Shiga toxin-producing *Escherichia coli* in humans and the food chain in Bangladesh

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Abstract

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are one of the most important foodborne pathogens. Infection with STEC in humans can lead to mild diarrhea, bloody diarrhea or even in certain cases to the severe hemolytic-uremic syndrome (HUS). Domestic ruminants appear to be important reservoirs for STEC and play a significant role in the epidemiology of human infections. The main objective of the research described in this thesis was to gain insight in the epidemiology of STEC infection in humans and the ecology of STEC in the human food chain in Bangladesh.

We found that the prevalence of STEC among diarrheal patients was relatively low compared with other enteric pathogens. Around 0.5% (n = 2) of patients in the hospital and 1.9% (n = 3) of patients in the community were found positive for STEC with no case (0/570) of STEC O157 infection. To identify an effective technique for isolation of STEC O157 from animal and food sources, we evaluated the efficiency of different methods. Immunomagnetic separation (IMS) using Dynabeads anti-*E. coli* O157 proved to be more efficient than the Vitek Immunodiagnostic Assay System (VIDAS) Immuno-Concentration *E. coli* O157 (ICE) kit (VIDAS ICE) in case of animal feces and the use of CHROMagar O157 with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) as plating media resulted in more positive samples than sorbitol–MacConkey agar with cefixime (0.05 mg/l) and tellurite (2.5 mg/l). In addition, IMS was found more sensitive than PCR to detect STEC O157. In order to estimate the prevalence of these organisms in animal feces and foods we used both IMS and PCR techniques. Of the fecal samples collected from buffalo (n = 174), cows (n = 139), and goats (n = 110), 82.2%, 72.7%, and 11.8% tested positive for stx_1 and/or stx_2, respectively. STEC could be isolated from 37.9%, 20.1%, and 10.0% of the buffalo, cows, and goats, respectively. STEC O157 strains were isolated from 14.4% of the buffalo, 7.2% of the cows, and 9.1% of the goats. In case of foods, more than 71% of the raw meats, 10% of the raw milk and 8% of the fresh juice samples were found positive for the *stx* genes. STEC O157 strains were isolated from 8% (n = 7) of the meat samples. In order to explain the lack of STEC O157 infection in humans, we investigated the presence of antibodies against these organisms among the healthy population in Bangladesh. We collected serum samples from different groups of people comprising butchers, and people with other occupations living in urban and rural areas. We found that around 50% (n = 116) of the samples were positive for antibodies (IgG, IgA and/or IgM) to *E. coli* O157 lipopolysaccharide. Using separate analysis, we found that all the 116 samples positive for polyvalent antibodies were positive for the IgG-class and 87 were also positive for IgM-class antibodies. A statistically significantly higher number of nonbutcher participants (63%; n = 57) were positive than the butcher participants (41%; n = 54) ($P < 0.05$). No statistically significant difference in antibody response was found between the urban and rural populations.

Finally, we compared the occurrences of STEC in diarrheal patients, animal reservoirs and foods in Bangladesh with similar data available in other countries from both developed and developing parts of the world in order to get a global perspective. A major difference between our findings and findings from developed countries was observed in the prevalence of STEC O157-associated human infections. Unlike developed countries, no case of STEC O157 infection was found among diarrheal patients in Bangladesh. We concluded that the lack of STEC O157 infection among Bangladeshi population might be attributable to the protective immunity against these pathogens acquired by the frequent exposure to the antigens.

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Mohammad Aminul Islam

Wageningen, January 2009

Dedicated to my late father
Md. Akter Hossain Talukder

Contents

Chapter 1

General introduction and outline of the thesis

Escherichia coli is probably the most studied organism in microbiology. Since its first description (48, 49), the bacterium has become the model organism for much microbiological research, such that it is often forgotten that its main ecological niche is the alimentary tract of humans and most warm-blooded animals. The bacterium is shed in the feces of warm-blooded animals and humans; however, they only comprise a very small percentage of the total fecal flora. The organisms typically colonize the gastrointestinal tract of human infants within a few hours after birth (44). Usually *E. coli* and its human hosts coexist in good health and with mutual benefits. The niche of commensal *E. coli* is the mucous layer of the mammalian colon. The bacterium is a highly successful competitor at this crowded site, comprising the most abundant facultative anaerobe of the human intestinal microflora. *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection.

Over the last half-century it has become increasingly obvious that there are a number of different enteropathogenic groups of *E. coli.* At least six known pathotypes associated with gastrointestinal infections have been recognized, apart from those opportunistic “nonpathogenic strains” causing urinary tract infections, septicemia, and meningitis in humans and a number of similar diseases in animals. The pathotypes associated with gastrointestinal infections currently recognized are:

• Enteropathogenic *E. coli* (EPEC)
• Enterotoxigenic *E. coli* (ETEC)
• Enterohemorrhagic *E. coli* (EHEC), which are a subgroup of Verocytotoxigenic *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC)
• Enteroinvasive *E. coli* (EIEC)
• Enteroaggregative *E. coli* (EAggEC)
• Diffuse-adherent *E. coli* (DAEC)

Each of these pathotypes has unique features in their interaction with eukaryotic cells, which is schematically represented in Fig. 1.1.

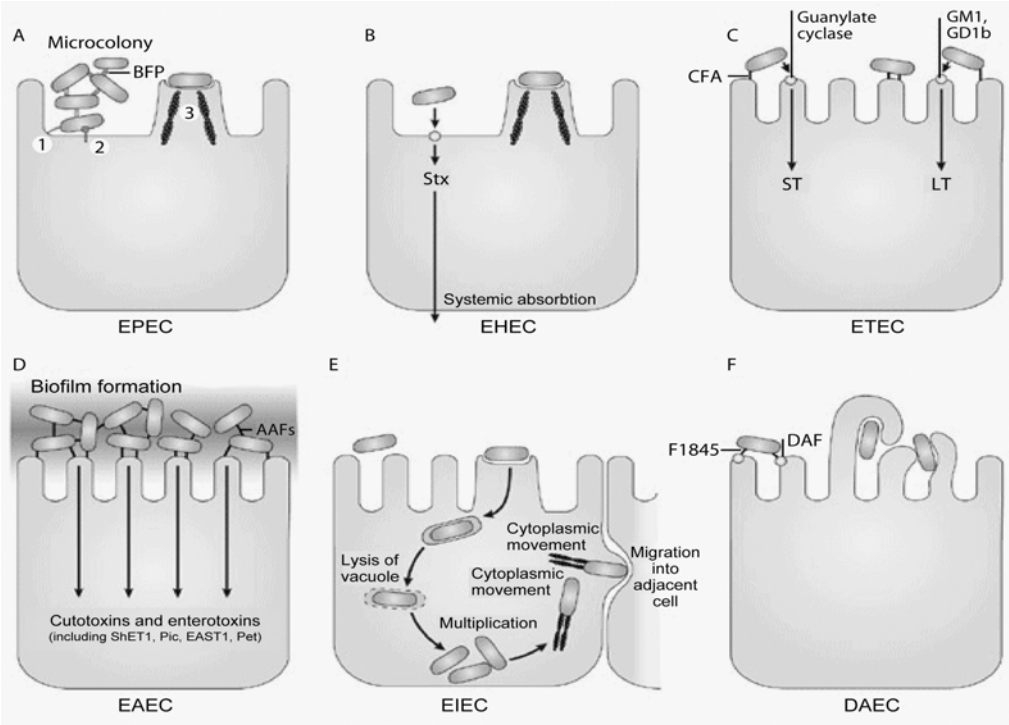

Fig. 1.1 These illustrations are largely the result of *in vitro* studies and might not completely reflect the phenomena that occur in infected humans. (A) EPEC adhere to small bowel enterocytes and, destroy the normal microvillar architecture. Cytoskeletal derangements are accompanied by an inflammatory response and diarrhea. 1. Initial adhesion, 2. Protein translocation by type III secretion, 3. Pedestal formation. (B) The distinguishing feature of EHEC is the elaboration of Shiga toxin (Stx), systemic absorption of which leads to potentially life-threatening complications. (C) Similarly, ETEC adhere to small bowel enterocytes and induce watery diarrhea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins. (D) EAEC adhere to small and large bowel epithelia in a thick biofilm and elaborate secretory enterotoxins and cytotoxins. (E) EIEC invade the colonic epithelial cell, lyse the phagosome and move through the cell by nucleating actin microfilaments. The bacteria might move laterally through the epithelium by direct cell-to-cell spread or might exit and re-enter the baso-lateral plasma membrane. (F) DAEC elicit a characteristic signal transduction effect in small bowel enterocytes that manifests as the growth of long finger-like cellular projections, which wrap around the bacteria. AAF, aggregative adherence fimbriae; BFP, bundle-forming pilus; CFA, colonization factor antigen; DAF, decay-accelerating factor; EAST1, enteroaggregative *E. coli* ST1; LT, heat-labile enterotoxin; ShET1, *Shigella* enterotoxin 1; ST, heat-stable enterotoxin. Adopted from Kaper et al. (79).

1.1 Shiga toxin-producing *E. coli*

1.1.1 Historical background

The history of Shiga toxin-producing *E. coli* (STEC) dates back to late 1970, when Konowalchuk and colleagues showed that culture filtrates of some strains of *E. coli* produced a striking, irreversible cytopathic effect on cultured Vero cells, a cell line derived from African green monkey kidney cells (101). The cytotoxins were termed as verocytotoxins or verotoxins (VTs) and the *E. coli* strains as verocytotoxin-producing *E. coli*, verotoxin-producing *E. coli*, or verotoxigenic *E. coli* (VTEC). At the same time, O'Brien et al. (131) reported that extracts of certain *E. coli* strains were cytotoxic for HeLa cells and that this cytotoxic activity could be neutralized by antitoxin prepared against crude *Shigella dysenteriae* type 1 (Shiga) toxin (Stx). They subsequently reported that many *E. coli* strains isolated from diarrheal illness produced a Shiga-like toxin (SLT), including one of the strains reported by Konowalchuk et al. (101) to produce the VT (132). O'Brien et al. (133) subsequently showed that SLT and the VT were the same toxin.

The recognition of STEC as a distinct class of pathogenic *E. coli* resulted from two key epidemiological observations. The first was in 1982 reported by Riley et al. (153), who investigated two outbreaks of a distinctive gastrointestinal illness characterized by severe crampy abdominal pain, watery diarrhea followed by grossly bloody diarrhea, and little or no fever. This illness, designated hemorrhagic colitis (HC), was associated with the ingestion of undercooked hamburgers at a fast-food restaurant chain. No evidence was found for infection with classic bacterial pathogens such as *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., and *Yersinia* spp. Stool cultures from these patients yielded a previously rarely isolated *E. coli* expressing O antigen 157 and H antigen 7 from 9 of 20 cases and not from any healthy controls. The second key observation was by Karmali et al. (84), also in 1982, who reported the association of sporadic cases of hemolytic-uremic syndrome (HUS) with fecal cytotoxin and cytotoxin-producing *E. coli* in stools. HUS was already known to be preceded typically by a bloody diarrheal illness indistinguishable from HC. Later, it was proposed (85) that VT/SLT was the common virulence factor between HC and HUS and was responsible for damage to both intestinal and renal tissue. These, two key clinical microbiological observations, one based on a rare *E. coli* serotype and the other based on production of a specific cytotoxin, led to the recognition of a novel and increasingly important class of enteric pathogens causing intestinal and renal disease.

1.1.2 Nomenclature

In the past decade the understanding of the VT/SLT has rapidly increased and the identification, purification, cloning and sequencing of several immunologically related toxin variants led to the insight that these toxins constitute a family with major sequence homology at both the nucleotide and peptide level. Therefore, Calderwood et al. (30) proposed a new, more rational nomenclature, in which VT and SLT have been renamed Stx, after the prototype toxin of the family, and the related VTEC and SLT-producing *E. coli* (SLTEC) organisms have been renamed STEC. VTEC, SLTEC, and STEC are equivalent terms, and all three refer to *E. coli* strains that produce one or more toxins of the Stx family. These names are used interchangeably but the term STEC is used throughout this thesis.

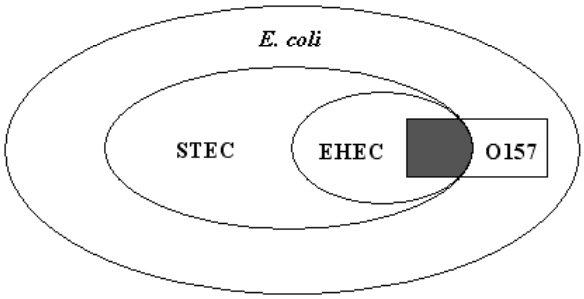

Fig. 1.2 Schematic representation of STEC, EHEC and *E. coli* O157 within *E. coli*.

The term "enterohemorrhagic *E. coli*" (EHEC) has been used to designate the subset of STEC that is considered to be pathogenic to humans (Fig. 1.2). The EHEC term was originally conceived to denote strains of *E. coli* that cause HC and HUS, produce Stx, induce a characteristic histopathologic lesion defined as "attaching and effacing" (A/E lesions) on epithelial cells, and possess an approximately 60-MDa "EHEC plasmid" (109). The term "atypical" EHEC has been used to define STEC strains that do not produce A/E lesions and/or do not possess the large "EHEC plasmid" (126). There is however no clear definition of the EHEC group except that all EHEC strains by definition are considered to be human pathogens, while this is not necessarily the case with all STEC strains. A simple definition of EHEC is therefore that it serves as a proxy for human pathogenic STEC, albeit the clinical outcome of an EHEC infection might not necessarily be HC.

The serotype that has been considered as one of the most important among the EHEC and/or STEC group is *E. coli* O157:H7 because of its association with severe human infection (Fig. 1.2).

1.2 Methods for detection of STEC

There are no common biochemical characteristics associated with the great majority of STEC serotypes. The only way to identify all types of STEC in any kind of test sample is the detection of Stx produced by the bacteria. STEC can also indirectly be detected by examining *E. coli* strains or samples for the genes encoding Stx. The gold standard for the detection of Stx employs Vero cells, but other cell lines can also be used, e.g., HeLa cells (132). Toxin production can also be detected by the use of immunological methods. Since a relatively small number of STEC serotypes are responsible for the majority of human STEC infections, serotype-specific detection methods have been developed, where strains are isolated on the basis of their O-antigen and are subsequently analyzed for Stx production or presence of *stx* genes. Generally, the diagnosis of STEC is laborious, and currently there are no simple, inexpensive methods available for routine isolation of all STEC serotypes.

1.2.1 Tissue culture methods

The Vero cell assay has been used widely for the detection of free Stx in fecal specimens and in enrichment cultures inoculated with foods, animal feces or environmental samples. However, the most common application of the Vero cell assay is for confirmation of toxin production by pure cultures. The Vero cell assay is performed by addition of cell-free supernatants to tissue culture monolayers and preliminary results are obtained after 24 h with final results after 3-4 days. The specificity of the tissue culture cytotoxicity tests can be considerably improved by employment of Stx1 and Stx2 specific neutralizing antisera in order to determine if the cytotoxic effect is caused by Stxs or by another nonneutralizable toxin present in the sample (88). This assay is sensitive and regarded as the ‘gold standard’ to which other methods should be validated. However, since maintenance of tissue culture is costly, specialized in nature and labor-intensive, immunological and DNA-based methods have been largely supplanted the Vero cell assay for confirmation of Stx production.

1.2.2 Immunological methods

Over the years, a number of immunological methods for the detection of Stxs have been developed such as enzyme immunoassays, colony blot and passive agglutination assays. The methods utilize Stx specific poly- or monoclonal antibodies. These assays can be applied to pure and mixed cultures (enrichment cultures of food or feces, usually incubated overnight).

When Stx is detected, the broth can be subcultured onto isolation media and pure or pooled colonies can be further examined. Immunoassays are generally reliable and most assays are easy to implement in laboratories and do not require expensive equipment. Several immunological assays are today available as commercial 'ready to use test kits', which is of advantage for routine microbiological laboratories. The most commonly used commercially available test kits are Premier EHEC (Meridian Diagnostics Inc., Cincinnati, Ohio), VTEC-RPLA toxin detection kit (a reverse passive latex agglutination test for the detection of Stx1 and Stx2) (Denka Seiken Co. Ltd., Japan), the Ridascreen® Verotoxin enzyme immunoassay technique (R-Biopharm, Darmstadt, Germany), the ProSpecT® Shiga toxin microplate assay (Alexon-Trend, Ramsey, Minn.) and the VTEC-Screen 'SEIKEN' (Denka Seiken). Most of these tests are used for nondiscriminative detection of Stx in supernatants from stool, or from bacterial cultures on microtitre plates coated with Stx1/Stx2-specific (monoclonal) antibodies.

1.2.3 DNA-based methods

DNA-based methods are considered as a good alternative to the culture or immunological methods because these methods detect the presence of small amounts of species- or strain-specific DNA rather than unique aspects of target organisms to be in a specific physiological state. For STEC, presence of *stx*-specific gene sequences in the fecal or food samples is an indication that STEC is present in the sample, which should always be confirmed by detecting the gene in subsequently isolated organisms. The presence of a particular serotype of STEC is determined by targeting serotype-specific gene fragments in the organism or in the sample. Detection of these targets is attained by the use of DNA-DNA hybridization probes or by amplification of target-specific DNA. Numerous DNA-DNA hybridization assays, using oligo- or polynucleotide probes have been described and different formats have been used, including dot-blot and replica assays, liquid-based assays, and more recently micro-array chips. Amplification of specific DNA is most frequently achieved by polymerase chain reaction (PCR), but other DNA amplification techniques like Nucleic Acid Sequence-based Amplification (NASBA) are also applicable for STEC detection. A range of different PCR formats is being used, and additional techniques to ensure the identity of the amplicon beyond measurement of the size of the amplified DNA are necessary. These techniques include the use of internal sequence-based probes (particularly in real-time PCR), full DNA sequencing, and the use of fragment analysis following restriction endonuclease digestion.

