitive enterotoxin, with a molecular weight of 4.1 kDa and a calculated isoelectric point of 9.25 (Savarino et al. 1993). It contains four cysteines (at positions 17, 20, 24 and 27), forming two disulphide bridges with a C1–C2 and C3–C4 conformation (Menard et al. 2004). EAST-1 is homologue to STa, as they share the small molecular weight, the disulphide bridges and 50 % identity in their enterotoxic domain. However, they differ immunologically as no cross-neutralisation occurs. Moreover, unlike STa, EAST-1 is only partially heat-stable, as after heating at 65 °C

for 15 min it retains 63 % of activity. Two main variants of EAST-1 have been described, one present in 17–2 strain and the other in strain O 42 (Yamamoto et al. 1997). Other variants have been described but they are considered rare since they have been isolated only once in epidemiological studies (Menard and Dubreuil 2002). Because of the similarities between EAST-1 and STa, they are considered to act through the same mechanism, with diarrhoea effects.

The *Shigella* enterotoxin 1 (ShET-1) has been firstly described in *Shigella flexneri* 2a but is also found as well in many EAEC strains. This toxin is encoded by chromosomal genes and has an A–B_5 configuration consisting of a single 22 kDa- A subunit associated with a pentamer of five 7 kDa- B subunits.

Several other virulence factors are associated with EAEC, as haemolysin E (Mueller et al. 2009). Another relevant class of virulence factors is the serine protease autotransporter of *Enterobacteriaceae* (SPATE) class of cytotoxins, which is present in the overwhelming majority of EAEC strains (Boisen et al. 2009). SPATE proteins are produced by *E. coli* and *Shigella* species.

SPATE are subdivided in two classes: I SPATE all have a cytotoxic effect in epithelial cells (Henderson and Nataro 2001). Class II SPATE consists of more phenotypes that are diverse and include different proteins and compounds (Boisen et al. 2009).

SPATE production is generally regulated by temperature, as more protein is observed in cultures grown at 37 °C than both at lower and higher temperatures. In some situation, pH is also relevant in SPATE expression, with the optimum at quite alkaline values (Dautin 2010).

Within the EAEC, identified serogroups are O3, O7, O15, O44, O77, O86, O111, O126 and O127 (Stenutz et al. 2006).

EAEC infection usually elicits watery diarrhoea, often with mucus, with or without blood, vomiting and low-grade fever. The pathogenesis of EAEC is complex due to the high heterogeneity of the strains and it involves an increased mucus secretion with the formation of thick biofilms. These biofilms could be responsible for the persistency of the colonisation (Nataro and Kaper 1998). Moreover, EAEC infection causes also cytotoxic effects on the intestinal mucosa, and a mild inflammatory response. EAEC strains are increasingly recognised as an emerging diarrheagenic pathogen, both in industrialised and in developing countries. They are a highly common cause of acute diarrhoea, but they may also provoke a persistent (>14 days) diarrheal illness above all in newborns, children and in Human Immunodeficiency Virus- infected patients. In addition, EAEC is actually the second most common cause of traveller's diarrhoea. Besides asymptomatic carriers, food is also a vehicle of transmission of EAEC. Some restaurant- and canteen-associated outbreaks have occurred, for instance, in Great Britain, Mexico and Japan, even if the type of food has not been identified. In Italy, two consecutive EAEC outbreaks are linked to contaminated unpasteurised cheese (Feng 2013; Kaur et al. 2010). Interestingly, in 2011 an unusual EAEC strain has been involved in one of the largest HUS outbreaks ever recorded.

4.2.5   *Enteroinvasive* E. coli *(EIEC)*

EIEC strains are characterised by the invasiveness of the colonic epithelium. They are closely related by the biochemical, genetic and pathogenic point of view with *Shigella spp*. In fact, they share a 140-MDa plasmid that carries the genes necessary for invasiveness and virulence.

The pathogenic scheme has yet to be fully understood, though it seems that the first stages of infection coincide with those of *Shigella* and are represented by epithelial cell penetration, lysis of the endocytic vacuole, multiplication in the intracellular environment, directional movement through the cytoplasm and extension into adjacent epithelial cells (Sansonetti 2002). These stages determine a strong inflammatory reaction. EIEC and *Shigella* infections cause in most cases a watery diarrhoea, even if occasionally they induce the dysentery syndrome, with blood and mucus in the stool.

Among the EIEC, some of the most common serogroups are O28ac, O29, O112ac, O124, O136, O143, O144, O152, O159, O164 and O167 (Stenutz et al. 2006).

Various outbreaks have occurred due to EIEC, even if the incidence in developed countries is lower. The transmission is primarily food-borne or water-borne; inter-human transmission has been reported. It is currently unknown as to what type of food may carry EIEC, but any food contaminated with human faeces directly or via contaminated water could elicit the disease. Outbreaks have been associated with hamburger meat, cheeses and vegetables in the United States, Europe and Japan (Feng 2013; Meng et al. 2001).