DNA-based detection methods can be applied to nucleic acid from pure or mixed cultures (enrichment cultures of food or feces), as well as colonies growing on solid isolation media. DNA probes are used to detect STEC by the use of replica plating techniques, whereas PCR-based methods can be used to investigate single colonies or pools of colonies. Generally, the amplification-based techniques are rapid and will give a result within hours (after enrichment or directly from colonies). However, when testing mixed cultures the detected genes might not originate from the same STEC strain. DNA-based methods have the disadvantage of being unable to distinguish between DNA from viable and nonviable cells, although this may only be important in specific situations.

1.3 Isolation and enrichment of STEC O157

1.3.1 Isolation

Usually STEC do not possess phenotypic characteristics that are distinguishable from those of other *E. coli*. However, an important exception to this is that STEC O157 are usually both unable to ferment sorbitol within 24 h of incubation and lack β-D-glucuronidase activity (120, 151). These characteristics are utilized in the routine selective isolation of STEC O157. The most widely used solid medium for the isolation of non-sorbitol-fermenting STEC O157 is sorbitol-MacConkey (SMAC) agar. This medium contains 1% sorbitol in place of lactose in the standard MacConkey agar. Because of the inability to ferment sorbitol, STEC O157 grow after overnight incubation as colorless colonies and can be distinguished from most of the remaining intestinal *E. coli* strains (around 75 to 94%) that ferment sorbitol and grow as pink colonies (120). However, some *Enterobacteriaceae* present in human stools, such as *Proteus* spp., *Providencia* spp., *Hafnia* spp., *Enterobacter* spp., and *Escherichia hermanii*, also grow in colorless colonies (120). Moreover, some of these species share common epitopes with the *E. coli* O157 antigen (113).

The inability of the majority of STEC O157 strains to produce β-D-glucuronidase is exploited by supplementation of agar media, e.g., SMAC agar, with the fluorogenic 4-methylumbelliferyl-β-D-glucuronide (MUG) or with the chromogenic 5-bromo-6-chloro-3-indolyl-β-D-glucuronide (BCIG). Cleaving of MUG or BCIG by β-D-glucuronidase-positive strains results in the formation of fluorescent (at 365 nm) and blue-colored colonies, respectively. No fluorescence or change in color is seen for strains negative for β-D-glucuronidase. Commercially available agar media based on this property are Rainbow® Agar

O157 (Biolog, Inc., Hayward, Calif.), Fluorocult® *E. coli* O157:H7 agar (Merck, Darmstadt, Germany) and CHROMagar O157. In a recent study, CHROMagar O157 has been shown to have a higher sensitivity (96.3%) and negative predictive value (100%) and a better diagnostic efficiency than SMAC agar for the isolation of STEC O157 from human stool samples (36).

The selectivity of solid media can be improved by the use of selective supplements. The most frequently used supplements are: cefixime, a third generation cephalosporine; and potassium tellurite (e.g., in CT-SMAC) (193). Cefixime inhibits *Proteus* spp. at a concentration not inhibitory to *E. coli* and tellurite inhibits many other non-sorbitol-fermenters such as *Aeromonas* spp., *Pleisomonas* spp., *Morganella* spp., *Providencia* spp., and most other *E. coli* strains. However, some STEC O157 strains are sensitive to cefixime and potassium tellurite and therefore may not be detected on CT-SMAC agar (118).

The use of only CT-SMAC for isolation of STEC O157 has become contentious with recent isolation of sorbitol-fermenting STEC O157 from patients with HUS or diarrhea in many countries, including Germany, Austria, and the Czech Republic (83). Such strains can be overlooked by the diagnostic procedures recommended for the isolation of non-sorbitol-fermenting STEC O157 strains.

Conventional culture methods are both time-consuming and laborious. Their sensitivity can significantly be increased by the application of an immunoconcentration step. A commonly used immunoconcentration method, which is also implemented in the international standard for the detection of *E. coli* O157 in food and feeding stuffs (6), is immunomagnetic separation (IMS). The procedure involves mixing of enrichment cultures with paramagnetic particles coated with anti-O157 antibodies. The target organisms in the sample bind to the immunomagnetic beads, which are then separated from other sample material and microorganisms in a magnetic field. Following an extensive washing procedure to remove nonspecifically bound bacteria and sample particles, the beads are plated onto solid media. The IMS procedure can be performed both manually and automatically. IMS increases the sensitivity by relatively concentrating *E. coli* O157 compared with background microflora, which may overgrow or mimic STEC O157 cells on selective agars. Example of a fully automatic immunoconcentration system is the Vitek Immunodiagnostic Assay System (VIDAS) (bioMérieux, Marcy l'Etoile, France).

Recently, a relatively new microbial capture system called “Recirculating Immuno Magnetic-capture System (RIMS)” has been developed for the detection of very low numbers

of pathogens, including *E. coli* O157, in food and environmental samples. Conventional IMS, which has been employed in the analysis of food and environmental samples, only processes 1 ml of enrichment broth. To maximize the chance of microbial capture, the RIMS procedure re-circulates the entire volume of enrichment broth past anti-O157-conjugated paramagnetic beads at 37°C for 30 min. However, there are several protocols available depending on the amount of sample to be examined and the accepted time to the results. The target organisms can be detected and isolated from the captured and concentrated sample by direct plating onto appropriate selective media or using for example the colortrix (a colorimetric assay), fluratrix (fluorescence microscopy), serology, PCR, ELISA, and/or DNA probes. In previous studies, it has been shown that RIMS coupled with real-time PCR can result in high recovery rates with low levels of *E. coli* O157:H7 in ground beef (7), fresh leafy produce and surface water after short enrichment periods (66). The method provided detection and isolation of *E. coli* O157:H7 at levels as low as 0.07 CFU/g of romaine lettuce and 0.1 CFU/g of spinach and isolation of *E. coli* O157:H7 in water samples at levels of 6 CFU/100 ml of surface water (66).

1.3.2 Enrichment

While human clinical stool specimens are examined mostly by direct plating onto selective and differential agars, animal feces, food and environmental samples usually contain low numbers of STEC O157 together with an abundant microbial flora, and therefore require a selective enrichment step. However, enrichment methods may also be applied to human feces, which can contain low levels of STEC. The most widely used media for the enrichment of STEC O157 are tryptone soya broth (TSB) (mainly for food) and buffered peptone water (for human and animal feces) (181). These broths may be supplemented with different selective agents such as bile salts, novobiocin, vancomycin, cefsulodin, and cefixime (33). Bile salts inhibit the growth of non-*Enterobacteriaceae* strains, which make up the majority of food background microflora (181). Among the antibiotics, novobiocin has been used most widely. Novobiocin is mainly active against Gram-positive bacteria (especially Gram-positive cocci) and against some Gram-negative bacteria frequently present as background microflora in various samples (181). STEC O157 organisms are generally resistant to novobiocin, which might also explain its relatively higher use in enrichment broths compared with other antibiotics. Vancomycin, cefsulodin and cefixime suppress the growth of Gram-positive bacteria, *Aeromonas* spp. and *Proteus* spp., respectively (107).

The occurrence of heat-, freeze-, acid-, or salt-stressed STEC O157 in food makes it important to be able to detect cells that are in a stressed state, since injured cells mostly retain their pathogenic properties. The detection and isolation of stressed STEC O157 by direct selective enrichment or direct plating onto selective agar may not allow the recovery of these strains (3, 169). Probably the best approach for the recovery of stressed STEC O157 cells is a nonselective pre-enrichment for at least 18 to 24 h (169).

There is currently no consensus on optimal incubation temperature (37°C versus 41.5°C) and time (6-8 h incubation versus overnight incubation) for all types of samples. The incubation period required will depend on the competing microflora. Standard methods for food include the analysis of both the 6- and 18-h incubating enrichment cultures (6, 181). A 6-8 h incubation of the enrichment broth increases the sensitivity when analyzing matrices with a high number of background flora. However, when stressed or sublethally injured STEC O157 are present there are difficulties in reaching a detectable level after 6-8 h of enrichment. Therefore, this short period of incubation can only be recommended when testing matrices where *E. coli* has a short-lag time before onset of growth, as for example with minced meat products.

1.4 Isolation and enrichment of STEC non-O157

There is no internationally accepted standard method for the isolation of STEC non-O157. Over 200 O:H serotypes have been recognized as STEC from different sources (45). Unlike STEC O157 most of the other serotypes of STEC show similar biochemical characteristics as commensal *E. coli*. This limits the development of selective culture media applicable for the isolation of all serotypes of STEC.

Following successful application in STEC O157 isolation, methods based on IMS have been developed for a few predominant STEC non-O157 serogroups. Magnetic beads coated with antibodies for STEC non-O157 have been developed for serogroups O26, O103, O111, and O145. IMS-based detection of serogroups other than O157 is similar to that for the detection of *E. coli* O157; enrichment, and IMS followed by plating onto selective indicative agars. However, these methods have not yet been sufficiently validated.

There is no recommended selective enrichment or plating medium for STEC non-O157. Selective agents to improve the isolation of STEC O157 (e.g., novobiocin) may inhibit the growth of some STEC non-O157 (182). Several studies showed that some STEC non-O157

serotypes (O5:H-, O26:H-, O26:H11, O91:H21, O111:H-, O111:H8, O104:H11, O113:H21 and O157:H8) are capable of growing on media supplemented with vancomycin, cefixime, and cefsulodin (68, 107).

A nonselective, but differential plating medium is enterohemolysin agar (Oxoid Ltd., Basingstoke, United Kingdom) (washed sheep blood agar supplemented with calcium), which may be suitable for isolation of all human pathogenic STEC strains, including STEC O157 (14). Nearly all (ca. 90%) STEC O157 strains and proportion of human pathogenic STEC non-O157 strains (ca. 70%) produce enterohemolysin. Enterohemolytic *E. coli* are characterized on this medium by small turbid zones of hemolysis around the colonies occurring after 18 to 24 h incubation at 37°C. α-hemolytic *E. coli* form large, clear zones of hemolysis after only 3 to 6 h of incubation. To improve the selectivity of the medium, antibiotics such as novobiocin and cefsulodin may be used. By combining culture on enterohemolysin agar and Stx detection using the VTEC-RPLA kit, Beutin et al. (15) were able to isolate STEC O157:H7 and non-O157 strains from stools of HUS patients that constituted as little as 0.03% of the total coliform flora. Enterohemolysin agar is easy to use and commercially available (Oxoid), and therefore suitable for routine application. However, it has some limitations: 1) enterohemolysin-positive colonies must be tested for Stx production (15); 2) a proportion of STEC non-O157 (14) and sorbitol-fermenting STEC O157 (16) fail to produce the enterohemolytic phenotype and can be missed; and 3) the presence of a large number of non-STEC α-hemolytic colonies or overgrowth with other enteric bacteria (*Proteus* spp., *Pseudomonas* spp., and Gram-positive cocci) can interfere with the detection of fewer enterohemolytic colonies (15).

1.5 Characterization and typing of STEC

Characterization of STEC isolates is extremely valuable since this allows comparisons between isolates from human, animal and food origin and also provides information on changes in their prevalence over time and in different geographical locations. Characterization of STEC with respect to the presence of a range of virulence properties may further identify markers that confer the capacity to cause serious infections and so identify strains with increased risk of causing disease. These data are essential for an evolving definition of human pathogenic STEC. Some typing and fingerprinting methods inform

epidemiological investigations that link human cases to each other and to specific sources of infection.

1.5.1 Serotyping

The serotype of an *E. coli* isolate is based on the O-(Ohne) antigen determined by the polysaccharide portion of cell wall lipopolysaccharide (LPS) and the H-(Hauch) antigen due to flagella protein. It is an important basis for differentiating STEC and is often the starting point in characterization. Strains of *E. coli*, including STEC are serotyped by an internationally recognized and evolving scheme comprising over 180 O-types and 56 H-types. Full serotyping is generally performed in national reference laboratories although antisera for some common STEC O-groups are available commercially. Agglutination kits, generally based on antibody-coated latex particles, are used widely in the identification of presumptive STEC, particularly O157, isolated from human and non-human samples.

Due to restricted use of antisera-based serotyping of *E. coli* in some reference laboratories, DNA-based serotyping has been evolved, which attempts to avoid the dependency on antisera and make serotype characterization more widely available. This method targets unique sequences involved in the biosynthesis of O-antigens specific for O-groups such as O157, O26, O111, O113 and O145 by PCR. PCR-RFLP (PCR-restriction fragment length polymorphism) and PCR combined with sequencing have also been used. Determination of the H-type has been directed mainly at the *fli*C gene that is present even if the isolate is nonmotile. The large number of O-types of *E. coli* means that in the short to medium term, DNA-based tests are unlikely to replace conventional serotyping in the reference laboratory setting for comprehensive characterization of isolates. Such developments require sequence data to become available on a more extensive range of O-groups than the present.

1.5.2 Stx production

STEC are defined by their ability to produce either one or both antigenically-distinct Shiga toxins termed Stx1 and Stx2 that were first recognized by their ability to cause an irreversible cytopathic effect on Vero cells and other cell lines in culture (101). Although very good tests in general, cell line assays are labour-intensive and time consuming and not really appropriate for many routine diagnostic laboratories, which may lack tissue culture facilities. Moreover, neutralizing antisera against Stxs are not commercially available. It is however essential for reference laboratories to continue to use this test, because it will reveal

the presence of unknown variants of Stxs, which the exclusive use of specific immunological or DNA-based methods would miss.

1.5.3 Stx typing and subtyping

Typing and subtyping of Stxs has a great importance in the epidemiology and ecology of these organisms. Two major types of Stxs, called Stx1 and Stx2, which share 56% homology to each other, have been described (141). The genetic analysis of the *stx* genes found in different STEC isolates resulted in the detection of an increasing number of genetic variants of both stx_1 and stx_2. A large number of PCR assays have been evolved for the detection of stx_1 and stx_2 genes and their variants. Until now, five genetic variants of stx_1 and 12 variants of stx_2 have been described and were summarized in a new proposal for an adapted nomenclature for the Stx family by Scheutz et al. (158). This nomenclature organizes 6 groups of toxin types (1, 2, 2c, 2d, 2e and 2f) according to antigenic variability, differences in toxicity for cells or animals, capacity to be activated by mouse elastase (mucus) and by differences in DNA or amino acid sequences.

1.5.4 Presence of other virulence genes

Most STEC included in the EHEC group colonize the intestinal mucosa with a mechanism that subverts the epithelial cell function (52) and induces A/E lesions. The complex mechanism of A/E lesions is genetically governed by a large pathogenicity island defined as the Locus of Enterocyte Effacement (LEE) (126). A/E lesions have been characterized routinely by the presence of the *eae* gene, which encodes for the adherence factor intimin. *eae* exists in a large number of sequence subtypes due to variation at the C-terminal end. PCR assays are usually directed at the conserved region of the sequence. The presence of the *eae* gene has been shown to be strongly associated with some STEC serotypes that can cause serious complications, including HC and HUS (19).

LEE-negative STEC strains have also been associated with serious illness indicating that other factors enhance the virulence potential of these strains. Several studies have investigated the role of other genomic islands identified in STEC O157 for their potential contribution to virulence of other STEC. One island, termed O122, is contiguous to LEE in many strains and is strongly associated with *E. coli* that can cause A/E lesions. In STEC O157, it contains the 5′ end of the *efa*1 (*E*HEC *f*actor for *a*dherence) gene but this region is complete in many STEC non-O157 and in sorbitol-fermenting STEC O157 and may be linked to colonization of the bovine intestine (45, 89).

Sorbitol-fermenting STEC O157 strains that have caused outbreaks of HUS are characterized by the presence of plasmid-coded fimbriae and the plasmid-borne pilin subunit gene (*sfp*A) has been used as a potential diagnostic marker for these strains by PCR.

Most of the STEC O157 and some STEC non-O157 strains contain a virulence plasmid called "pO157". This plasmid contains several genes encoding for proteins implicated in STEC pathogenesis. These include: hly_{EHEC} encoding for EHEC hemolysin; *espP* encoding for an autotransported serine protease involved in the cleavage of human coagulation factor V (27); *toxB* encoding for a 362-kDa protein sharing amino acid sequence similarity with the large *Clostridium* toxin family, which is involved in adherence to epithelial cells in culture (173); *saa* encoding for STEC autoagglutinating adhesin; and *stcE* encoding for a zinc metalloprotease. StcE is secreted by the *etp* type II secretion system, cleaves the C1 esterase inhibitor (C1-INH) of the complement pathway, has mucinase activity, and is thought to be involved in colonization and tissue damage (60, 103).

1.5.5 Phage typing

Phage typing is an important internationally standardized subtyping method, which is used only for STEC O157. Phage typing schemes are not available for STEC non-O157. Phage types are determined by the lysis pattern obtained when a test isolate is subjected to a panel of established standard lytic phages. STEC O157 strains are differentiated into about 90 types by a scheme of 16 bacteriophages (96). The method is rapid, epidemiologically valuable in real time and gives information about the emergence and distribution of new strains. It is performed routinely in a limited number of laboratories worldwide. Although phage typing is relatively easy to perform, the biggest drawback of using phage typing as the sole typing method is the occurrence of common phage types. There is a close association between phage type and the presence of stx_1 and stx_2 gene subtypes. Some phage types (e.g., PT2, PT21/28) appeared to be more strongly associated with HUS than others. Generally, the same phage types that cause human illness are recovered from animals, although the proportions may differ. To obtain maximum strain discrimination, it has been recommended to use phage typing in conjunction with *stx* subtyping and pulsed-field gel electrophoresis (PFGE) analysis (154).

1.5.6 Subtyping and fingerprinting for epidemiology and population studies

One of the most widely applied methods of subtyping is PFGE, a technique in which fragments of the bacterial chromosome generated by digestion with a restriction enzyme

selected to cut the DNA into 20 to 25 pieces are separated by agarose gel electrophoresis (58, 176). The procedure for this technique is relatively similar to performing a standard gel electrophoresis except that instead of constantly running the voltage in one direction, the voltage is periodically switched among three directions. One that runs through the central axis of the gel and two that run at an angle of 120° either side. The pulse times are equal for each direction resulting in a net forward migration of the DNA. Numerical analysis of digitally captured banding patterns can be carried out to build dendrograms, with the ultimate potential of using the technique comparatively to trace and identify outbreaks through a central database (172). PFGE has been applied to both STEC O157 and non-O157. However, there is considerably more information on the application of PFGE to STEC O157.

In 1995, the Centers for Disease Control and Prevention (CDC) set up "PulseNet" (www.cdc.gov/ncidod/dbmd/pulsenet/pulsenet.html), a national network of public health laboratories in the United States for rapid comparison of fingerprints generated by PFGE subtyping of *E. coli* O157 isolates with an electronic database at the CDC. Rigorous standardization of a protocol involving digestion of genomic DNA with the enzyme *Xba*I has resulted in patterns that may be compared across the PulseNet system in the United States (58) and in other countries (176). Strong epidemiological data are essential in the application of PFGE as strains that share profiles may be obtained from unlinked cases separated geographically and over long periods of time.

Sequence-based typing methods include multi-locus variable number of tandem repeat analysis (MLVA) has been evaluated as an alternative to PFGE for STEC O157 (72). It has the advantage that the output appears as a numeric sequence that can be generated automatically. MLVA methods have not yet been applied to STEC non-O157 strains because of lack of sequence data. Multi-locus sequence typing (MLST), another sequence-based typing method is more appropriate for studying the relationships between *E. coli* populations rather than for typing and epidemiological analysis (130).

1.5.7 Seropathotype classification of STEC

The concept of seropathotype that has recently been proposed classifies STEC into five groups based on the incidence of serotypes in human disease, association with outbreaks versus sporadic infection, their capacity to cause HUS or HC, and the presence of virulence markers (90). This approach attempts to combine these inputs to understand better the apparent differences in virulence of different STEC. Seropathotype A strains (STEC O157) have a high relative incidence, commonly cause outbreaks and are associated with HUS.

STEC O26:H11, O103:H2/NM, O111:NM and O145:NM together with O121:H19 fall into seropathotype B, as they have a moderate incidence and are uncommon in outbreaks but are associated with HUS. Seropathotype C includes STEC O91, O104 and O113 strains, all of H-type 21, are associated with HUS, but these strains are of low incidence and rarely cause outbreaks. Seropathotypes A and B possess LEE and genes of O-island 122 but strains belonging to seropathotype C may be LEE-negative and only some of the strains possess O122 virulence genes. Seropathotypes D and E are not HUS-associated and are uncommon in human or found only in nonhuman sources. Surveys targeting isolation of STEC (not specifically O157) from nonhuman sources generally result in isolates from groups C and D.

Substantial differences in virulence potential have been observed among isolates belonging to the same STEC serotypes. For example, studies based on subtyping of *stx* from STEC O157 isolated from human patients and healthy cattle have indicated that there are differences among the relative frequency of the seropathotypes that are predominating among patients with severe disease (HC and HUS) and seropathotypes that are predominating in the bovine reservoir (155). The same observation is made for STEC O26. Strains of STEC O26:H11 that cause HUS are usually identified as Stx2- and *eae*-positive whereas infections caused by Stx1- and *eae*-positive STEC O26:H11 usually are characterized by causing relatively mild diarrheal symptoms in most patients (50). The concept of seropathotype is likely to be further refined and will provide a valuable tool in the future for the assessment of the human pathogenic potential of different STEC serotypes.

1.6 Epidemiology of STEC infection

The epidemiology of STEC infections has remarkably changed during the past ten years. The organisms have been reported in a large variety of domestic and wild animal species, and an increasing number of unusual food vehicles have been associated with human infections. New routes of transmission have emerged, like contact with animals during farm visits and a wide variety of environment-related exposures.

STEC infections are still a much greater problem in developed countries than in developing countries. The true incidence of STEC-associated illness is difficult to estimate since many persons presenting only mild symptoms may not seek medical attention. The vast majority of reported outbreaks and sporadic cases of HUS in humans have been caused by serotype O157:H7. However, more than 100 different STEC serotypes have been associated

with disease in humans (159, 190) and STEC non-O157 serotypes (especially O26:H11, O103:H2, O111:H-, and O145:H-) are increasingly being associated with outbreaks of foodborne illness and HUS (126).

Table 1.1 Reported cases of Shiga toxin-producing *E. coli* infections in humans in European countries during the period of 2003 to 2006 (confirmed cases in 2004) and the incidence for confirmed cases in 2006 (EU-total incidence is based on the population in reporting countries). Adopted from EFSA (46)

Country	2006[a]				2005	2004	2003
	Report Type[b]	Total cases	Confirmed cases	Confirmed cases/100,000 population	Total cases[a]		
Austria	C	41	41	0.5	53	45	28
Belgium	A	47	47	0.4	ND	36	39
Czech Republic	C	1,561	1,558	15.2	ND	1,743	ND
Denmark	C	146	146	2.7	154	163	128
Estonia	C	8	8	0.6	19	0	ND
Finland	C	14	14	0.3	21	10	14
France	C	67	67	0.1	ND	ND	ND
Germany	C	1,183	1,183	1.4	1,162	903	1,100
Greece	C	1	1	<0.1	ND	ND	ND
Hungary	C	3	3	<0.1	5	12	20
Ireland	C	158	153	3.6	125	61	95
Italy	ND	17	17	<0.1	ND	3	5
Lithuania	A	0	0	<0.1	ND	ND	ND
Luxembourg	C	2	2	0.4	8	ND	ND
Malta	C	21	21	5.2	23	ND	ND
Netherlands	C	41	41	0.3	64	30	51
Poland	C	4	4	<0.1	4	3	ND
Slovakia	C	8	8	0.1	61	4	1
Slovenia	C	30	30	1.5	ND	2	ND
Spain	C	13	13	<0.1	16	ND	ND
Sweden[c]	C	265	265	2.9	336	149	52
United Kingdom	C	1,294	1,294	2.1	1,171	926	974
EU-Total		4,924	4,916	1.1	3,222	4,090	2,507
Iceland	C	1	1	0.3	ND	ND	ND
Norway	C	50	50	1.1	18	12	15
Switzerland	C	64	48	0.6	62	45	56

[a] ND, Not determined.
[b] A, Aggregated data; C, Case-based.
[c] In Sweden, in July 2004 the reporting system changed so all serovars became notifiable, before this date only STEC O157 were notifiable.

STEC infections are reported from different parts of the world, being found in 75 to 100% of episodes of sporadic HUS in Europe, North America, Canada, and Latin America especially in Argentina (20, 59, 154). From 1982-2002, there were 350 STEC O157 outbreaks reported in the United States, resulting in 8,500 clinical cases, 1,500 hospitalizations and 40 deaths (151). Human STEC infection is also considered as a significant public health problem in continental Europe. In 2006, a total of 4,916 confirmed

human STEC cases were reported from 22 member states of the EU (46), giving an incidence of 1.1 per 100,000 populations. There has been a statistically significant decreasing trend in the EU incidence since 2004 (Table 1.1). The most commonly identified STEC serogroup was O157. Overall, more than one half of the reported STEC cases occurred in 0-4 year old children. There was, however, increased reporting of human cases caused by non-O157 serogroups.

In Australia and New Zealand the annual incidence of HUS is approximately 1.0 to 1.3 per 100,000 children less than five years old. Interestingly, the predominant STEC serotypes associated with HUS in these two countries differ. In New Zealand, STEC O157 strains make up around half of the isolates, and in Australia STEC O111 strains account for most HUS cases, with STEC O157 being associated with fewer than 20% (108).

Table 1.2 Reports of the isolation of Shiga toxin-producing *E. coli* from various sources in Asian countries

Country	Serotype	Sources	Reference
India	Non-O157	Diarrheal patients	97
	Non-O157	Healthy cattle	140
	Non-O157	Animal, human and food	35
	Non-O157	Cow feces, raw beef	97
	Non-O157	Fish (seafood), patient with bloody diarrhea	102
	Non-O157+O157	Diarrheic calves and lambs	187
	Non-O157	Healthy goats	188
	O157	Slaughtered cattle, diarrheic calves	119
Thailand	Non-O157	Children with bloody diarrhea and without diarrhea	25
	Non-O157	Adult patients with diarrhea	13
	Non-O157	Marketed beef, fresh beef in slaughterhouse, cattle feces	171
	O157	Retail beef, bovine feces	183
	Non-O157	Child with non-bloody diarrhea, adult with bloody diarrhea, normal child	106
	Non-O157	Children with acute diarrhea	150
Vietnam	Non-O157	Diarrheal patients, healthy cow	128
	O157	Cow feces	125
	Non-O157	Buffaloes, cattle, and goats	184
Korea	O157	HUS patients	98
	O111	HUS patients	99
	O104:H4	HUS patients	11
	O111	High school participants (outbreak)	91
	O157	Cattle	76
	O157	Cattle	192
Malaysia	O157	Beef	147
	O157	Tenderloin beef, chicken burger	148
Iran	Non-O157	Healthy population	9
	Non-O157	Randomly selected inhabitants	10
	Non-O157+O157	Acute diarrheal patients, healthy controls	156
	Non-O157	Children with and without diarrhea	5

In most of the Asian countries, STEC are not yet considered as a major health problem, except in Japan, where 29 outbreaks were reported during the period 1991 to 1995 (123). Several STEC outbreaks were also reported from China and Korea, where cases of HUS patients infected with STEC O157 have been reported. Xu et al. reviewed the isolation of STEC from different places in China (191). A review of literature on STEC isolation from Asian countries is given in Table 1.2.

1.7 Reservoirs of STEC

STEC represent the only pathogenic group of *E. coli* that has a definite zoonotic origin, although not all STEC strains have been demonstrated to cause disease in humans. STEC rarely cause disease in animals, and ruminants are recognized as their main natural reservoir. Cattle are considered to be the major animal source of STEC that are virulent to humans, in particular STEC O157, and the ecology of these microorganisms in cattle farming have been extensively studied (32). Cattle are asymptomatic excretors of STEC O157, which are transient members of their gut microflora. The presence of STEC O157 appears to be influenced by the age of the animals and by the season. Shedding is usually longer and more intense in calves than in adult cattle, and increases after weaning. It is also much higher during the summer period (32). The reported prevalence of STEC and/or STEC O157 in cattle is also clearly influenced by the sampling and detection methods adopted in the investigations. STEC O157 and other serotypes associated with human infection have also been isolated frequently from the intestinal content of other ruminant species, including sheep, goat, water buffalo, and wild ruminants, while pigs and poultry have not been identified to be major sources of STEC.

Fecal testing of dairy cattle worldwide showed prevalence rates for STEC O157 and STEC non-O157 ranging from 0.2 to 48.8% and 0.4 to 74.0%, respectively (70). Global testing of beef cattle feces revealed prevalence rates for STEC O157 and STEC non-O157 of 0.2 to 27.8% and 2.1 to 70.1%, respectively (69).

1.8 Transmission of STEC

Although the ultimate source of STEC is the feces of ruminants, there are four main transmission routes whereby these organisms may be transmitted to humans: 1) foodborne

transmission; 2) waterborne transmission; 3) person-to-person transmission; and 4) direct contact with animals.

During the 1980s, most of the outbreaks of STEC O157 infection were foodborne and food vehicles implicated were mostly inadequately cooked hamburgers or other beef products, and unpasteurized milk (32). Over the years numerous studies on transmission routes of human pathogenic STEC have identified many other types of food vehicles for these organisms. In addition to foods of bovine origin, several outbreaks have been associated with low pH products like fermented salami, apple juice/cider, mayonnaise and yogurt (122). This has highlighted the tolerance of STEC O157 to acidic pH and its ability to survive the processes of fermentation and drying. In addition, waterborne outbreaks and outbreaks associated with other types of environment related exposures have been increasingly reported (124). The dispersion of untreated manure in the environment can cause the contamination of different items, which can then act as secondary vehicles for human infections (32). A detailed scheme of the routes of transmission of STEC has been depicted in Fig. 1.3.

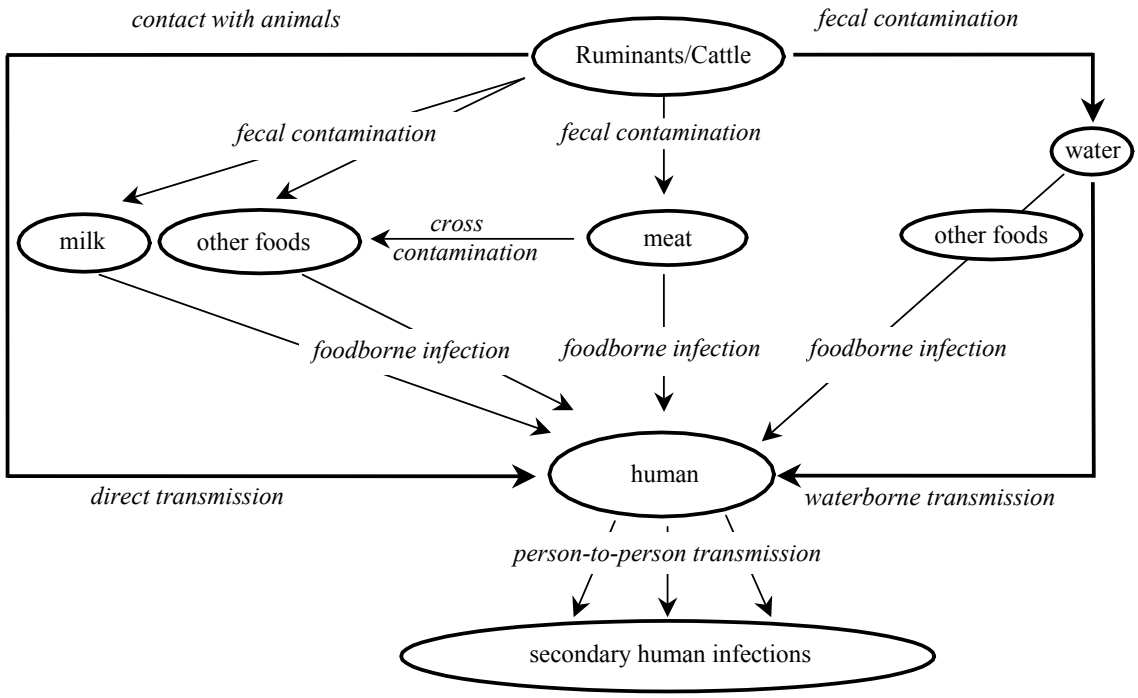

Fig. 1.3 Possible transmission routes of STEC.

Data on transmission routes have been established by the investigation of outbreaks and individual cases of infection; in many outbreaks more than one route may be involved, such that primary infection acquired from a food or animal sources may be disseminated to

secondary cases in families or the wider community. The high infectivity and intrinsic properties of STEC (e.g., acid tolerance and the ability to survive well in the environment) have made investigation of infection increasingly complex. Case-control studies of varying design have been performed to assess risk factors for sporadic infection with STEC (mainly O157) in several European and other countries. Results show differences between countries and the risk factors may be age-related. In several studies specific food vehicles were not identified but contact with animals and/or the rural environment were identified as the major risk factor for STEC O157 infection (115, 136). A risk analysis study conducted on STEC O157 outbreaks in Scotland from 1994 to 2003 showed that approximately 40% of the outbreaks were foodborne, 54% were environmental, and 6% involved both transmission routes (170).

1.8.1 Foodborne transmission

Descriptive epidemiology of STEC infection in Ontario, Canada during the period of 1996-2005 identified that food (35%) was the most frequent mode of transmission of STEC, followed by person-to-person (5%) and waterborne (3%) transmission (114). In the United States, 52% of the outbreaks of STEC O157 infection between 1982 and 2002 were associated with consumption of contaminated food (149). The food vehicle for 75 (41%) outbreaks was ground beef, and for 38 (21%) outbreaks, produce (149).

Contamination of carcasses with STEC usually occurs during slaughter and subsequent processing through fecal material originating directly or indirectly from the rectal-caecal area (47). Dairy products (e.g., milk, cheese, cream) associated with infection have included those that are unpasteurized, have had a pasteurization failure or have been contaminated post-pasteurization. Ready-to-eat foods have also been associated with infection, particularly cooked meats contaminated by raw materials during processing, in catering establishments, at retail sale and in the home.

Vegetables and fruits, fertilized with ruminants' manure or contaminated during irrigation, harvesting or processing have also been implicated in transmitting STEC especially STEC O157 (32). Examples are lettuce, potatoes, radish sprouts, alfalfa sprouts, cantaloupe, and unpasteurized fruit juice. Such 'produce' items are now recognized in the United States as a major cause of outbreaks requiring increased biosecurity and changes in processing practices.

STEC O157 can survive for substantial periods of time on stainless steel (51) and plastic (4). Hence, these surfaces can serve as intermediate sources of contamination during food

processing operations. Processing equipment and utensils used in the preparation of fruit juices have also been linked to cross-contamination events. A meat grinding unit can also be a significant contributor to cross contamination resulting from multiple contacts with surfaces in the mixing, blending, cutting and forming actions (47).