4.2.6   *Diffusely Adherent* E. coli *(DAEC)*

DAEC is a category of diarrheagenic *E. coli* (Kaper et al. 2004). However, little is known about pathogenesis of DAEC.

Epidemiological studies do not always confirm the association between the presence of DAEC strains and watery diarrhoea. In fact, in some studies, certain DAEC strains have been isolated from diarrheal patients and asymptomatic controls in similar frequencies; however, a certain age-dependent susceptibility has been observed. In particular, the association is stronger in children older than 1 year. However, the hypothesis of the presence of additional factors triggering the disease is to be considered. Besides being isolated from developing countries, DAEC strains have been isolated in France in several diarrhoeal cases among hospitalised patients (Jallat et al. 1993), showing that they may account for a number of cases also in the developed world. Currently, no food vehicles have still been reported.

4.3   Detection Methods

Culture methods have represented the 'gold standard' for a long time but they are time-consuming. In addition, they do not allow to discern among pathogenic forms and commensal and/or environmental *E. coli*. Moreover, phenotypic traits are often equivocal because of their high variability; consequently, they may lead to evaluation errors. For instance, many *E. coli* strains—about 90 %—are lactose-positive; as a result, the presumptive identification on agar plates can be misleading in the residual 10 %, given that many laboratories assume that all *E. coli* strains are lactose-positive. An exception could be O157:H7, for which particular biochemical characteristics have been observed. In fact, *E. coli* O157 strains do not produce β-glucuronidase, do not grow well at the temperature commonly used for environmental strains (44 °C) and do not ferment sorbitol rapidly. The last feature is used to isolate O157 in sorbitol-MacConkey (SMAC) agar, where it grows without production of coloured pigments. SMAC selectivity is improved by adding cefixime and tellurite that are supposed to inhibit the growth of most other strains.

More specific methods are based on immunology. Immunological methods use specific antibodies to identify homologous antigens. Latex agglutination is one of the fastest and most common techniques and it involves the in vitro aggregation of microscopic carrier particles (i.e. latex), mediated by the reaction with the suspected antigen. In addition, antibodies can be labelled with a fluorescent compound to emit fluorescence if they are bound by the complementary antigen, or with a radioactive label. However, these techniques are not widely used for the advent of molecular techniques and require costly equipment as the fluorescence microscope. Enzyme-linked immunosorbent assay (ELISA) is another immunoassay: the antibody is bound to an enzyme, typically peroxidase or alkaline phosphatase, and it gives a quantitative colorimetric reaction depending on the amount of enzyme. ELISA technique is extensively used to identify microorganisms, their serotype and their metabolites such as toxins in food: for instance, LT, ST and Stx (Cryan 1990; Downes et al. 1989; Yolken et al. 1977).

In some situations, strains are present in the sample with a low concentration—especially for O157:H7 serotype—making it more difficult to detect bacteria. A very sensitive method that allows to isolate microorganisms, particularly used for *E. coli* O157, is immunomagnetic separation (IMS), requiring numbers as low as 10^2 CFU/g (Karch et al. 1996). IMS employs paramagnetic beads conjugated with antibodies against *E. coli* O157 and exploits the formation of a complex between the antigen and antibodies when bound to beads. The complex is separated from the rest of the sample by using a magnet. The complex is then suspended again and cultivated in appropriate media. Beads coated with antibodies against serogroups O103, O111 and O145 are commercially available.

Traditional methods are based on the phenotypic identification of the microorganism of interest by means of procedures such as culture and biochemical methods. However, these methods suffer principally from a major drawback: they often do not consider the interspecific variability. In fact, some strains exhibit

different biochemical characteristics that do not fit into the predetermined pattern. Indeed, the current trend in research is oriented towards an increasing use of molecular methods, which involve examining the DNA of the bacterium. In addition, molecular detection has two main advantages over traditional techniques. The identification can be completed using a very small amount of material and is more accurate than with previous methods, allowing reliable differentiation of species and pathotypes, when speaking of *E. coli*.

Two major approaches are used in identification: probes (sequences that bind to the DNA of targeted organisms only) and polymerase chain reaction (PCR). Probes are used for a quick detection and identification of bacteria and rely on the hybridisation of a designed species-specific oligonucleotide probe with a precise region of the targeted gene. Each probe is tagged with a radioisotope, enzymes (e.g. alkaline phosphatase) or fluorescent dyes that give a detectable reaction upon hybridisation. For instance, the detection of ETEC may be performed using probes for LT and ST enterotoxins (Moseley et al. 1982). In relation to EHEC, the detection of Stx1 and Stx2 genes has been made easier thanks to the development of probes (Willshaw et al. 1987).