In 2006, a multistate outbreak of STEC O157:H7 infection occurred in the United States (39). A total of 183 persons infected with the outbreak strain of *E. coli* O157:H7 had been reported to the CDC, from 26 states. Among the ill persons, 95 (52%) were hospitalized, 29 (16%) had HUS, and one person died. Fresh spinach was identified as the source of the outbreak. One hundred and twenty-three of the 130 patients (95%) reported consuming uncooked fresh spinach within the 10 days before illness onset. In addition, STEC O157:H7 with a PFGE pattern matching the outbreak strain had been isolated from three open packages of fresh spinach consumed by patients.

1.8.2 Waterborne transmission

Water is a very efficient vehicle for the dissemination of STEC. Surface waters may be subjected to STEC contamination through run-off from organic wastes applied to agricultural land and from direct fecal deposition. Fresh water close to livestock farming systems may therefore represent a potential reservoir for enteric pathogens, allowing cycles of livestock re-infection and increasing the potential for the organism to spread (121).

STEC may be present in manure heaps and surrounding run-off puddles, which provide another opportunity for the pathogen to be washed into surface waters. Besides in many countries, river water is readily contaminated with huge load of treated and untreated sewage (178). Recently, *E. coli* O157:H7 has been detected in the Ganges River, which is an extensively used water source in India (63).

There have been a number of water-associated outbreaks following contamination of water by STEC O157 (34). Swimming-associated outbreaks (1, 144) and outbreaks linked with contamination of private water supplies have been described (8). The largest STEC O157 outbreak in the United States occurred in 1999 at a county fair due to contaminated drinking water (37). A total of 921 persons reported diarrhea after attending the fair. Stool cultures yielded *E. coli* O157:H7 from 116 persons; 13 of these persons were co-infected with *Campylobacter jejuni*. Sixty-five persons have been hospitalized; 11 children have developed HUS; and two persons died: a 3-year-old girl from HUS and, a 79-year-old man from HUS/thrombotic thrombocytopenic purpura. The implicated water was from a temporary unregulated well at the fairground. Recently, one small-scale outbreak of STEC

O157 infection has been reported in England, which was associated with unchlorinated water in a swimming pool (180).

1.8.3 Person-to-person transmission

Because of the low infectious dose (1 to 100 CFU) of STEC O157 (141), person-to-person fecal-oral transmission can easily occur in settings of poor hygiene and close contacts. Accordingly, person-to-person transmission has emerged as the predominant route of infection in outbreaks of STEC O157 infection in daycare facilities (137), and in institutional settings (2, 86). During outbreaks, transmission from asymptomatically infected individuals may also be a source of secondary infections and can further amplify the outbreak (2). This mode of transmission is also considered responsible for the spreading of STEC infection within families (165). However, rates of STEC transmission by person-to-person and resultant illness are largely unknown (117).

1.8.4 Contact with animals

During the past decennia, the role of animals in health and social care has significantly increased. Animals are brought in nursing homes and hospitals and increasing number of farms combine agriculture and care (65). Certain venues encourage or permit the public to contact animals, resulting in millions of human-animal interactions each year. Contact with animals in public settings provides opportunities for entertainment and education. These settings include county or state fairs, petting zoos, animal swap meets, pet stores, zoological institutions, circuses, carnivals, farm tours, livestock-birthing exhibits, educational exhibits at schools and wildlife photo opportunities. Although multiple benefits of human-animal contact exist, inadequate understanding of disease transmission and animal behavior can increase the likelihood of zoonotic infectious diseases in these settings (127).

Transmission through direct animal contact has been documented in outbreaks and sporadic infections by *E. coli* O157:H7 (38, 64). Although reports often document cattle, sheep, or goats as sources for infection, poultry, rodents (93), and other domestic and wild animals also are potential sources. The primary mode of transmission for enteric pathogens is fecal-oral. Because animal fur, hair, skin, and saliva (92) can become contaminated with fecal organisms, transmission can occur when people touch, feed, or are licked by animals.

1.9 Pathological features of STEC disease

It is now recognized that STEC are associated with a very broad spectrum of clinical manifestations ranging from symptom-free infection through mild uncomplicated diarrhea, to severe HC and HUS. The most extensive clinical observations have been made with STEC of serogroup O157. The average interval between exposure and illness is three to four days, although incubation times as long as five to eight days or as short as one to two days have been described in some outbreaks (141). In uncomplicated cases of infection, the diarrheal symptoms usually resolve within two weeks. Excretion of the organisms usually continues for about one to two weeks. However, prolonged fecal shedding of organisms has also been observed (80, 163). Some individuals infected with STEC may be completely asymptomatic, in spite of the presence of large numbers of organisms as well as free toxin in the feces (23).

The most common clinical presentation of STEC (especially *E. coli* O157:H7) infection is HC. HC as defined earlier this section is characterized by severe abdominal cramps, bloody diarrhea, and endoscopic evidence of colonic mucosal edema or hemorrhage. This stage usually lasts between four and ten days and fecal specimens are described as all blood and no stool. Most of the patients with HC recover within a week (Fig. 1.4), without specific therapy or complications, but in a proportion of patients especially in children younger than ten years, the illness progresses to HUS.

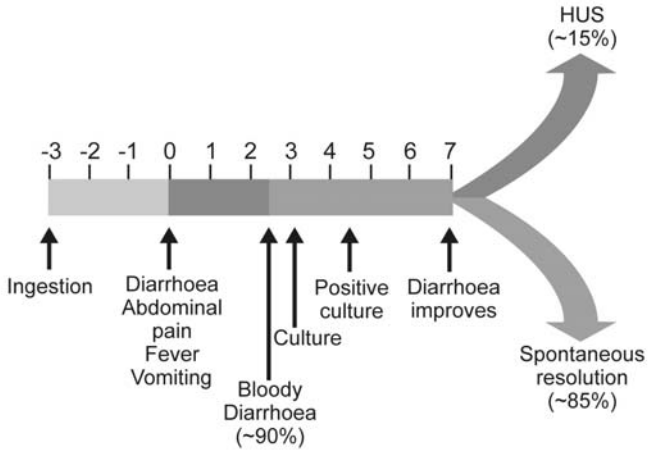

Fig. 1.4 Progression of *E. coli* O157:H7 infections in children. Adopted from Tarr et al. (175).

Three to 15 percent of patients infected with STEC O157:H7 can progress to HUS, but this figure may reach 50% if antibiotics are used (162). Young children and older persons with altered immune response (189) are particularly vulnerable. The classic triad of features for HUS consists of microangiopathic hemolytic anaemia, thrombocytopenia, and acute renal failure (152). Clinical features identifying patients at high risk for HUS are vague and may mimic common gastroenteritis, including bloody diarrhea occurring from three days to more than two weeks before HUS is diagnosed. Ten percent of cases are associated with rectal prolapse and colitis (152).

HUS cannot be diagnosed without evidence of hemolytic anaemia. Hematologic findings include destruction and fragmentation of erythrocytes that results in microangiopathic hemolytic anaemia. This develops in all patients within around a day of infection and may result in respiratory and cardiovascular compromise. Mean hemoglobin concentration of 6 g per dL (60 g per L) is common and requires red blood cell transfusion (152). Ninety-two percent of patients with HUS develop thrombocytopenia, which results from entrapment of platelets in the organs (152). Clotting times are normal, and petechiae and purpura are uncommon features of HUS (152). Acute renal failure results when microthrombi are deposited in kidney parenchyma. This manifests in the form of hypertension associated with oliguria and anuria. Approximately 12% of patients who contract HUS either develop end-stage renal disease or die (57). The central nervous system is another organ system that could become involved. Thirty-three percent of patients with HUS experience neurologic complaints such as irritability, seizures, and altered mental status (152). Approximately 10% of patients with HUS develop central nervous system problems and subsequent coma, hemiparesis, or stroke (57, 173). Currently, the overall mortality rate for patients with HUS is around 10% (152).

1.10 Pathogenesis

The pathogenesis of STEC is considered to be a multistep process, involving a complex interaction between a range of bacterial and host factors.

1.10.1 Survival in the acidic environment

Orally ingested STEC must initially survive the harsh environment of the stomach and then compete with other microorganisms in the gut to establish intestinal colonization. There

are several mechanisms that enable *E. coli* to resist acidic conditions: a) an acid-induced oxidative system; b) an acid-induced arginine-dependent system; and c) a glutamate-dependent system (105). At pH 2.0 the arginine-dependent system provided more protection in STEC strains than in commensal *E. coli* strains, although the glutamate-dependent system appears to be equally effective in all strains (110). The oxidative resistance mechanism is regulated by an alternate sigma factor rpoS. This regulates genes required for survival at low pH (pH below 2.5 for over 2 h).

1.10.2 Colonization

1.10.2.1 A/E adherence

Once survived in the harsh conditions of the stomach, STEC must establish colonization of the gut by adhering to intestinal epithelial cells. One of the most important characteristics of STEC O157 and some other STEC is the ability to produce A/E lesions. This lesion is characterized by the destruction of the microvilli and the rearrangement of the cytoskeleton to form a pedestal-like structure that cups the bacteria individually (79). The genes required for the formation of the A/E lesions are located on the LEE pathogenicity island, which is encoded in both pathogenic STEC and EPEC strains but not in commensal *E. coli* (74).

The LEE is composed of 41 genes, the majority of which are organized into five polycistronic operons (*LEE1* to *LEE5*) (Fig. 1.5) (186).

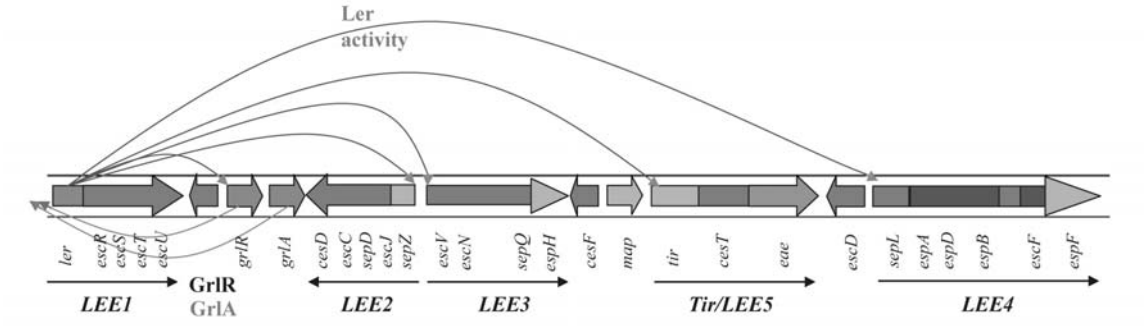

Fig. 1.5 Summary of the regulation of the LEE genes in *Escherichia coli* O157:H7. Adopted from Spears et al. (167).

Several studies have shown that Ler (*L*EE-*e*ncoded *r*egulator), a 15-kDa protein encoded by the first gene of the *LEE1* operon, is a central positive regulator needed for the expression

of the LEE genes (61, 168). Recently, two other novel LEE-encoded regulators, GrlA (*g*lobal *r*egulator of *L*EE *a*ctivator; formerly called Orf11) and GrlR (Grl *r*epressor; formerly called Orf10) have been identified, which are highly conserved in all pathogens with the A/E property (41). These proteins are encoded by the *grlRA* operon located between the *rorf3* gene and the *LEE2* operon in the LEE. GrlA is a positive regulator of *ler* expression (41). It has been shown that Ler and GrlA regulate each other, forming a transcriptional positive regulatory loop (12). GrlR has a significant negative effect on LEE gene expression, probably acting as a negative regulator of *ler* (112), although its mechanism of action remains to be defined. The majority of the remaining genes in *LEE1*, as well as the *LEE2* and *LEE3* operons, encode structural and secondary proteins required for the formation of the type III secretion system (TTSS) (74). *LEE5* contains genes encoding an adhesin (intimin) and its cognate receptor (Tir) that is translocated through TTSS into the host cell (186). *LEE4* encodes several *E. coli* secreted proteins (Esp proteins) that make up the translocon portion of the TTSS (186). The TTSS in STEC facilitates the translocation of the LEE-encoded effector proteins Tir, EspH, EspG, EspF, SepZ, and Map (186) as well as several nonLEE-encoded (Nle) effectors into eukaryotic target cells.

When STEC are in close proximity to the epithelial lining, the translocated intimin receptor (Tir) is secreted through the TTSS and inserted into the plasma membrane of the host cells. Once inside the host cell, it embeds itself into the eukaryotic membrane in a hairpin loop formation, and its extracellular domain serves as a bacterial receptor that binds the adhesin intimin, allowing the bacteria to attach tightly to the eukaryotic cell (94). Inside the host cell, the cytoplasmic portion of Tir initiates a signaling cascade that leads to recruitment of Arp2/3 and N-WASP, eliciting actin nucleation to form pedestals characteristic of the A/E lesion (31, 77).

The most important effector protein, intimin is expressed by most, if not all, STEC O157 isolates. The *eae* gene coding for intimin is also frequently found in STEC non-O157 commonly isolated from humans (105). Animal isolates of STEC non-O157 carry the *eae* gene less frequently and this may in part explain the reduced incidence of these STEC in human disease. However, several STEC non-O157 serotypes are *eae*-positive, but these are associated with only sporadic cases of human illness (105). Besides, it has been suggested that the presence of the LEE is not always essential for pathogenesis, as a number of cases of severe STEC disease, including HUS, as well as occasional outbreaks were caused by LEE-negative strains (95, 142). With more LEE-negative STEC strains being reported, investigations of adhesins encoded outside of the LEE have been carried out (131, 143).

1.10.2.2 Other adhesions

Several proteins were proposed to be novel adhesion factors; these include ToxB (a protein identified from a large, 93-kb plasmid pO157 and required for full expression of adherence of O157:H7 strain Sakai) (173), Saa (an autoagglutinating adhesin identified in LEE-negative strains) (143), Sfp (sorbitol-fermenting EHEC O157 fimbriae) (29), Iha (adherence-conferring protein similar to *Vibrio cholerae* IrgA) (174), Efa1 (EHEC factor for adherence) (129), and LPF (long polar fimbriae; closely related to LPF of *Salmonella* enterica serovar Typhimurium) (43). These putative adhesins are encoded either in the large plasmid harbored by STEC strains or in unique DNA segments called O-islands (OIs). ToxB, Sfp fimbriae, and Saa are plasmid-encoded. Iha is encoded in OI-43 and OI-48, which are identical and contain 106 open reading frames (ORFs). Efa1 is encoded in OI-122, which was recently reported by Karmali et al. (89) to be associated with STEC serotypes that are linked to epidemic or severe human disease. In STEC O157:H7 strains, the LPF OI-141 and OI-154 operons are present. $LPF_{O157/OI-141}$ was reported by Torres et al. (177) to play a role in adherence. On the other hand, Doughty et al. (43) suggested that LPF of STEC O113:H21, encoded in OI-154 (LPF_{O113}), functions as an adhesin in LEE-negative isolates of STEC.

1.10.3 Release of Stx

After successful colonization in the gut, STEC organisms release the potent Stxs. Investigations on Stxs led to the discovery of two major toxin types: Stx1 and Stx2, which were shown to be genetically and immunologically (not cross-reactive) different from each other (87). Stx1 is genetically and immunologically related to Stx, which is produced by *S. dysenteriae* type 1 strains (134).

1.10.3.1 Structure of Stx

Members of the Stx family are compound toxins (the holotoxin is approximately 70 kDa), comprising a single catalytic A-subunit (~32 kDa) in noncovalent association with a pentamer of identical B-subunits (the molecular mass of each B-subunit is ~7.7 kDa) that is involved in the binding of the toxin to specific glycolipid receptors on the surface of target cells (42, 134). X-ray crystallographic analysis of the Stx holotoxin demonstrated that the B-subunit pentamers form a doughnut-shaped structure with the carboxy terminus of the A-subunit inserted into the central pore of B-subunits (53). This was consistent with the results of mutational analysis of the carboxy terminal region of the Stx A-subunit (293-residue), which demonstrated that a sequence of nine nonpolar amino acids from residues 279 to 287 was essential for holotoxin assembly (62). These residues form an α-helix that penetrates the

pore in the center of the B pentamer; flanking charged residues appear to stabilize this interaction (75).

1.10.3.2 Mode of action

The eukaryotic cell surface receptor for members of the Stx family is a galactose-α-1, 4-galactose containing neutral glycolipid designated as globotriaosyl-ceramide (Gb_3) (111). Gb_3 is expressed on epithelial and endothelial cells derived from a variety of sites in humans and animals, including human renal tissue, human lymphocytes, and human erythrocytes (the Pk antigen) (18, 21, 40). However, human intestinal epithelial cells have been shown to lack the Gb_3 receptor for Stx binding (67). Although Stx2e has some binding affinity to Gb_3, it preferentially binds to Gb_4 with a terminal β-N-acetyl-galactosamine residue, also known as the P-antigen on human erythrocytes (40).

Once Stxs bind their glycolipid receptors, the toxins are internalized via clathrin-coated pits and transported through the trans-Golgi network and Golgi apparatus to the endoplasmic reticulum (ER) and nuclear membrane. This pattern of intracellular trafficking is referred to as retrograde transport (157). During retrograde transport, the A-subunit is cleaved by furin, a calcium-sensitive serine protease localized to the Golgi network. The resultant A-subunit fragments, A1+A2, remain associated by a disulfide bond. An alternative mechanism of A-subunit processing involving the action of the protease calpain has also been described (138). Once in the ER, the disulfide bond linking A1+A2 is reduced, and the A1 fragment is translocated across the ER membrane into the cytoplasm. The A1 fragments of Stxs possess N-glycosidase activity and act to catalytically cleave a single adenine residue from the 28S rRNA component of the eukaryotic ribosomal 60S subunit. Following depurination, elongation factor 1-dependent aminoacyl-tRNA binding is inhibited and peptide elongation ceases (135), resulting in cell death.