PCR has become one of the major advances in molecular diagnostics thanks to its high specificity and sensitivity, including tests for several pathogens as *E. coli*, *Salmonella*, *Staphylococcus* and *Listeria*. PCR is based on gene amplification that multiplies, creating millions of copies, specific regions of the particular segment of a DNA strand of interest. There are several variants among PCR and the most useful adaptations include multiplex PCR. In the last method, the amplification reaction is directed towards two or more target genes. There is also real-time PCR that allows determining the presence and quantity of DNA. PCR may be performed both on stool, food or environmental samples and on pure subcultures; target genes can be located in the chromosome or in plasmids, even if plasmids can be lost during subcultures and storage (O'Sullivan et al. 2007).

Several PCRs have been developed to identify the different pathotypes among *E. coli* (Tornieporth et al. 1995), based on the principal genes responsible for pathogenic activity (Cerna et al. 2003; Fratamico et al. 2000; Stacy-Phipps et al. 1995).

Other detection methods are sometimes used in laboratories, including animal assays (e.g. the suckling mouse assay for detection of ST in ETEC) and assays on specific cellular lines, but they fall outside the scope of this book.

4.4 Regulatory Norms and *E. coli*

Coliform bacteria, including *E. coli*, are the most extensively used microbiological indicators of faecal contamination both in water for human consumption and in foodstuffs. Nevertheless, the term 'coliform' includes many bacterial species, ranging from enteric commensals to environmental members as *Serratia* and *Aeromonas*. Therefore, recovery of coliforms in food is not always easy to interpret.

E. coli, which is regarded as a permanent member of intestinal flora, can grow in a variety of extra-intestinal niches, including processing plants (Cox et al. 1988). However, the presence of *E. coli* in raw materials is often due to a direct or indirect contact with human or animal faeces. Specifically, bacteria enter roots or leaf surfaces and can colonise the internal plant compartment (Solomon et al. 2002), making it difficult to remove them by washing and disinfection. Microorganisms can further spread from raw materials to uncontaminated products during processing and/or packaging steps. In fact, it is objectively difficult to isolate completely environments where raw materials arrive from processing areas: anything may serve as vehicle, even operators clothing. *E. coli* may also be present in processed foods, preserved through lethal treatments (pasteurisation), due to an inadequate thermal process or to a re-entry of bacteria in the product via cross-contamination. In addition, a number of *E. coli* can undergo a rapid increase in processing environments starting initially from a minimum contamination, depending on the presence of favourable environmental factors. *E. coli's* principal limiting factor is the availability of water (Ottaviani 1996). The minimum water activity required for growth is 0.95, even if the optimum is 0.995; tolerance to lower values diminishes as other factors become sub-optimal, as temperature and pH. As said in Sect. 4.2.3, *E. coli* has a wide range of pH tolerance, growing at pH 4.4–10.0 (but with optimum around neutral pH values, between 6.0 and 7.0). Temperature may be important: *E. coli* is able to grow between 7–8 and 46 °C. The optimum temperature is 35–40 °C, but its heat resistance increases when it is in the stationary phase of growth (Desmarchelier and Fegan 2003). Finally, *E. coli* does not require oxygen for growth being a facultative anaerobe, even if aerobic conditions are preferred.

However, the presence of *E. coli* in processing rooms and in the proximity of equipments is rather controllable—even if not completely eliminable—through the application of Good Manufacturing Practices and Good Sanitation Practices and does not necessarily imply the simultaneous presence of pathogens.

In fact, the dilemma whether hygiene deficiency is associated or not to a real risk of the presence of pathogens is not determined by the presence of *E. coli* itself, but by the severity of the productive defect and by epidemiological considerations, depending on the type of raw materials.

Currently, the tendency in European legislation regards prevention and control according to hazard analysis and critical control points (HACCP), which has been reassessed with Regulation (EC) No. 852/2004, and to microbiological criteria expressed in the Regulation EC No. 2073/2005 and subsequent amendments. These regulatory protocols have defined different limits for *E. coli* in food; in addition, *E. coli* can be considered as a faecal indicator when speaking of live fishery products, as bivalves and gastropods, and meat products (e.g. minced meat and preparations). On the other hand, this bacterium is also a hygiene indicator, specifically in cheeses that have undergone heat treatment, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation. Other products are cooked fishery products, ready-to-eat (RTE) pre-cut fruits and vegetables, unpasteurised RTE fruit and vegetable juices.

Therefore, *E. coli* can play a pivotal role in pointing out the need to perform corrective actions. On the other side, RTE-sprouted-seed sprouts are a high-risk food, as the sprouting process provides the ideal condition for bacterial multiplication, like humidity and temperature. The recent outbreak of diarrheagenic *E. coli*, occurred in 2011, is one of the biggest events in Europe. A total number of 4,321 cases, including 852 HUS cases and 50 deaths, have been reported (RKI 2011). It has been concluded that many fenugreek seeds, imported from Egypt, were the most likely source of the outbreak (Soon et al. 2013). The factor responsible was a particular O104:H4 *E. coli* strain, which can combine virulence properties of enteroaggregative and enterohaemorrhagic pathotypes. As a result, a heightened virulence of the strain has been observed: in fact, the number and combination of SPATE and the enhanced adherence due to the EAEC phenotype may have had a role in the high prevalence of the HUS syndrome, by facilitating the adsorption of Shiga-toxin (Bielaszewska et al. 2011). After the outbreak, the European Food Safety Authority has published a scientific opinion on the risk posed by the potential presence of Shiga-toxin-producing *E. coli* and other pathogens in seeds and sprouted seeds, suggesting that contamination should be detected and mitigated earlier in the production chain, with the aim of avoiding bacterial multiplication. In fact, short shelf-life values of these products would not consent an in-time withdrawal from the market. The new Regulation (EC) No. 209/2013 was then issued amending the Regulation (EC) No. 2073/2005 as regards microbiological criteria for sprouts and the sampling rules for poultry carcases and fresh poultry meat. The new protocol states that the following STEC: O157, O26, O111, O103, O145 pathotypes and O104:H4 must be absent in 25 g in sprouts for the whole shelf life, adding a new step for the consumer protection.

References

Abdul-Raouf UM, Beuchat LR, Zhao T, Ammar MS (1995) Growth and verotoxin 1 production by *Escherichia coli* O157:H7 in ground roasted beef. Int J Food Microbiol 23(1):79–88. doi:10.1016/0168-1605(94)90223-2

Alderete JF, Robertson DC (1978) Purification and chemical characterization of the heat-stable enterotoxin produced by porcine strains of enterotoxigenic *Escherichia coli*. Infect Immun 19 (3):1021–1030

Bergthorsson U, Ochman H (1998) Distribution of chromosome length variation in natural isolates of *Escherichia coli*. Mol Biol Evol 15(1):6–16

Bielaszewska M, Mellmann A, Zhang W, Kock R, Fruth A, Bauwens A, Peters G, Karch H (2011) Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany 2011: a microbiological study. Lancet Infect Dis 11(9):671–676. doi:10.1016/S1473-3099(11)70165-7

Boisen N, Struve C, Scheutz F, Krogfelt KA, Nataro JP (2008) New adhesin of enteroaggregative *Escherichia coli* related to the Afa/Dr/AAF family. Infect Immun 76(7):3281–3292. doi:10.1128/IAI.01646-07

Boisen N, Ruiz-Perez F, Scheutz F, Krogfelt KA, Nataro JP (2009) Short report: high prevalence of serine protease autotransporter cytotoxins among strains of enteroaggregative *Escherichia coli*. Am J Trop Med Hyg 80(2):294–301

Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA (2005) Non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States, 1983–2002. J Inf Dis 192(8):1422–1429. doi:10.1086/466536

Bürk C, Dietrich R, Acar G, Moravek M, Bulte M, Märtlbauer E (2003) Identification and characterization of a new variant of *Shiga* toxin 1 in *Escherichia coli* ONT:H19 of bovine origin. J Clin Microbiol 41(5):2106–2112. doi:10.1128/JCM.41.5.2106-2112.2003

Byappanahalli MN, Fujioka RS (1998) Evidence that tropical soil can support the growth of *Escherichia coli*. Water Sci Technol 38(12):171–174. doi:10.1016/S0273-1223(98)00820-8

Cerna JF, Nataro JP, Estrada-Garcia T (2003) Multiplex PCR for detection of three plasmid-borne genes of Enteroaggregative *Escherichia coli* strains. J Clin Microbiol 41(5):2138–2140. doi:10.1128/JCM.41.5.2138-2140.2003

Connell TD (2007) Cholera toxin, LT-I, LT-IIa and LT-IIb: the critical role of ganglioside binding in immunomodulation by type I and type II heat-labile enterotoxins. Expert Rev Vaccines 6(5):821–834. doi:10.1586/14760584.6.5.821

Cox LJ, Keller N, van Schothorst M (1988) The use and misuse of quantitative determinations of Enterobacteriaceae in food microbiology. J Appl Bacteriol Symp Suppl 17:237S–249S. doi:10.1111/j.1365-2672.1988.tb04654.x

Cryan B (1990) Comparison of three assay systems for detection of enterotoxigenic *Escherichia coli* heat-stable enterotoxin. J Clin Microbiol 28(4):792–794

Danielsson ML, Mollby R, Brag H, Hansson N, Jonsson P, Olsson E, Wadstrom T (1979) Enterotoxigenic enteric bacteria in foods and outbreaks of food-borne diseases in Sweden. J Hyg (Lond) 83(1):33–40. doi:10.1017/S0022172400025808

Dautin N (2010) Serine protease autotransporters of enterobacteriaceae (SPATEs): biogenesis and function. Toxins 2(6):1179–1206. doi:10.3390/toxins2061179