In addition to inhibition of protein synthesis, Stxs have been demonstrated to induce apoptosis or programmed cell death in many human cell types *in vitro*, including epithelial cell lines, primary renal epithelial cells, Burkitt's lymphoma cells, microvascular endothelial cells, and myelogenous leukemia cell lines (164).

1.10.3.3 Translocation of Stx into the circulation

Microvascular endothelial damage underlies the pathological changes in HC and HUS caused by STEC. Stxs are presently the best-characterized STEC virulence factors that cause the microvascular endothelium injury. In order to cause this injury the toxins need to be translocated across the gut epithelium to the systemic circulation. STEC are generally considered to be noninvasive and thus are thought not to penetrate intestinal epithelial tissue,

but rather to produce Stxs in the intestinal lumen. Several studies have addressed the pathway by which Stx translocates from the intestinal lumen into the circulation. Stx1 translocation through intestinal epithelial cells occurs via a transcellular route, whereas that of Stx2 occurs through a paracellular pathway (71, 146). In different culture cell line assays, it has been demonstrated that translocation of Stx is energy dependent and directional, with greater toxin movement from the apical side to the basolateral side than vice versa (78, 146). The toxins may also bind to intestinal endothelial cells (73) and, by damaging both epithelial and endothelial cells, induce HC and gain access to the bloodstream.

According to the concept of the HUS pathogenesis, after entrance into the bloodstream, the toxins are targeted to the microvascular endothelial cells of the kidney. The mechanism of toxin delivery to the endothelial cells is still a matter of debate, although the role of polymorphonuclear leukocytes as an Stx carrier has been indicated (24). At the renal endothelial cells, Stxs bind, through their B-subunits, to the Gb_3 receptor and internalize by the mechanism as described above.

1.10.3.4 Influence of Stx type on pathogenesis

Epidemiological and clinical investigations on the association of toxin types with the animal reservoir of STEC and with human pathogenicity revealed remarkable differences. Epidemiological studies have indicated that STEC strains producing Stx2 only are more commonly associated with serious human disease, such as HUS, than those producing Stx1 alone or Stx1 and Stx2 (100, 139). One possible explanation for this is that the level of transcription of *stx_2 in vivo* is higher than that of *stx_1* (141). The link between Stx2 production and HUS may be a direct consequence of increased *in vivo* toxicity of Stx2, or, alternatively, carriage of *stx_2* may simply be a clonal marker of STEC strains producing some additional virulence factors (141). This is consistent with *in vitro* studies, which demonstrated that human renal microvascular endothelial cells were approximately 1,000 times more sensitive to the cytotoxic action of Stx2 than of Stx1 (116). Increased *in vivo* toxicity of Stx2 is also supported by studies involving a streptomycin-treated mouse model of toxin-induced renal tubular damage (185). It should also be emphasized that the link between Stx2 production and the capacity of an STEC strain to cause HUS is not absolute. STEC strains producing only Stx1 are capable of causing HUS, as are strains of *S. dysenteriae* type 1, which also produce the same toxin.

Several studies have indicated that there is a strong association between the variants of Stx1/Stx2 produced by the organism and the development of serious disease (55, 145). Among the variants of Stx2, Stx2c has been found to be less frequently associated with

bloody diarrhea and HUS than Stx2 (55). Stx2 and the elastase (mucus)-activatable Stx2d type, are associated with the high virulence of STEC and with HC and HUS (17). Other toxin variants, such as Stx1c, Stx2-O118 (formerly Stx2d-Ount), Stx2e, and Stx2f, were found to be associated mainly with uncomplicated diarrhea or asymptomatic excretion (55, 56). It has also been demonstrated that there is an association between the type of Stx and the origin of the strains. For example, STEC strains producing Stx1c and/or Stx2d-O118 were associated with sheep (22, 179), Stx2e with pigs (54), and Stx2f with pigeons (166) as natural reservoirs.

1.10.4 Release of other potential virulence factors

1.10.4.1 Enterohemolysin

A high proportion of STEC strains posses a novel hemolytic phenotype, which is distinct from that associated with the *E. coli* α-hemolysin (hly) (14). Nearly all STEC O157 strains and a wide range of STEC non-O157 strains are positive for enterohemolysin (subsequently designated EHEC-hly) (82). Strains producing this enterohemolysin are not hemolytic on standard blood agar but produce small, turbid hemolytic zones on washed sheep red blood cell agar (supplemented with calcium) after overnight incubation. Unlike α-hemolysin, which is chromosomally encoded, EHEC-hly is encoded by the 60-MDa "virulence plasmid" of the STEC O157 (pO157). The role of enterohemolysin in the pathogenesis of STEC infection is still subject to speculation. One possibility is that hemoglobin released by the action of EHEC-hly provides a source of iron, thereby stimulating the growth of STEC in the gut (104).

1.10.4.2 EHEC type II secretion system (EtpD)

Upstream of the EHEC-hly operon in pO157 is a gene cluster showing similarities to genes of members of the type II secretion pathway systems of Gram-negative bacteria (81). The highest similarities were found to the pullulanase secretion pathway of *Klebsiella oxytoca* and *Klebsiella pneumoniae* in which at least 14 genes and proteins are involved (81). The genetic determinants of this type II secretion pathway system are encoded in 13 ORFs. These ORFs are termed as etpC through etpO. The significance of this gene cluster in the pathogenesis of STEC infection is yet to be elucidated. The distribution of the genetic determinants of this type II secretion pathway system is not uniform. Karch et al. (81) showed that all 50 isolates of STEC O157:H7 (100%) tested possessed this pathway, but that it was present in only 52% of 50 STEC non-O157. In another study, the presence of one of the genes in the pathway, *etpD*, was detected in all the 13 STEC O103 strains but not in any of the STEC strains tested belonging to O26 or O111 (161). The *etp* genes were rarely

detected in STEC isolated from bovine feces (160). Moreover, it was found not to occur in other pathotypes of *E. coli* (160).

1.10.4.3 The bifunctional catalase peroxidase (KatP)

The STEC bifunctional catalase peroxidase is termed as KatP (26). The functional role of this enzyme remains unclear. It has been suggested that bacterial catalases and superoxide dismutases detoxify cytotoxic oxidant products during the oxidative bursts of macrophages and neutrophils, thereby assisting the bacterium in escaping host defenses (26). The distribution of KatP genetic determinants is not uniform. Brunder et al. (26) found that KatP was present in 66% of the STEC O157:H7 isolates and 38% of the STEC non-O157 isolates. Besides, absence of KatP in STEC isolates causing HC or HUS suggested that the enzyme does not have a major role in virulence.

1.10.4.4 The secreted serine protease (EspP)

One of the most important virulence factors of STEC strains is the secreted serine protease (EspP), which is also encoded on the large plasmid (pO157) but is not involved in the production of the A/E lesions (27). *E. coli* has several secreted proteins (Esps). The entire sequence of EspP was homologous to that of EspC (110 kDa protein secreted by EPEC strains), SepA (the major extracellular protein of *Shigella flexneri*) and Tsh (a haemagglutinin from an avian pathogenic *E. coli* strain) (81). The EspP is subjected to N- and C-terminal processing during the secretion process. EspP is known as an autotransporter as it mediates its own secretion through the outer membrane. It also has proteolytic activity against a narrow range of substrates, one of which is human coagulation factor V (81). This surprising activity indicates that EspP secreted by STEC may influence the blood clotting cascade. Cleavage of factor V could result in a decreased coagulation reaction leading to prolonged bleeding. Local degradation of factor V by STEC attached to the intestinal mucosa could increase hemorrhage in the gastrointestinal tract (81). Children suffering from STEC infection produce an antibody response to EspP indicating its expression *in vivo* (27), although this does not necessarily indicate a role in pathogenesis. The distribution of the EspP toxin in STEC strains causing infection is not uniform (27, 28). Therefore, the significance of EspP in disease is unclear, but its frequent absence in STEC O157:H7 and non-O157 strains indicates that it is not vital to the development of the disease process in humans.

1.11 Outline of the thesis

The main objective of the work presented in this thesis was to gain insight in the epidemiology of STEC infection in humans and the ecology of STEC in the human food chain in Bangladesh. Although Bangladesh is an endemic zone for diarrheal diseases caused by different enteric pathogens, no systematic study has yet been done on the epidemiology of STEC infection in diarrheal patients. In **chapter 2** we described the prevalence and characteristics of STEC isolated from patients with diarrhea attending the hospital as well as a community clinic in Dhaka city in Bangladesh. The next two chapters deal with the evaluation of detection and isolation methods for STEC O157 from animal feces and raw meats. There are several methods available for the detection and isolation of STEC O157 but a single method may not be equally effective for all different kinds of samples. In **chapter 3**, we compared the efficiency of two commonly used immunoconcentration methods, Dynabeads anti-*E. coli* O157 and the Vitek Immunodiagnostic Assay System (VIDAS) Immuno-Concentration *E. coli* O157 (ICE) kit, by analyzing retrospectively the results of isolation of STEC O157 from animal feces and raw meats. In this study, we also compared two commonly used selective plating media CT-SMAC and 1/2CT-CHROMagar for isolation of STEC O157 in combination with the Dynabeads anti-*E. coli* O157 method and the VIDAS ICE method. When the Dynabeads anti-*E. coli* O157 method turned out to be more effective than the VIDAS ICE method for the isolation of STEC O157 from animal feces, we further compared this method with a PCR-based method and this has been described in **chapter 4**. In order to study the occurrence of STEC in the food chain, we investigated the prevalence of this organism in the reservoirs and in food. We focused on animals and food, which have the greatest chance of being positive for STEC and therefore can play an important role in the transmission of these pathogens to humans. In **chapter 5**, we described the prevalence and characteristics of STEC in different slaughtered animals in Bangladesh, including buffalo, cows and goats. Similarly in **chapter 6**, we described the occurrence and characteristics of STEC in different types of food, including raw meat, raw milk and freshly prepared juices in Bangladesh. In order to interlink the results obtained from the investigations of STEC in each of the components of the food chain, we analyzed the antibody responses against STEC O157 among the healthy population in Bangladesh, which has been described in **chapter 7**. Finally, in **chapter 8**, the findings of this thesis were summarized with a more general discussion and placed in perspective with future directions of the research.

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Chapter 2

Shiga toxin-producing Escherichia coli *isolated from patients with diarrhea in Bangladesh*

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Abstract

The prevalence of Shiga toxin-producing *Escherichia coli* (STEC) and its characteristics were determined among hospitalized patients with diarrhea and children with diarrhea in an urban slum community of Dhaka city using sensitive culture and PCR methods. Stool samples were collected from 410 patients with diarrhea enrolled in the 2% surveillance system (every 50th patient attending the hospital with diarrheal disease is included) at the ICDDR,B hospital and from 160 children of 2–5 years of age with diarrhea living in an urban slum in Dhaka, between September 2004 and April 2005. Shiga toxin genes (*stx*) were detected by multiplex PCR in the enrichment broth of nine samples (2.2%) from hospitalized patients and 11 samples (6.9%) from the community patients. STEC were isolated from five stool samples with positive PCR results using a colony patch technique. All five isolates were positive in the Vero cell assay and PCR fragments of *stx* genes were confirmed by sequencing. Two isolates were positive for the *E. coli* attaching and effacing (*eae*) gene and four were positive for the enterohemolysin (hly_{EHEC}) gene and enterohemolysin production. The five isolates belonged to five different serotypes: O32:H25, O2:H45, O76:H19, ONT:H25 and ONT:H19. It can be concluded that STEC is not a common pathogen in Bangladesh among hospitalized patients with diarrhea nor among mild cases of diarrhea in the community.

2.1 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important causative agents of hemorrhagic colitis and diarrhea-associated hemolytic–uremic syndrome (HUS) with or without neurological complications (13, 22, 26). STEC are serologically diverse group of foodborne, zoonotic pathogens. Serotype O157:H7 has been the predominant type worldwide (34). However, altogether more than 200 STEC serotypes have been reported and more than 100 have been linked with human infection (7). In some geographic areas, STEC non-O157 are more commonly isolated from persons with diarrhea or HUS than STEC O157 strains (28). Although most sporadic cases and outbreaks have been reported from developed countries, human infections associated with STEC strains have also been described in Latin America, India and other developing countries (11, 17). In Bangladesh, the predominant group of *E. coli* associated with childhood diarrhea is enterotoxigenic *E. coli*, accounting for approximately 20% of all diarrheal cases (29). The burden of STEC-associated diarrhea in Bangladesh is unknown. In the present study, we investigated the prevalence of STEC among hospitalized patients with diarrhea as well as among patients with diarrhea attending the community clinics. The STEC isolates were characterized in detail.

2.2 Materials and methods

2.2.1 Stool samples

Between September 2004 and April 2005, stool samples were collected from patients with diarrhea enrolled in an ongoing active surveillance system at the Dhaka treatment centre operated by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). In this surveillance system, every 50th patient attending the hospital with diarrheal disease is included. A detailed history of the patients is obtained, including information on the age, sex and clinical presentation. Stool samples were collected in sterile McCartney bottles and transported to the laboratory within 1 h.

In the same period, stool samples were collected from children having complaints of diarrhea attending the Centre's community clinic at Mirpur, an urban slum in Dhaka. The inhabitants of Mirpur are of Bihari ethnic origin and settled there after the separation of Bangladesh from Pakistan in 1971. The area is densely populated and has poor sanitary and hygiene conditions. Clinical information was collected by health care workers who visited the

children and their parents every other day. Stool samples were kept on ice, and transported to the laboratory for processing within 2–4 h after collection.

2.2.2 Isolation procedure for STEC O157

A loopful of stool sample was inoculated into 3 ml modified tryptone soy broth (Oxoid Ltd., Basingstoke, United Kingdom) and incubated overnight at 37°C. Immunomagnetic separation using Dynabeads anti-*E. coli* O157 (Dynal, Oslo, Norway) was performed with 1 ml broth culture, following the manufacturer's instructions. The immunoconcentrated samples were inoculated onto two selective isolation media: sorbitol-MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg/l) and tellurite (2.5 mg/l) (Oxoid) (CT-SMAC); and CHROMagar O157 (ITK Diagnostics BV, the Netherlands) with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) (1/2CT-CHROMagar). The agar plates were incubated for 18–24 h at 37°C. Typical colonies (colorless colonies on CT-SMAC and mauve on 1/2CT-CHROMagar), up to 12 per plate, were selected and streaked onto tryptone soy agar (TSA) plates. The colonies on TSA plates were tested for agglutination with an *E. coli* O157 latex test kit (Murex) and a PCR for the *rfb*E_{O157} gene, which is specific for *E. coli* O157 (Table 2.1).

2.2.3 Detection and isolation of STEC non-O157 types

The overnight incubated enrichment broth was also examined by PCR using *stx*$_1$ and *stx*$_2$ primers (Table 2.1). DNA was extracted from 1 ml broth culture by thermal cell lysis using Chelex-100 resin (Bio-Rad Laboratories Ltd., Richmond, Calif.) (21) and 5 µl of the DNA extract was used in the PCR. Touchdown multiplex PCR for *stx*$_1$ and *stx*$_2$ was carried out in a PTC-200 peltier thermal cycler (Bio-Rad). After initial incubation at 94°C for 5 min, a 40-cycle amplification protocol was implemented as follows: 94°C for 30 s, 64°C for 30 s and 72°C for 60 s for two cycles followed by eight cycles with decreasing annealing temperatures of 2°C in every two cycles. When the annealing temperature of 54°C was reached at cycle 10, the PCR was continued with these cycling parameters followed by a final extension of 10 min at 72°C. All PCR fragments for *stx*$_1$ and *stx*$_2$ genes were subjected to sequencing. After electrophoresis, bands of PCR products were extracted and purified using the PCR product purification kit (Roche Diagnostics, Mannheim, Germany). Subsequently, a cycle-sequence reaction was performed using a kit (BigDye Terminator v 3.1 cycle-sequencing kit; Applied Biosystems) according to the manufacturer's protocol. Purified PCR products were sequenced on an automated sequencer (ABI Prism 3100-Avant Genetic Analyzer; Applied

Biosystems). The chromatogram sequencing files were inspected using Chromas 2.23, and contigs were prepared using SeqMan II (DNASTAR). Nucleotide sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) server on the GenBank database, release 138.0. Broth cultures that yielded positive PCR results for stx_1 and stx_2 or both were streaked onto tryptone bile x-glucuronide (TBX) agar (Oxoid) supplemented with cefixime (20 mg/l), cefsulodin (3 mg/l) and vancomycin (30 mg/l) (CCV-TBX). Single colonies, including different morphological types were transferred from the CCV-TBX and plated onto Luria agar (Difco, BD diagnostics, USA) to create a grid pattern of 96 colonies (12×8). The plates were incubated overnight at 37°C. The isolates were subjected to PCR for the detection of stx_1 and stx_2 as described above. The number of PCR reactions was reduced to 20 by pooling the colonies per row and per column in 500 µl sterile distilled water. DNA was extracted by boiling the suspensions for 10 min and 5 µl of the supernatant was used in the PCR. The isolates with positive PCR results were identified as *E. coli* by an API 20E test (bioMérieux, Marcy l'Etoile, France).

2.2.4 Characterization of STEC isolates

All isolates were sent to the National Institute of Public Health and the Environment (RIVM) in Bilthoven, the Netherlands, for O and H typing. Production of Shiga toxin was determined by Vero cell culture assay (14). The isolates were tested by PCR for the presence of the *E. coli* attaching and effacing (*eae*) and the enterohemolysin (hly_{EHEC}) genes (10, 35). The oligonucleotide primers used for PCR are listed in Table 2.1. Enterohemolytic activity was examined by streaking the isolates onto tryptone soy agar supplemented with 10 mM $CaCl_2$ and 5% defibrinated sheep blood cells washed three times in PBS (pH 7.2), as described previously (3). The plates were observed for hemolysis after 4 h (α-hemolysis) and after overnight incubation in ambient air at 37°C (enterohemolysis or nonhemolysis).

2.2.5 Isolation of other enteric pathogens

All samples from hospitalized patients were examined for other enteric pathogens in addition to STEC, including *Vibrio cholerae*, *Salmonella* spp., *Shigella* spp. and *Aeromonas* spp., using standard microbiology procedures (36).

2.2.6 Statistical analysis

Data were entered and statistical analyses were performed using SPSS and Epi Info. The significance of difference in proportions was evaluated by the chi-square test, and Fisher's exact test was applied when appropriate. $P < 0.05$ was considered statistically significant.

Table 2.1 PCR primers used in this study

Primer	Sequence (5′-3′)	Target	PCR product (bp)	Reference
stx$_1$F	CAC AAT CAG GCG TCG CCA GCG CAC TTG CT	*stx*$_1$	606	9
stx$_1$R	TGT TGC AGG GAT CAG TCG TAC GGG GAT GC			
stx$_2$F	CCA CAT CGG TGT CTG TTA TTA ACC ACA CC	*stx*$_2$	372	10
stx$_2$R	GCA GAA CTG CTC TGG ATG CAT CTC TGG TC			
*eae*F	TGCGGCACAACAGGCGGCGA	*eae*	629	10
*eae*R	CGGTCGCCGCACCAGGATTC			
hly$_{EHEC}$F	GAGCGAGCTAAGCAGCTTG	*hly*$_{EHEC}$	889	35
hly$_{EHEC}$R	CCTGCTCCAGAATAAACCACA			
*rfb*E$_{O157}$F	CGGACATCCATGTGATATGG	*rfb*E$_{O157}$	259	27
*rfb*E$_{O157}$R	TTGCCTATGTACAGCTAATCC			

2.3 Results and Discussion

Over the years, STEC has been found to be associated with all forms of diarrheal infection ranging from watery to severe bloody diarrhea. Bangladesh is an endemic zone for diarrheal diseases: every year, more than 5% of deaths of children below five years of age are attributed to diarrhea (2). In Bangladesh, no STEC infection among the patients with diarrhea has been reported (1). The reasons might be: (1) the lack of proper surveillance for STEC; (2) STEC is not present; or (3) STEC is present but relatively few infections occur due to acquired immunity in the population. A study on the prevalence of STEC in neighboring Calcutta, India, showed a very low prevalence of STEC among hospitalized patients with diarrhea (1.4% and 0.6% of bloody and watery stool samples, respectively) (16). The present study was aimed at measuring the burden or occurrence of STEC infection among the patients with diarrhea in the hospital and in the community using sensitive culture and PCR methods.

2.3.1 Prevalence of STEC

Of 410 stool samples collected from hospitalized patients, nine (2.2%) were positive by PCR: four for *stx*$_2$ only, three for *stx*$_1$ only and two for both *stx*$_1$ and *stx*$_2$ (Table 2.2).

Table 2.2 Presence of *stx* genes in stool samples, clinical manifestation and stool characteristics of patients positive for *stx* genes

Patient code	Presence of *stx* genes[a]		Patient history								Stool characteristics			
	stx$_1$	*stx*$_2$	Age (years)	Sex[b]	Fever	Vomiting	Abdo-minal pain	Conv ulsion	Chemo-therapy before arrival[c]	Diagnosis[d]	Watery	Bloody	Mucus	Presence of other pathogen in stool[e]
Hospitalized patients														
Am 3	+	-	70	F	-	<10 times	-	-	Metro	UCD	+	-	++	None
Am 35	-	+	7	M	-	<10 times	-	-	None	UCD	+	-	+	*V. cholerae* 01
Am 84	+	+	22	F	-	>10 times	+	-	Metro	UCD	+	-	+	*V. cholerae* 01
Am 107	+	+	35	M	-	<10 times	-	-	Metro	UCD	+	-	++	*V. cholerae* 01
Am 113	-	+	25	F	-	<10 times	-	-	UID	UCD	+	-	+	None
Am 167	-	+	2	F	-	-	+	-	UID	UCD	+	-	+	*V. cholerae* 01
Am 181	+	-	6	F	+	<10 times	+	-	UID	UCD	+	-	++	*V. cholerae* 01
Am 281	-	+	18	F	-	-	+	-	None	UCD	+	-	+	*V. cholerae* 01
Am 403	+	-	18	F	-	>10 times	-	-	None	UCD	+	-	+	*V. cholerae* 01
Community patients														
AI 1	+	+	10	F	+	-	+	-	Cotri	UCD	+	-	-	ND
AI 3	+	+	7	M	-	-	+	-	None	UCD	+	-	-	ND
AI 9	+	-	10	M	-	-	+	-	Ery	UCD	+	-	-	ND
AI 11	+	-	10	F	-	-	+	-	None	UCD	+	-	-	ND
AI 25	+	-	11	F	-	-	-	-	None	UCD	+	-	+	ND
AI 48	+	+	8	F	-	-	-	-	None	UCD	+	-	-	ND
AI 101	-	+	8	F	+	-	+	-	None	UCD	+	-	-	ND
AI 103	-	+	8	F	+	-	-	-	None	UCD	+	-	-	ND
AI 130	+	-	10	M	+	-	+	-	None	UCD	+	-	-	ND
AI 132	-	+	11	M	-	-	-	-	None	UCD	+	-	-	ND
AI 160	+	-	10	M	-	-	+	-	None	UCD	+	-	-	ND

[a] *stx*, Shiga toxin.
[b] F, female; M, male.
[c] Metro, metronidazole; UID, unidentified; Cotri, cotrimoxazole; Ery, erythromycin.
[d] UCD, uncomplicated diarrhea.
[e] ND, not done.

No statistically significant correlation between the age of patients and the presence of *stx* genes was observed. All *stx* positive patients were clinically diagnosed as having uncomplicated diarrhea. All patients had watery diarrhea and mucus was present in the stool samples. Vomiting was recorded for seven patients (78%). Interestingly, seven of the nine patients were primarily diagnosed with a *V. cholerae* O1 infection (Table 2.2). The relative frequency of other enteric pathogens detected in the hospital samples is shown in Table 2.3.

Table 2.3 Prevalence of other bacterial enteropathogens in stool samples from hospitalized patients examined for Shiga toxin-producing *E. coli*

Enteropathogen	Prevalence (%)
Vibrio cholerae O1 El Tor Inaba	19.0
Vibrio cholerae O1 El Tor Ogawa	18.0
Shigella flexneri	2.2
Shigella boydii	0.7
Shigella dysenteriae	0.2
Salmonella Typhi	0.2
Salmonella paratyphi B	0.2
Salmonella Group C1	0.7
Salmonella Group D	0.2
Salmonella Group C2	0.2
STEC O157	0.0
STEC non-O157	0.5

Of 160 samples collected from the community patients with diarrhea, 11 (6.9%) were found to be positive: five for stx_1, three for stx_2 and three for both stx_1 and stx_2 (Table 2.2). All patients had mild diarrhea and were diagnosed as having uncomplicated diarrhea. STEC could be isolated from two (22%) among nine PCR positive samples from the hospitalized patients and three (27%) of 11 PCR positive samples from community patients. The prevalence of STEC in hospitalized patients with diarrhea was very low (0.5%), and was somewhat higher among the community patients with diarrhea (1.9%). Failure to isolate the STEC non-O157 from PCR positive stool samples is still a common problem encountered by researchers around the world (5). According to previous studies, the nonisolation of viable STEC from PCR-positive samples might be due to the presence of very low numbers of bacteria (16), the presence of free *stx* phages in the sample (15) and loss of *stx* genes upon subcultivation of strains (12). In routine diagnostics, there is no definitive biochemical characteristic, such as sorbitol fermentation in the case of serogroup O157, which can identify STEC irrespective of serotypes from the commensal flora. Therefore, selection of suitable culture media for isolation of STEC non-O157 serogroups is always difficult. In this study, we used TBX agar; this medium allows basic differentiation of *E. coli* colonies but

does not discriminate between STEC and non-STEC. In order to make it more selective we supplemented TBX agar with cefixime (20 mg/l), cefsulodin (3 mg/l) and vancomycin (30 mg/l). Antibiotics at these concentrations were used previously with blood agar in order to isolate STEC non-O157 serogroups (18). These antibiotic supplements suppress the growth of Gram-positive bacteria, *Proteus* spp. and *Pseudomonas* spp., and other *E. coli*.

2.3.2 Characterization of STEC isolates

No STEC O157 organisms were isolated from patients in this study. The serotypes of STEC isolates were different: O32:H25, O2:H45, O76:H19, ONT:H25, ONT:H19. The characteristics of the O76:H19 isolate in the present study were similar to those described previously (33) for O76:H19 isolates from asymptomatic human carriers, except for the stx_2 gene, which was additionally present in our isolate. STEC ONT:H25 was previously isolated from healthy cattle in Canada with a higher prevalence (22.5%) compared to the prevalence of *E. coli* O157:H7 (15%). The virulence properties of our isolate and the isolates from Canada were similar except for the type of *stx* gene. In contrast to the isolate in the study, which was positive for the stx_1, all isolates from Canada were positive for stx_2 (32). One isolate from the hospitalized patient was of serotype O32:H25. This serotype has not previously been described as being associated with STEC infection in humans or isolated from animals. The other two serotypes in the current study, O2:H45 and ONT:H19, were previously isolated from cattle in Hong Kong (19) and India (25), respectively.

All five isolates were positive in the Vero cell cytotoxicity test. One isolate from a hospitalized patient and one isolate from a community patient were positive for the *eae* gene (Table 2.4). All three isolates from the community patients and one isolate from the hospitalized patients were positive for the hly_{EHEC} gene (Table 2.4). It has been suggested in a previous study that there is an association among the locus of enterocyte effacement (i.e., the location of the *eae* gene), the enterohemorrhagic *E. coli* hemolysin plasmid, and the hemolysin itself of STEC non-O157 isolates (6). *E. coli* possessing the *eae* gene were statistically more likely to be enterohemolytic than *E. coli* that did not carry this gene (7). STEC having both the *eae* gene and hemolytic properties (hly_{EHEC} gene) is more virulent and causes more human infection than strains carrying only the *stx* genes (7, 24, 30). However, we did not find a more severe clinical presentation in those patients from whom STEC were isolated.

Table 2.4 Shiga toxin-producing *E. coli* isolated from patients with diarrhea in Bangladesh

No. of strain	Source[a]	Serotype	PCR result[b]	Ehly[c]
Am 181	A	O32:H25	*stx*$_1$+, *eae*-, *hly*$_{EHEC}$-	-
Am 281	A	O2:H45	*stx*$_2$+, *eae*+, *hly*$_{EHEC}$+	+
AI 3	B	O76:H19	*stx*$_{1,2}$+, *eae*-, *hly*$_{EHEC}$+	+
AI 130	B	ONT:H25	*stx*$_1$+, *eae*+, *hly*$_{EHEC}$+	+
AI 132	B	ONT:H19	*stx*$_2$+, *eae*-, *hly*$_{EHEC}$+	+

[a] Samples were collected from two different groups of diarrheal patients. A, hospitalized patients; B, community patients.

[b] Carriage of *stx*$_1$ or *stx*$_2$ or *eae* or *hly*$_{EHEC}$ gene. +, positive; -, negative.

[c] Ehly, enterohemolysin production. +, enterohemolysin positive; -, no hemolysis or α-hemolysis.

Although STEC have been isolated sporadically from different regions of developing countries, it was never implicated as a major causative agent of diarrhea. In a previous study in Bangladesh, a total of 452 children with diarrhea and 602 matched control children without diarrhea were investigated for the presence of diarrheagenic *E. coli* (1). In children (up to five years of age) with diarrhea, enteropathogenic *E. coli* (EPEC) was the most prevalent (15.5%), followed by enterotoxigenic *E. coli* (12%), enteroaggregative *E. coli* (9.5%) and diffuse adherent *E. coli* (8.2%). Enterohemorrhagic *E. coli* possessing *stx* was not detected in any of the children with diarrhea but was detected in five children without diarrhea. No further characterization of these five isolates was performed. STEC-identified diarrheal cases in developing countries are often infected with other pathogens as well; for example, in India, 58% of the STEC positive patients were co-infected with other enteric pathogens (16). This trend was also observed in the current study; seven of the nine PCR positive samples from hospitalized patients were also positive for *V. cholerae*. In fact, *V. cholerae* was the most common pathogen isolated from the hospitalized patients included in this study, which accounted for 37% of samples (Table 2.3). Therefore, the specific role of STEC in causing diarrheal illness in this area is difficult to estimate. The reasons for the low prevalence of STEC-associated diarrhea among hospitalized and community patients are still not clear. However, protective immunity against STEC could be an explanation, and was addressed by most of the studies done in developing countries (8, 20, 23, 31). The low prevalence of STEC in Calcutta and possibly other places in India was explained by the fact that Indians acquire protective antibodies at an early age or their cooking practices effectively eliminate STEC (16). According to previous studies, this immunity can be O group-specific or cross-reactive (mucosal) and is normally acquired during infancy (20, 23). This could be associated with the repeated antigenic stimulation in a contaminated environment where diarrheal diseases are considered endemic (23). It has also been suggested (4) that EPEC infections in early childhood confer cross-reacting protective immunity against STEC types that share common

antigens (such as LPS and intimin) with classical EPEC strains. However, a detailed study of the immune status of patients with diarrhea as well as healthy controls in areas where enteric pathogens are considered to be endemic should be carried out in order to explain these phenomena.

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Chapter 3

Immunoconcentration of Shiga toxin-producing Escherichia coli *O157 from animal feces and raw meats by using Dynabeads anti-*E. coli *O157 and the VIDAS system*

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Abstract

To identify the reservoirs and routes of transmission of Shiga toxin-producing *Escherichia coli* (STEC) O157, sensitive detection and isolation methods are necessary. The sensitivity of traditional culture methods can be improved significantly by the inclusion of an immunoconcentration step, resulting in less false-negatives. In this report, we evaluated the results of two commercially available test systems: Dynabeads anti-*E. coli* O157 and the Vitek Immunodiagnostic Assay System (VIDAS) Immuno-Concentration *E. coli* O157 (ICE) kit. Additionally, we compared two selective isolation media for STEC O157. Statistical analysis of the results obtained for animal fecal samples (n = 637) examined by both immunoconcentration methods showed that by the manual Dynabeads anti-*E. coli* O157 procedure systematically more samples were identified as positive than by the VIDAS ICE. In case of meat samples (n = 360), no difference between the results of the two methods was found. In addition to being accurate, the Dynabeads anti-*E. coli* O157 method is a less expensive method than the VIDAS ICE. But, the Dynabeads method is laborious and there is a risk of cross-contamination. The VIDAS ICE procedure on the other hand is fully automated with a standardized performance; fast and safe for the user. Irrespective of the type of sample (feces or meat) and the immunoconcentration technique applied (Dynabeads anti-*E. coli* O157 or VIDAS ICE) more samples were found positive after plating onto CHROMagar O157 with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) than after plating onto sorbitol–MacConkey agar with cefixime (0.05 mg/l) and tellurite (2.5 mg/l). However, only in case of meat samples examined by the VIDAS ICE the difference between the isolation media was not statistically significant.

3.1 Introduction

Since the early 1980s, it is known that an infection with Shiga toxin-producing *Escherichia coli* (STEC) strains of serogroup O157 are the major cause of hemorrhagic colitis and the hemolytic–uremic syndrome (5, 9, 14). The infections frequently have been associated with the consumption of undercooked (minced) beef and raw milk (15). Epidemiological studies have shown that cattle, but also other farm animals can excrete the bacteria with their feces, without showing any clinical symptoms (11). In addition to (fecally contaminated) foods, direct transmission from animal to human can result in infection (13).

To elucidate further the reservoirs of STEC O157, sensitive methods are needed since these pathogens may be present in food and environmental samples in only small numbers. Furthermore, sensitive and rapid detection methods are necessary for the food industry to ensure a safe supply of foods. Conventional culture methods are both time-consuming and laborious. Their sensitivity can significantly be increased by the application of an immunoconcentration step, which will result in less false-negatives. A commonly used method, which is also implemented in the international standard for the detection of *E. coli* O157 in food and feeding stuffs (2), is immunomagnetic separation (IMS). Prior to plating onto selective isolation media, *E. coli* O157 cells present in the enrichment culture are selectively concentrated by magnetic beads with *E. coli* O157-specific antibodies covalently bound onto their surface. The IMS procedure can be performed both manually and automatically. Another example of immunoconcentration is the fully automated Vitek Immunodiagnostic Assay System (VIDAS) (bioMérieux, Marcy l'Etoile, France). The VIDAS Immuno-Concentration *E. coli* O157 (ICE) kit includes two ready-to-use components. One is a Solid Phase Receptacle (SPR), which serves as the solid phase as well as the pipetting device for the assay. The interior of the SPR is coated with anti-*E. coli* O157 antibodies adsorbed on its surface. The other component is a strip which contains all the wash and release solutions. An aliquot of the enrichment culture is manually transferred into the strip and subsequently the sample is cycled in and out of the SPR. *E. coli* O157 present in the broth will bind to the anti-*E. coli* O157 antibodies coating the interior of the SPR. Unbound sample components are then washed away. A final enzymatic step releases the captured *E. coli* O157 into a specific well from which they can be plated onto selective agar.

In our laboratory, samples of animal feces and meats had been examined regularly for the presence of STEC O157 by using a manual IMS procedure as well as the VIDAS ICE

system. In this study, we retrospectively evaluated the performance of both methods. Additionally, two selective agars used to finally isolate STEC O157 were compared.