Desmarchelier PM, Fegan N (2003) Enteropathogenic Escherichia coli. In: Hocking AD (ed) Foodborne microorganisms of public health significance, 6th edn. Australian Institute of Food Science and Technology (NSW Branch), Sydney, pp 267–310

Downes FP, Green JH, Greene K, Stockbine N, Wells JG, Wachsmuth IK (1989) Development and evaluation of enzyme-linked immunosorbent assays for detection of Shiga-like toxin I and Shiga-like toxin II. J Clin Microbiol 27(2):1292–1297

Dreyfus LA, Frantz JC, Robertson DC (1983) Chemical properties of heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli* of different host origins. Infect Immun 42(2):539–548

Fegan N, Vanderlinde P, Higgs G, Desmarchelier P (2004) The prevalence and concentration of *Escherichia coli* O157 in faeces of cattle from different production systems at slaughter. J Appl Microbiol 97(2):362–370. doi:10.1111/j.1365-2672.2004.02300.x

Feng P (2013) Escherichia coli. In: Jebbé RG, Garcia S (eds) Guide to foodborne pathogens, 2nd edn. Wiley, New York, pp 222–240

Fraser ME, Chernaia MM, Kozlov YV, James MN (1994) Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 A resolution. Nat Struct Biol 1(1):59–64. doi:10.1038/nsb0194-59

Fratamico PM, Bagi LK, Pepe T (2000) A multiplex polymerase chain reaction assay for rapid detection and identification of *Escherichia coli* O157:H7 in foods and bovine feces. J Food Prot 63(8):1032–1037

Fremaux B, Prigent-Combaret C, Vernozy-Rozand C (2008) Long-term survival of shiga toxin-producing *Escherichia coli* in cattle effluents and environment: an updated review. Vet Microbiol 132(1–2):1–18. doi:10.1016/j.vetmic.2008.05.015

Fujii Y, Okamuro Y, Hitotsubashi S, Saito A, Akashi N, Okamoto K (1994) Effects of alterations of basic amino acid residues of *Escherichia coli* heat-stable enterotoxin II on enterotoxicity. Infect lmmun 62(6):2295–2301

Girón JA, Ho ASY, Schoolnik GK (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science 254(5032):710–713. doi:10.1126/science.1683004

Gonzales L, Ali ZB, Nygren E, Wang Z, Karlsson S, Zhu B, Quiding-Järbrink M, Sjöling Å (2013) Alkaline pH is a signal for optimal production and secretion of the heat labile toxin, LT in enterotoxigenic *Escherichia coli* (ETEC). PLoS ONE 8(9):1–12. doi:10.1371/journal.pone.0074069

Guarino A, Capano G, Malamisura B, Alessio M, Guandalini S, Rubino A (1987) Production of *Escherichia coli* STa-like heat-stable enterotoxin by *Citrobacter freundii* isolated from humans. J Clin Microbiol 25(1):110–114

Guth BE, Twiddy EM, Trabulsi LR, Holmes RK (1986) Variation in chemical properties and antigenic determinants among type II heat-labile enterotoxins of *Escherichia coli*. Infect Immun 54(2):529–536

Gyles CL (2007) Shiga toxin-producing *Escherichia coli*: an overview. J Anim Sci 85 Suppl 13: E45–E62. doi:10.2527/jas.2006-508

Schmidt H, Beutin L, Karch H (1995) Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 Strain EDL 933. Infect Immun 63(3):1055–1061

Hajishengallis G, Connell TD (2013) Type II heat-labile enterotoxins: structure, function, and immunomodulatory properties. Vet Immunol Immunopathol 152(1–2):68–77. doi:10.1016/j.vetimm.2012.09.034

He X, Quinones B, McMahon S, Mandrell RE (2012) A single-step purification and molecular characterization of functional Shiga toxin 2 variants from pathogenic *Escherichia coli*. Toxins 4(7):487–504. doi:10.3390/toxins4070487

Henderson IR, Nataro JP (2001) Virulence functions of autotransporter proteins. Infect Immun 69 (3):1231–1243. doi:10.1128/IAI.69.3.1231-1243.2001

Hussein HS (2007) Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. J Anim Sci 85:E63–E72. doi:10.2527/jas.2006-421

Jackson MP, Neill RJ, O'Brien AD, Holmes RH, Newland JW (1987) Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933. FEMS Microbiol Lett 44:109–114. doi:10.1111/j.1574-6968.1987.tb02252.x

Jallat C, Livrelli V, Darfeuille-Michaud A, Rich C, Joly B (1993) *Escherichia coli* strains involved in diarrhea in France: high prevalence and heterogeneity of diffusely adhering strains. J Clin Microbiol 31(8):2031–2037

Johnson WM, Lior H, Johnson KG (1978) Heat-stable enterotoxin from *Escherichia coli*: factors involved in growth and toxin production. Infect Immun 20(2):352–359

Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140. doi:10.1038/nrmicro818

Karch H, Janetzki-Mittmann C, Aleksic S, Datz M (1996) Isolation of enterohemorrhagic *Escherichia coli* O157 strains from patients with hemolytic-uremic syndrome by using immunomagnetic separation, DNA-based methods, and direct culture. J Clin Microbiol 34 (3):516–519

Karmali MA, Mascarenhas M, Shen S, Ziebell K, Johnson S, Reid-Smith R, Isaac-Renton J, Clark C, Rahn K, Kaper JB (2003) Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. J Clin Microbiol 41(11):4930–4940. doi:10.1128/JCM.41.11.4930-4940.2003

Kaur P, Chakraborti A, Asea A (2010) Enteroaggregative *Escherichia coli*: an emerging enteric food borne pathogen. Interdiscip Perspect Infect Dis. doi:10.1155/2010/254159

Klipstein FA, Engert RF, Houghten RA (1983) Immunological properties of purified *Klebsiella pneumoniae* heat-stable enterotoxin. Infect Immun 42(2):838–841

Kunkel SL, Robertson DC (1979) Purification and chemical characterization of the heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli*. Infect Immun 25(2):586–596

Leyer GJ, Wang LL, Johnson EA (1995) Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl Environ Microbiol 61(10):3752–3755

Lortie LA, Dubreuil JD, Harel J (1991) Characterization of *Escherichia coli* strains producing heat-stable enterotoxin b (STb) isolated from humans with diarrhea. J Clin Microbiol 29 (3):656–659

Louise CB, Obrig TG (1995) Specific interaction of Escherichia coli O157:H7-derived Shiga-like toxin II with human renal endothelial cells. J Infect Dis 172(5):1397–1401

Matecko I, Burmann BM, Schweimer K, Kalbacher H, Einsiedel J, Gmeiner P, Rösch P (2008) Structural characterization of the *E. coli* heat-stable enterotoxin STh. Open Spectrosc J 2:34–39

Mellies JL, Navarro-Garcia F, Frederickson J, Nataro JP, Kaper JB (2001) The espC pathogenicity island of enteropathogenic *E. coli* encodes an enterotoxin. Infect Immun 69(1):315–324. doi:10.1128/IAI.69.1.315-324.2001

Ménard LP, Dubreuil JD (2002) Enteroaggregative *Escherichia coli* heat-stable enterotoxin1 (EAST1): a new toxin with an old twist. Crit Rev Microbiol 28(1):43–60

Menard LP, Lussier JG, Lepine F, Paiva de Sousa C, Dubreuil JD (2004) Expression, purification and biochemical characterization of enteroaggregative Escherichia coli heat-stable enterotoxin 1. Protein Expr Purif 33(2):223–231. doi:10.1016/j.pep.2003.09.008

Meng J, Feng P, Doyle MP (2001) Pathogenic Escherichia coli. In: Pouch Downes F, Ito KA (eds) Compendium methods for the microbiological examination of foods, 4th edn. American Public Health Association, Washigton DC, pp 331–342

Meng J, Schroeder CM (2007) *Escherichia coli*. Chapter 1 In: Simjee S (ed) Foodborne diseases. Humana Press, Totowa, pp 1–25

Molina PM, Parma AE, Sanz ME (2003) Survival in acidic medium of shiga toxin-producing *Escherichia coli* O157:H7 and non-O157:H7 isolated in Argentina. BMC Microbiol 3:17. doi:10.1186/1471-2180-3-17

Moseley SL, Echeverria P, Seriwatana J, Tirapat C, Chaicumpa W, Sakuldaipeara T, Falkow S (1982) Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. J Infect Dis 145(6):863–869. doi:10.1093/infdis/145.6.863

Mueller M, Grauschopf U, Maier T, Glockshuber R, Ban N (2009) The structure of acytolytic alpha-helical toxin pore reveals its assembly mechanism. Nature 459(7247):726–730. doi:10.1038/nature08026

Nardi AR, Salvadori MR, Coswig LT, Gatti MS, Leite DS, Valadares GF, Neto MG, Shocken-Iturrino RP, Blanco JE, Yano T (2005) Type 2 heat-labile enterotoxin (LT-II)-producing *Escherichia coli* isolated from ostriches with diarrhea. Vet Microbiol 105(3–4):245–249. doi:10.1016/j.vetmic.2004.11.005

Nataro JP, Deng Y, Maneval DR, German AL, Martin WC, Levine MM (1992) Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect Immun 60(6):2297–2304

Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11(1):142–201

Nawar HF, King-Lyons ND, Hu JC, Pasek RC, Connell TD (2010) LT-IIc, a new member of the type II heat-labile enterotoxin family encoded by an *Escherichia coli* strain obtained from a nonmammalian host. Infect Immun 78(11):4705–4713. doi:10.1128/IAI.00730-10