3.2 Materials and methods

3.2.1 Samples

The following samples were examined for the presence of STEC O157 by performing a manual IMS procedure as well as the VIDAS ICE system: (1) feces from farm animals that previously tested positive and had been stored at -20°C (*n* = 144); (2) rectal contents of different farm animals in petting zoos sampled individually by rectal palpation (*n* = 73); (3) fecal droppings from different animals collected at different petting zoos (*n* = 95); (4) rectal contents of sheep collected at the slaughterline (*n* = 325); (5) swab samples from sheep carcasses (*n* = 325); and (6) different retail raw meats that previously tested positive and had been stored at -20°C (*n* = 35).

For the comparison of two selective isolation media, in addition to the results obtained for the samples listed above, results of another 679 samples of animal feces were included, which were analyzed with either IMS or VIDAS ICE. These samples, both rectal contents and droppings, had been collected from different animals at petting zoos and cattle farms, either in order to trace sources of human infection (*n* = 177) or to study the occurrence of STEC O157 in petting zoos (*n* = 502).

With the exception of the positive feces and meat samples that were kept in the freezer, all samples were fresh and the microbiological examination had been started within 72 h after their collection. As a rule, the carcass swabs were processed within the day of sampling.

3.2.2 Selective enrichment

The samples were, if applicable after being thawed at 2 to 4°C, diluted 10 times in modified tryptone soy broth (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with novobiocin (20 mg/l) (Sigma Chemical Co., St. Louis, MO) (mTSB+n). To the plastic stomacher bags containing the sponges used to sample the sheep carcasses, 90 ml of mTSB without novobiocin was added. After homogenization (1 min) in a stomacher, the samples were incubated at 41.5±0.5°C for 18 to 24 h. In case of the sheep feces collected at slaughter, after 6 to 8 h of incubation about 5 ml of enrichment culture was taken for the purpose of the IMS procedure. For all the other analyzes overnight cultures were used.

3.2.3 Immunoconcentration

*3.2.3.1 Dynabeads anti-*E. coli *O157*

For the IMS procedure Dynabeads anti-*E. coli* O157 (Dynal, Oslo, Norway) were used and the instructions of the manufacturer were followed.

3.2.3.2 VIDAS Immuno-Concentration E. coli *O157*

The manufacturer of the VIDAS ICE kit (bioMérieux, Marcy l'Etoile, France) prescribes a first selective enrichment of the sample in mTSB+n (6–7 h at 41±1°C) followed by a second selective enrichment (1:10 dilution) in MacConkey broth supplemented with cefixime (0.05 mg/l) and tellurite (2.5 mg/l) (18±1 h at 35–37°C) before testing with the VIDAS ICE kit. Deviating from this protocol, 500 µl from the overnight cultures in mTSB+n was pipetted into the strips. This choice has been made based upon the results of an extensive study on methods for detection and isolation of *E. coli* O157, previously performed in our laboratory (16).

3.2.4 Selective isolation and confirmation

The immunoconcentrated samples were inoculated onto two selective isolation media: sorbitol–MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg/l) and tellurite (2.5 mg/l) (Oxoid) (CT-SMAC) and CHROMagar O157 (ITK Diagnostics BV, Uithoorn, The Netherlands) with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) (1/2CT-CHROMagar). The agar plates were incubated for 18 to 24 h at 37°C. Typical colonies (colorless colonies on CT-SMAC and purple on 1/2CT-CHROMagar), up to 12 per plate, were selected and inoculated onto Levine's eosin methylene blue agar (Oxoid) (L-EMB) agar and sorbitol–MacConkey agar with 4-methylumbelliferyl-β-D-glucuronide (0.1 g/l) (Sigma) (SMAC-MUG) agar. The plates were read after 18 to 24 h of incubation at 37°C. Presumptive STEC O157 isolates (those with a typical *E. coli* metallic sheen on L-EMB, being both sorbitol-nonfermenting and β-glucuronidase-negative on SMAC-MUG) were tested for agglutination with an *E. coli* O157 latex test kit (Murex Biotech Ltd., Dartford, Kent, United Kingdom). Isolates that gave a positive latex test result were confirmed to be *E. coli* by using an API 20E biochemical test strip (bioMérieux). Finally, the isolates were confirmed with a number of PCR tests specific for identification of several characteristics of STEC O157, including the presence of *stx* genes (stx_1 and stx_2) (7), the *eae* gene (7), the enterohemolysin gene (hly_{EHEC}) (17) and a specific portion of the *rfb* (O-antigen encoding) region of *E. coli* O157 lipopolysaccharide ($rfbE_{O157}$) (12).

3.2.5 Statistical analysis

Since the international standard for the detection of *E. coli* O157 is based on IMS, the Dynabeads anti-*E. coli* O157 procedure was considered as the reference method for the statistical analysis. The relative sensitivity, specificity and diagnostic accuracy were calculated with the following formulas (3):

- Relative sensitivity=100%×(no. of samples positive with both methods)/(total no. of samples positive with the Dynabeads anti-*E. coli* O157 method).
- Relative specificity=100%×(no. of samples negative with both methods)/(total no. of samples negative with the Dynabeads anti-*E. coli* O157 method).
- Relative accuracy=100%×(no. of samples positive and negative with both methods)/(total no. of samples examined by both methods).

The diagnostic accuracy takes into account the target and non-target microorganisms in the presence of a biological matrix and consists of the terms sensitivity and specificity (10). The relative sensitivity is the ability of the alternative method (here the VIDAS ICE) to detect the target when it is also detected with the reference method. The relative specificity is the ability of the alternative method not to detect the target organism when it is also not detected with the reference method. The relative accuracy is the degree of correspondence between the responses obtained by the alternative method and the reference method on identical samples.

Finally, it was calculated whether the methods were statistically significantly different, according to Annex F of EN ISO 16140:2003(E) (3). The same calculations were performed to compare the isolation media CT-SMAC and 1/2CT-CHROMagar using CT-SMAC as the reference.

3.3 Results and discussion

Since their first identification as an enteropathogen, strains of STEC O157 have emerged as a serious threat to public health, especially in developed countries (15). Various factors may affect the prevalence findings of *E. coli* O157 in different kind of samples, of which the method of detection and isolation has a major impact. A total of 997 samples had been examined by both the Dynabeads anti-*E. coli* O157 method and the VIDAS ICE. Of the 637 fecal samples, 50 (7.8%) were found positive: 29 with both methods, 17 only with the Dynabeads anti-*E. coli* O157 method, and four only with the VIDAS ICE. Therefore, by using the Dynabeads anti-*E. coli* O157 method systematically more samples were identified

as being positive (Binomial test, $P < 0.05$). However, for the 360 meat samples no systematic difference was found between the methods (Binomial test, $P \geq 0.05$); in total 12 (3.3%) samples were positive, five with both methods, four only with the Dynabeads anti-*E. coli* O157 method and three only with the VIDAS ICE.

Table 3.1 Comparison of the results of the VIDAS ICE with the Dynabeads anti-*E. coli* O157 procedure (reference method) concerning the isolation of Shiga toxin-producing *E. coli* O157 from samples of feces and meat onto sorbitol–MacConkey agar with cefixime (0.05 mg/l) and tellurite (2.5 mg/l)

Sample	Origin	Total no. of samples	IMS[a]/CT-SMAC		VIDAS ICE/ CT-SMAC	
			Positive	Negative	False-negative	" False-positive"[b]
Feces	Petting zoo[c]	95	0	95	0	1
	Petting zoo[d]	73	15	58	2	1
	Frozen[e]	144	11	133	4	2
	Sheep slaughterhouse[f]	325	5	320	4	0
	Total	637	31	606	10	4
Meat	Sheep slaughterhouse[g]	325	0	325	0	2
	Frozen[h]	35	1	34	0	0
	Total	360	1	359	0	2

[a] IMS, immunomagnetic separation with Dynabeads anti-*E. coli* O157.
[b] "False-positive" means in this case true-positive, because the target bacteria are being isolated.
[c] Droppings taken from the ground.
[d] Individual sampling (rectal palpation) living animals.
[e] Diverse origin (droppings and feces from individually sampled animals); all 144 samples were found positive in a previous study, stored at -20°C and after some years examined again.
[f] Rectal contents from sheep, sampled individually at the slaughterline.
[g] Swab of a sheep carcass, sampled individually immediately after entrance in the cooling room.
[h] All 35 samples were found positive in a previous study, stored at -20°C and after some years examined again.

The relative sensitivity, specificity, and accuracy of the VIDAS ICE method in comparison with the Dynabeads anti-*E. coli* O157 method was 63.0%, 99.3%, and 96.7%, respectively, for the fecal samples and 55.6%, 99.1%, and 98.1%, respectively, for the meat samples.

When the results obtained with CT-SMAC (Table 3.1) and 1/2CT-CHROMagar (Table 3.2) were analyzed separately, a systematic difference between the two immunoconcentration systems was observed only in case of fecal samples examined in combination with 1/2CT-CHROMagar (Binomial test, $P < 0.05$) (Table 3.2). The relative accuracy of the VIDAS ICE method compared with the Dynabeads anti-*E. coli* O157 method was 97.8% when CT-SMAC was used as the final isolation medium (Table 3.1) and 96.7% when 1/2CT-CHROMagar was used (Table 3.2).

Table 3.2 Comparison of the results of the VIDAS ICE with the Dynabeads anti-*E. coli* O157 procedure (reference method) concerning the isolation of Shiga toxin producing *E. coli* O157 from samples of feces and meat onto CHROMagar O157 with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) (1/2CT-CHROMagar)

Sample	Origin	Total no. of samples	IMS[a]/1/2CT-CHROMagar		VIDAS ICE/1/2CT-CHROMagar	
			Positive	Negative	False-negative	" False-positive"[b]
Feces	Petting zoo[c]	95	1	94	0	0
	Petting zoo[d]	73	16	57	4	0
	Frozen[e]	144	17	127	3	1
	Sheep slaughterhouse[f]	325	12	313	12	1
	Total	637	46	591	19	2
Meat	Sheep slaughterhouse[g]	325	7	318	3	1
	Frozen[h]	35	2	33	1	1
	Total	360	9	351	4	2

[a] IMS, immunomagnetic separation with Dynabeads anti-*E. coli* O157.
[b] "False-positive" means in this case true-positive, because the target bacteria are being isolated.
[c] Droppings taken from the ground.
[d] Individual sampling (rectal palpation) living animals.
[e] Diverse origin (droppings and feces from individually sampled animals); all 144 samples were found positive in a previous study, stored at -20°C and after some years examined again.
[f] Rectal contents from sheep, sampled individually at the slaughterline.
[g] Swab of a sheep carcass, sampled individually immediately after entrance in the cooling room.
[h] All 35 samples were found positive in a previous study, stored at -20°C and after some years examined again.

A comparative analysis on overall performance of both methods is presented in Table 3.3. According to the manufacturer's instruction, the VIDAS ICE is only used in conjunction with VIDAS ECO for the confirmation of positive food samples.

Table 3.3 Comparative analyses on overall performance of the manual Dynabeads anti-*E. coli* O157 and VIDAS ICE procedure

Factors considered	Dynabeads anti-*E. coli* O157	VIDAS ICE
Time per 30 samples	~100 min	~40 min
Cost per sample[a]	Relatively low	Relatively high
Chances of cross-contamination	Relatively high	Relatively low
User application	Labor-intensive	User-friendly
In-process manipulation	Possible	Not possible

[a] Cost per sample (materials and reagents) is almost three fold higher in case of VIDAS ICE compared to that of Dynabeads anti-*E. coli* O157.

In the present study, we tested the performance of the VIDAS ICE kit as a detection and/or isolation method, without pre-screening by the VIDAS ECO kit. Instead of a two-step enrichment procedure, we performed a one-step, overnight enrichment in mTSB+n at 41.5°C. Then, the organism was detected and isolated by using 1/2CT-CHROMagar as a second

plating medium. Although the VIDAS system has been developed and validated for the examination of food samples, we also evaluated its performance for animal feces. All the changes that have been introduced in the VIDAS ICE in the present study were either preceding or following the immunoconcentration step implying the robustness of the system on one hand as well as its flexibility on the other. By using the VIDAS ICE, the entire immunoconcentration process is performed automatically within 40 min, for a maximum of 30 samples (including controls) in one run. The concentration of 30 samples with the Dynabeads anti-*E. coli* O157 procedure takes about 100 min (when using two magnetic particle concentrators, fitting six samples each). The Dynabeads procedure consists of several handlings, including a number of washing steps to remove non-(specifically) bound bacteria and matrix components. The risk of cross-contamination is being reduced by the use of filtertips and so-called tube-openers. Alternatively the BeadRetriever (Dynal) can be used which is an automated IMS instrument. With the BeadRetriever 15 samples can be tested within 20 min, not taking into account the time for pipetting the beads and the wash buffer. Advantages of automated systems like the BeadRetriever and the VIDAS are the standardized performance, which does not require any manipulation once the assay has started and the safety of the user.

The international standard for the detection of *E. coli* O157 prescribes the final immunoconcentrates being transferred to CT-SMAC and a second selective agar of own choice (2). In one of our previous studies, CHROMagar O157 turned out to be the best isolation medium (16). Compared with several other media, STEC O157 colonies could be best distinguished from background flora on CHROMagar O157. An additional benefit of the use of chromogenic media is the ability to recognize sorbitol-fermenting STEC O157 strains, while these cannot be distinguished from nonpathogenic *E. coli* on media containing sorbitol. These sorbitol-fermenting strains are increasingly being isolated from patients in Europe and sporadically in Australia (1, 4, 6, 8). A comparison between the performance of CT-SMAC and 1/2CT-CHROMagar was done for a total of 1676 samples: 1316 fecal and 360 meat samples (Table 3.4). In case of feces, systematically more samples were found positive when 1/2CT-CHROMagar was used as the plating medium (McNemar test, $P < 0.05$), irrespective of the method of immunoconcentration carried out. For meat samples, the use of 1/2CT-CHROMagar also resulted in more positives than CT-SMAC. However, this difference between the two plating media was statistically significant only in combination with the Dynabeads anti-*E. coli* O157 (Binomial test, $P < 0.05$) but not with the VIDAS ICE (Binomial test, $P \geq 0.05$). The relative accuracy of 1/2CT-CHROMagar compared with CT-

SMAC was 94.7% and 97.8% for fecal and meat samples, respectively when examined by the IMS procedure. In combination with the VIDAS ICE system, the relative accuracy of 1/2CT-CHROMagar was 97.4% and 98.3% for fecal and meat samples, respectively.

Table 3.4 Comparison of the results obtained with CHROMagar O157 supplemented with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) (1/2CT-CHROMagar) and sorbitol–MacConkey agar supplemented with cefixime (0.05 mg/l) and tellurite (2.5 mg/l) (CT-SMAC) (reference medium) for the selective isolation of Shiga toxin producing *Escherichia coli* (STEC) O157 from fecal and meat samples following immunoconcentration with the Dynabeads anti-*E. coli* O157 or the VIDAS ICE kit

Sample	Origin	Total no. of samples	Isolation method	CT-SMAC		1/2CT-CHROM	
				Pos	Neg	False-neg	"False-pos"[a]
Feces	Miscellaneous[b]	177	IMS[c]	67	110	11	14
	Petting zoo[d]	95 {	IMS	0	95	0	1
			VIDAS ICE	1	94	0	0
		27	IMS	1	26	1	3
		435	VIDAS ICE	6	429	2	12
	Petting zoo[e]	73 {	IMS	15	58	0	1
			VIDAS ICE	14	59	2	0
		15	IMS	2	13	1	0
		25	VIDAS ICE	2	23	0	2
	Frozen[f]	144 {	IMS	11	133	0	6
			VIDAS ICE	9	135	1	7
	Sheep slaughterhouse[g]	325 {	IMS	5	320	0	7
			VIDAS ICE	1	324	1	1
	Total	1316 {	IMS	101	755	13	32
			VIDAS ICE	33	1064	6	22
Meat	Sheep slaughterhouse[h]	325 {	IMS	0	325	0	7
			VIDAS ICE	2	323	1	4
	Frozen[i]	35 {	IMS	1	34	0	1
			VIDAS ICE	1	34	0	1
	Total	360 {	IMS	1	359	0	8
			VIDAS ICE	3	357	1	5

[a] "False-positive" means in this case true-positive, because the target bacteria are being isolated.

[b] Diverse origin (droppings and feces from individually sampled animals); collected because of notification of STEC O157 infection in humans.

[c] IMS, immunomagnetic separation with Dynabeads anti-*E. coli* O157.

[d] Droppings taken from the ground.

[e] Individual sampling (rectal palpation) living animals.

[f] Diverse origin (droppings and feces from individually sampled animals); all 144 samples were found positive in a previous study, stored at -20°C and after some years examined again.

[g] Rectal contents from sheep, sampled individually at the slaughterline.

[h] Swab of a sheep carcass, sampled individually immediately after entrance in the cooling room.

[i] All 35 samples were found positive in a previous study, stored at -20°C and after some years examined again.

In conclusion, for safety of the user and a saving of time, it is recommended to prefer the VIDAS ICE method to the manual Dynabeads anti-*E. coli* O157 procedure for meat samples.

To our knowledge, this is the first effort so far to use the VIDAS ICE system as a diagnostic method for routine isolation of *E. coli* O157 from the animal fecal sample. But, it can be a good choice only in case of prevalence studies at the farm level, where testing of more than one sample from the same origin (same farm, same herd etc.) is a prerequisite. Furthermore, it is recommended to use 1/2CT-CHROMagar as a standard isolation medium in addition to CT-SMAC, that currently counts as the golden standard according to the ISO 16654:2001 (E) (2), following immunoconcentration with both the Dynabeads anti-*E. coli* O157 and the VIDAS ICE kit.