Neill MA, Tarr PI, Taylor DN, Wolf M (2001) *Escherichia coli*. In: Hui YH, Pierson MD, Gorham JR (eds) Foodborne disease handbook: diseases caused by bacteria, 2nd edn. Marcel Dekker, New York, pp 169–212

Ochman H, Jones IB (2000) Evolutionary dynamics of full genome content in *Escherichia coli*. EMBO J 19(24):6637–6643. doi:10.1093/emboj/19.24.6637

Okamoto K, Fujii Y, Akashi N, Hitotsubashi S, Kurazono H, Karasawa T, Takeda Y (1993) Identification and characterization of heat-stable enterotoxin II-producing *Escherichia coli* from patients with diarrhea. Microbiol lmmunol 37(5):411–414. doi:10.1111/j.1348-0421.1993.tb03230.x

O'Sullivan J, Bolton DJ, Duffy G, Baylis C, Tozzoli R, Wasteson Y, Lofdahl S (2007) Methods for detection and molecular characterisation of pathogenic Escherichia coli. Ashtown Food Research Centre, Dublin. http://www.antimicrobialresistance.dk/data/images/protocols/e%20coli%20methods.pdf. Accessed 23 March 2015

Ottaviani F (1996) Enterobacteriaceae, coliformi totali ed Escherichia coli: microrganismi e acidità. In: Ottaviani (ed) Microbiologia dei prodotti di origine vegetale. Chiriotti Editori, Pinerolo, pp 203–242

Pradel N, Livrelli V, De Champs C, Palcoux JB, Reynaud A, Scheutz F, Sirot J, Joly B, Forestier C (2000) Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. J Clin Microbiol 38(3):1023–1031

Qadri F, Svennerholm AM, Faruque AS, Sack RB (2005) Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 18(3):465–483. doi:10.1128/CMR.18.3.465-483.2005

Rasooly R, Do PM (2010) Shiga toxin Stx2 is heat-stable and not inactivated by pasteurization. Int J Food Microbiol 136(3):290–294. doi:10.1016/j.ijfoodmicro.2009.10.005

RKI(2011) EHEC O104:H4—The outbreak 2011. The Robert Koch Institute (RKI), Berlin. http://www.rki.de/EN/Content/Prevention/EHEC_O104/ehec_O104_node_en.html;jsessionid=03F7BD05F267608E35C50EEB6C26E1EB.2_cid381. Accessed 23 March 2015

Sack RB, Sack DA, Mehlman IJ, Orskov F, Orskov (1977) Enterotoxigenic *Escherichia coli* isolated from food. J Infect Dis 135(2):313–317. doi:10.1093/infdis/135.2.313

Sansonetti P (2002) Host–pathogen interactions: the seduction of molecular cross talk. Gut 50 Issue supplement 3:S2–S8. doi:10.1136/gut.50.suppl_3.iii2

Sato T, Ozaki H, Hata Y, Kitagawa Y, Katsube Y, Shimonishi Y (1994) Structural characteristics for biological activity of heat-stable enterotoxin produced by enterotoxigenic *Escherichia coli*: X-ray crystallography of weakly toxic and nontoxic analogs. Biochemistry 33(29):8641–8650. doi:10.1021/bi00195a004

Sato T, Shimonishi Y (2004) Structural features of *Escherichia coli* heat-stable enterotoxin that activates membrane-associated guanylyl cyclase. J Pept Res 63(2):200–206. doi:10.1111/j.1399-3011.2004.00125.x

Savarino SJ, Fasano A, Watson J, Martin BM, Levine MM, Guandalini S, Guerry P (1993) Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. Proc Natl Acad Sci USA 90, 7:3093–3097

Sheikh J, Czeczulin JR, Harrington S, Hicks S, Henderson IR, Le Bouguenec C, Gounon P, Phillips A, Nataro JP (2002) A novel dispersin protein in enteroaggregative *Escherichia coli*. J Clin Invest 110(9):1329–1337. doi:10.1172/JCI16172

Sixma TK, Kalk KH, van Zanten BA, Dauter Z, Kingma J, Witholt B, Hol WG (1993a) Refined structure of *Escherichia coli* heat labile enterotoxin, a close relative of cholera toxin. J Mol Biol 230(3):890–918. doi:10.1006/jmbi.1993.1209

Sixma TK, Pronk SE, Kalk KH, van Zanten BA, Berghuis AM, Hol WG (1992) Lactose binding to heat-labile enterotoxin revealed by X-ray crystallography. Nature 355(6360):561–564. doi:10.1038/355561a0

Sixma TK, Pronk SE, Kalk KH, Wartna ES, van Zanten BA, Witholt B, Hol WG (1991) Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli*. Nature 351, 6325:371–377. doi:10.1038/351371a0

Sixma TK, Stein PE, Hol WG, Read RJ (1993b) Comparison of the B-pentamers of heat-labile enterotoxin and verotoxin-1: two structures with remarkable similarity and dissimilarity. Biochemistry 32(1):191–198. doi:10.1021/bi00052a025

Solo-Gabriele HM, Wolfert MA, Desmarais TR, Palmer CJ (2000) Sources of *Escherichia coli* in a coastal subtropical environment. Appl Environ Microbiol 66(1):230–237. doi:10.1128/AEM.66.1.230-237.2000

Solomon EB, Yaron S, Matthews KR (2002) Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl Environ Microbiol 68(1):397–400. doi:10.1128/AEM.68.1.397-400.2002

Soon JM, Seaman P, Baines RN (2013) *Escherichia coli* O104:H4 outbreak from sprouted seeds. Int J Hyg Environ Health 216(3):346–354. doi:10.1016/j.ijheh.2012.07.005

Stacy-Phipps S, Mecca JJ, Weiss JB (1995) Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during the course of infection. J Clin Microbiol 33(5):1054–1059

Stein PE, Boodhoo A, Tyrrell GJ, Brunton JL, Read RJ (1992) Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. Nature 355(6362):748–750. doi:10.1038/355748a0

Stenutz R, Weintraub A, Widmalm G (2006) The structures of *Escherichia coli* O-polysaccharide antigens. FEMS Microbiol Rev 30(3):382–403. doi:10.1111/j.1574-6976.2006.00016.x

Streatfield SJ, Sandkvist M, Sixma TK, Bagdasarian M, Hol WG, Hirst TR (1992) Intermolecular interactions between the A and B subunits of heat-labile enterotoxin from *Escherichia coli* promote holotoxin assembly and stability in vivo. Proc Natl Acad Sci USA 89(24):12140–12144

Takao T, Tominaga N, Shimonishi Y, Hara S, Inoue T, Miyama A (1984) Primary structure of heat-stable enterotoxin produced by *Yersinia enterocolitica*. Biochem Biophys Res Commun 125(3):845–851. doi:10.1016/0006-291X(84)91360-3

Taneike I, Zhang HM, Wakisaka-Saito N, Yamamoto T (2002) Enterohemolysin operon of Shiga toxin-producing *Escherichia coli*: a virulence function of inflammatory cytokine production from human monocytes. FEBS Lett 524(1–3):219–224. doi:10.1016/S0014-5793(02)03027-2

Tesh VL, Burris JA, Owens JW, Gordon VM, Wadolkowski EA, O'Brien AD, Samuel JE (1993) Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect Immun 61(8):3392–3402

Topp E, Welsh M, Tien YC, Dang A, Lazarovits G, Conn K, Zhu H (2003) Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. FEMS Microbiol Ecol 44(3):303–308. doi:10.1016/S0168-6496(03)00055-2

Tornieporth NG, John J, Salgado K, de Jesus P, Latham E, Melo MC, Gunzburg ST, Riley LW (1995) Differentiation of pathogenic *Escherichia coli* strains in Brazilian children by PCR. J Clin Microbiol 33(5):1371–1374

Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P et al (2009) Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. PLoS Genet. doi:10.1371/journal.pgen.1000344

van Elsas JD, Semenov AV, Costa R, Trevors JT (2011) Survival of *Escherichia coli* in the environment: fundamental and public health aspects. ISME J 5(2):173–183. doi:10.1038/ismej.2010.80

WHO (2011) Enterohaemorrhagic *Escherichia coli* (EHEC), Fact sheet No 125, December 2011. http://www.who.int/mediacentre/factsheets/fs125/en/. Accessed 23 March 2015

Willshaw GA, Smith HR, Scotland SM, Field AM, Rowe B (1987) Heterogeneity of *Escherichia coli* phages encoding Vero cytotoxins: comparison of cloned sequences determining VT1 and VT2 and development of specific gene probes. J Gen Microbiol 133(5):1309–1317. doi:10.1099/00221287-133-5-1309

Yamamoto T, Wakisaka N, Sato F, Kato A (1997) Comparison of the nucleotide sequence of enteroaggregative *Escherichia coli* heat-stable enterotoxin1genes among diarrhea-associated *Escherichia coli*. FEMS Microbiol Lett 147(1):89–95. doi:10.1111/j.1574-6968.1997.tb10225.x

Yamanaka H, Fuke Y, Hitotsubashi S, Fujii Y, Okamoto K (1993) Functional properties of pro region of *Escherichia coli* heat-stable enterotoxin. Microbiol Immunol 37(3):195–205. doi:10.1111/j.1348-0421.1993.tb03200.x

Yolken RH, Greenberg HB, Merson MH, Sack RB, Kapikian AZ (1977) Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. J Clin Microbiol 6(5):439–444

Zhang W, Bielaszewska M, Kuczius T, Karch H (2002) Identification, characterization, and distribution of a Shiga toxin 1 gene variant (stx(1c)) in *Escherichia coli* strains isolated from humans. J Clin Microbiol 40(4):1441–1446. doi:10.1128/JCM.40.4.1441-1446.2002