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Chapter 4

Evaluation of immunomagnetic separation and PCR for the detection of Escherichia coli *O157 in animal feces and meats*

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Abstract

Series of animal feces and meat samples artificially contaminated with strains of *Escherichia coli* O157 isolated from different sources were tested by both an immunomagnetic separation (IMS)-based method and a PCR method using primers specific for a portion of the *rfb*E gene of *E. coli* O157 ($rfbE_{O157}$). IMS is laborious and time consuming but ends up with the isolation of the pathogen. PCR is fast and less laborious, but it can only be used for screening purposes, so a further culture step is required to isolate the organism. For both fecal and meat samples, the IMS method was found to be more sensitive than the PCR. Furthermore, the detection efficiency of the PCR was influenced by the origin of the fecal sample and the type of meat. For sheep feces, the efficiency of the PCR appeared to be systematically lower than for cattle feces. And the efficiency of the PCR in detecting *E. coli* O157 in spiked samples of raw minced beef and dry-fermented sausages was systematically lower than in samples of *filet americain*. Based on this study, it can be concluded that both for animal feces and meat, IMS can be used more successfully to detect *E. coli* O157 than PCR, because IMS showed to be more sensitive and the outcome was not influenced by the type of animal feces or meat.

4.1 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are pathogens capable of causing sporadic and epidemic infections in humans. Symptoms of STEC infections range from mild diarrhea to the life-threatening hemolytic-uremic syndrome (17, 21, 25). STEC have zoonotic potential and a wide range of animals have been recognized as a transient reservoir for these pathogens (7). *E. coli* O157:H7 is still by far the most important serotype of STEC due to its high virulence profile and severe manifestation of illness. Contamination of meat with animal feces during slaughtering is one of the major routes of transmission of *E. coli* O157 into the human food chain (10, 13).

With the increase in reports of *E. coli* O157 cases of human infection, greater attention has been given to develop methods for detecting this pathogen, including cultural, immunological, and molecular methods. Traditional culture methods for the detection of *E. coli* O157 are time consuming, taking approximately three to four days and are often inefficient due to the large numbers of other flora that either overgrow or mimic *E. coli* O157 colonies (9, 22). The sensitivity of culture-based methods has significantly been increased by the application of an immunomagnetic separation (IMS) step (12), which is also adopted by the International Organization for Standardization for the detection of *E. coli* O157 in food and animal feeding stuffs (5). Prior to plating onto selective isolation media, *E. coli* O157 cells present in the enrichment culture are selectively concentrated by magnetic beads with *E. coli* O157–specific antibodies covalently bound onto their surface.

Although IMS offers significant advantages in the detection and isolation of *E. coli* O157, the successful application of this culture-based method is still dependent on the expression of the physiological characteristics and/or metabolic activities of the bacterium (26). One of the difficulties can be that bacteria suffer damage during food processing and preservation, which can inhibit expression of the phenotypic characteristics essential for their growth and ultimate detection (27).

PCR-based methods are considered as a good alternative because the presence of small amounts of species- or strain-specific DNA is being detected rather than unique aspects of target organisms to be in a specific physiological state. Recently, the O157 O antigen gene cluster has been sequenced containing certain genes highly specific to *E. coli* O157 (29) and a diagnostic PCR assay has been developed based on the amplification of sequences of the

rfbE_{O157} gene (2). This PCR assay was validated toward an international standard, by means of a multicenter validation study (1).

In the present study, we evaluated the performance of an IMS-based method and the PCR assay targeting the *rfb*E_{O157} gene by testing series of animal feces and meat samples artificially contaminated with strains of *E. coli* O157 isolated from different sources.

4.2 Materials and Methods

4.2.1 Test strains and samples

Seven *E. coli* O157 strains were used as follows: one isolate from cattle feces, one from minced beef, one from sheep feces, one from lamb meat, and three clinical isolates, including NCTC 12900. As negative control strain, we used the *E. coli* non-O157 strain ATCC 25922. With the exception of the two strains of the official culture collections, all of the strains were from our own collection (VWA, Zutphen, the Netherlands).

For the spiking experiments, we collected five samples of cattle feces and three samples of sheep feces on two different farms in The Netherlands, ruminants being the main reservoir of STEC O157. In addition, we purchased three samples of minced beef, two samples of dry-fermented sausages, and two samples of *filet americain* (minced beef with added spices and mayonnaise) from retail sellers. All of these types of meat products previously have been linked with human infection.

4.2.2. Artificial contamination

The complete scheme of the artificial contamination and detection strategy has been described in Fig. 4.1. The test strains were cultured in brain heart infusion broth (Oxoid Ltd., Basingstoke, United Kingdom) at 37°C for 18 to 20 h. Except for the negative control strain ATCC 25922, 10-fold serial dilutions of each of the overnight broth cultures were made in 0.1% peptone water. For each of the seven *E. coli* O157 strains one replicate of sample (animal feces or meat) was divided into five portions of 10 g each. Each portion was mixed with 90 ml of modified tryptone soy broth (Oxoid) supplemented with novobiocin (20 mg/l) (Sigma Chemical Co., St. Louis, Mo.). After homogenization in a stomacher for 1 min (eight strokes per s), each portion was inoculated with 1 ml of each dilution of the test strains ranging from dilution 10^{-5} to 10^{-9}. For every replicate of a sample, two more suspensions were prepared as described above; one was inoculated with 1 ml of overnight culture of the

negative control strain ATCC 25922 and the other served as blank. All of the samples were incubated at 41.5±0.5°C for 8 h and subsequently examined for the presence of *E. coli* O157 by performing IMS and PCR. The actual inoculation levels were determined by spread plating 0.1 ml of the serial dilutions onto tryptone soy agar (Oxoid). The plates were incubated at 37°C for 18 to 24 h.

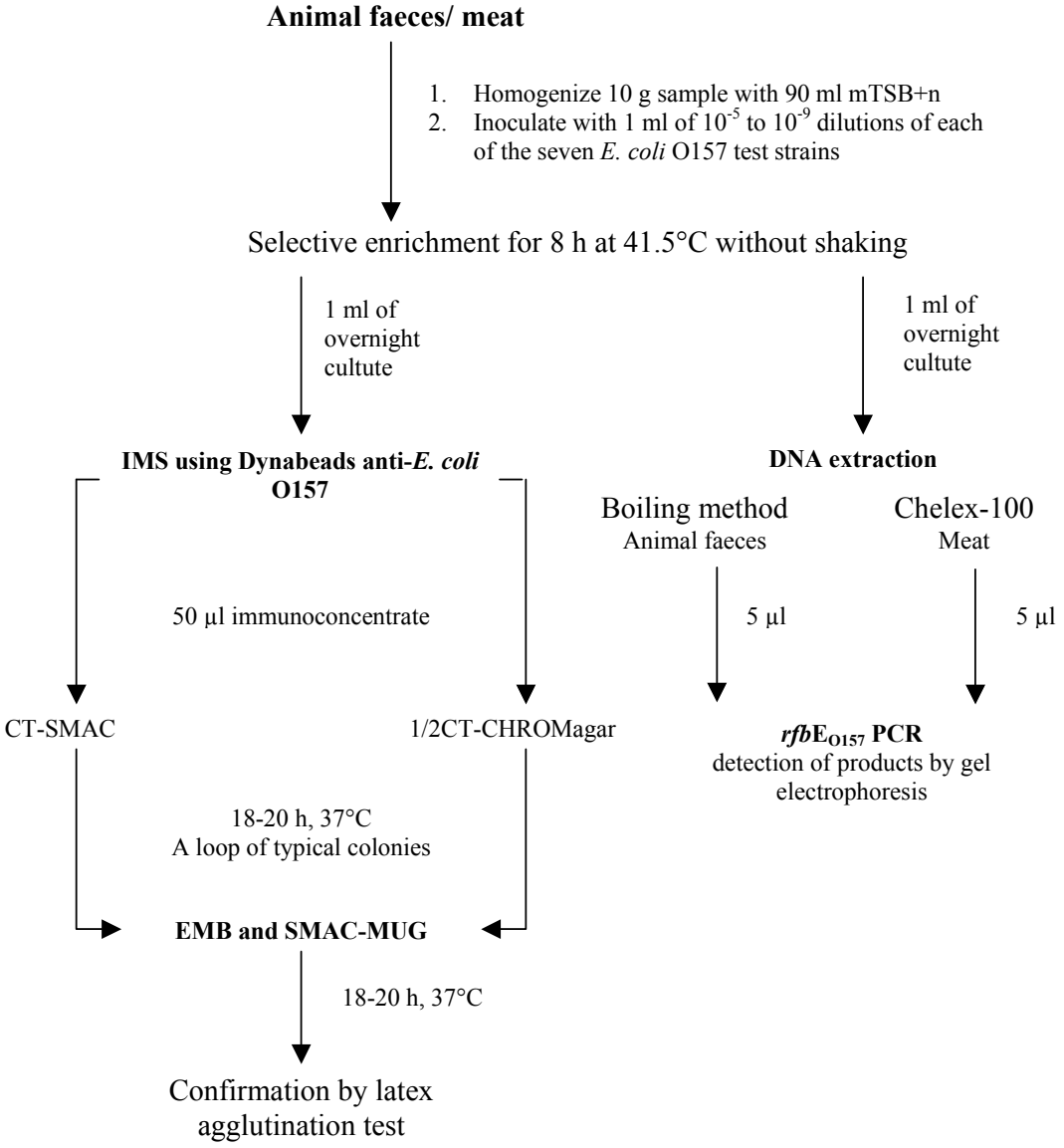

Fig. 4.1 Schematic flow diagram of the experimental protocol used for comparing the efficiency of IMS and PCR to detect *E. coli* O157 in artificially contaminated animal feces and meats.

4.2.3 Immunomagnetic separation

After 8 h incubation, IMS with Dynabeads anti-*E. coli* O157 (Dynal, Oslo, Norway) was performed using 1 ml aliquot of each enriched culture and following the instructions of the manufacturer. The concentrated samples were inoculated onto sorbitol–MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg/l) and tellurite (2.5 mg/l) (Oxoid) (CT-SMAC) and CHROMagar O157 (ITK Diagnostics BV, Uithoorn, the Netherlands) with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) (1/2CT-CHROMagar), 50 µl onto each plate. The plates were incubated at 37°C for 18 to 20 h. From each plate, up to 8 typical colonies (non-sorbitol-fermenting colonies on CT-SMAC; β-glucuronidase-negative colonies on 1/2CT-CHROMagar) were selected and inoculated onto eosine methylene blue (Oxoid) and sorbitol-MacConkey agar supplemented with 4-methylumbelliferyl-β-D-glucuronide (SMAC-MUG). Typical colonies with green metallic shine on eosine methylene blue and fluorescence-negative colonies on SMAC-MUG were then subjected to agglutination using an *E. coli* O157 latex agglutination kit (Murex Biotech Ltd., Dartford, Kent, United Kingdom).

4.2.4 DNA extraction

Based on the results of preliminary experiments comparing different methods of DNA extraction, we selected a simple boiling method to extract DNA from the enrichment cultures of animal feces and thermal cell lysis using Chelex-100 resin (Bio-Rad Laboratories Ltd., Richmond, Calif.) to extract DNA from the enrichment cultures of meat samples. In case of the boiling method, 1 ml of enrichment culture was centrifuged at 14,000× *g* for 5 min and the pellet was washed with 1 ml of fresh modified tryptic soy broth (14,000× *g* for 5 min). After discarding the supernatant, the pellet was resuspended in 200 µl of milliQ water, boiled for 10 min, and then immediately put on ice. The suspension was centrifuged (14,000× *g,* 10 min at 4°C) and 5 µl of the supernatant was used in the PCR. In case of the Chelex-100 method, we also used 1 ml of the enrichment cultures and followed the procedure described by Malorny et al. (20).

4.2.5 PCR assay

The *E. coli* O157-specific PCR assay evaluated is based on amplification of sequences of the *rfb*E_{O157} gene. The specific oligonucleotide primers GiO157-I and GiO157-II have been developed by Abdulmawjood et al. (2). For each sample, 25 µl of reaction mix was prepared containing 0.2 µl of each primer (50 pmol/µl), 0.5 µl of each deoxynucleoside triphosphate (10 mM) (Roche Diagnostics, Mannheim, Germany), 2.5 µl of 10 Fast Start buffer (Roche

Diagnostics), 1.5 µl of $MgCl_2$ (25 mM) (Roche Diagnostics), 1 µl (5.23 fg) of *rfb*E_{O157} internal amplification control (3), 0.2 µl of Fast Start *Taq* DNA polymerase (5 U/µl) (Roche Diagnostics), 2.5 µl of 10 mg/ml bovine serum albumin (BSA) fraction V, and 11.4 µl of molecular biology grade water. Finally, 5 µl of template DNA solution was added to each reaction mix. The PCR was carried out in a thermal cycler (Eppendorf Mastercycler Gradient, VWR, Amsterdam, The Netherlands) with the following program: denaturation at 94°C for 5 min, 30 cycles of 15 s at 94°C, 15 s at 60°C, and 30 s at 72°C, followed by a final extension incubation of 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in a 1.5% agarose gel with Tris-borate EDTA buffer. A 100-bp DNA ladder marker XIV was used as molecular marker (Roche Diagnostics).

4.2.6 Statistical analysis

To calculate whether the outcome (i.e., the total number of positive replicates of animal feces or meat divided by the total number of replicates of animal feces or meat examined, respectively) of the IMS and the PCR method were statistically significantly different, we performed the Fisher's exact test (double-sided). Furthermore, we analyzed whether the outcome of the IMS and the PCR method were influenced by the origin of the fecal sample or the type of meat by the Fisher's exact test (double-sided).

4.3 Results

For the detection of *E. coli* O157 in both animal feces and meat, IMS proved to be more sensitive than the PCR (Table 4.1). For example, when 10^1 CFU of the seven strains were inoculated per 10 g of animal feces, the organisms were recovered from 95% of the replicates of both cattle and sheep feces by using IMS and from only 33 and 5%, respectively, by using the PCR-based method (Table 4.1). The lowest level of inoculation at which at least 95% of the replicates of cattle and sheep feces were found positive by PCR was 10^3 CFU of *E. coli* O157 per 10 g of sample (Table 4.1). Whereas the outcome of the IMS was not influenced by the origin of the fecal samples, the detection efficiency of the PCR was systematically lower for sheep feces than for cattle feces (Table 4.1).

When 10^1 CFU of any of the seven strains were inoculated per 10 g of meat, the organisms were recovered from 100% of the replicates of both minced beef and dry-

fermented sausages by using IMS and from only 74 and 57%, respectively, by using the PCR (Table 4.1). The lowest level of inoculation at which all sample replicates of minced beef and dry-fermented sausages were detected as positive by the PCR-based method was 10^2 CFU per 10 g (Table 4.1). Although the overall result for meat showed the IMS method to be clearly more sensitive than the PCR, the methods showed similar detection efficiency when the *E. coli* O157 test strains were inoculated into *filet americain,* with all sample replicates detected as positive by both methods at the inoculation level of 10^1 CFU per 10 g (Table 4.1). Again, the outcome of IMS was not influenced by the type of meat, whereas the detection efficiency of the PCR was systematically lower for raw minced beef and dry-fermented sausages than for *filet americain* (Table 4.1). None of the samples of animal feces nor meat was found positive either by using IMS or the PCR when spiked with less than 10^0 CFU of organism per 10 g sample (Table 4.1). Results obtained for the uninoculated portions of animal feces and meat and the portions spiked with the negative control strain were all negative.

4.4 Discussion

With the increased interest in the occurrence of STEC O157, the demand for more reliable and easy detection methods for diversified sources has been intensified. To date, IMS and PCR have been evaluated as the most effective methods for routine diagnosis of *E. coli* O157 from a variety of sources (11, 12). IMS is laborious and time consuming but ends up with the isolation of the pathogen. On the other hand, PCR is fast and less laborious but it can only be used for screening purposes, so a further culture step is required to isolate the organism. The objective of this study was to evaluate the performance of IMS and PCR in detecting *E. coli* O157 in animal feces and meats. Although IMS is considered as the official standard method for detection and isolation of *E. coli* O157 from food and animal feeding stuffs (5), no standardized protocol has yet been described for animal feces.

For a PCR-based method to be completely suitable for implementation as an analytical tool, its diagnostic accuracy must be thoroughly evaluated and demonstrated (15, 20). The sensitivity of PCR detection systems depends on the choice of reaction conditions and interactions with the particular nature of the sample matrix (26). PCR is inhibited by numerous compounds often found in feces and food (6), providing a practical hindrance to the molecular-based detection of small numbers of contaminating pathogens (4, 23).

Table 4.1 Detection of *E. coli* O157 in artificially contaminated samples of animal feces and meat by using IMS and PCR

Samples	No. of positives/total no. of replicates (%)[a]					
	Detection method	10^3 CFU/10 g	10^2 CFU/10 g	10^1 CFU/10 g	10^0 CFU/10 g	<10^0 CFU/10 g	Total
Cattle feces (n = 3)	IMS	21/21 (100)	20/21 (95)	20/21 (95)	2/21 (10)	0/21 (0)	53/105 (60)
	PCR	21/21 (100)	14/21 (67)	7/21 (33)	0/21 (0)	0/21 (0)	42/105 (40)[b]
Sheep feces (n = 3)	IMS	21/21 (100)	21/21 (100)	20/21 (95)	1/21 (5)	0/21 (0)	63/105 (60)
	PCR	21/21 (100)	4/21 (19)	1/21 (5)	0/21 (0)	0/21 (0)	26/105 (25)[b]
Total animal feces	IMS	42/42 (100)	41/42 (98)	40/42 (95)	3/42 (7)	0/42 (0)	126/210 (60)[c]
	PCR	42/42 (100)	18/42 (43)	8/42 (19)	0/42 (0)	0/42 (0)	68/210 (32)[c]
Raw minced beef (n = 5)	IMS	35/35 (100)	35/35 (100)	35/35 (100)	14/35 (40)	0/35 (0)	119/175 (68)
	PCR	35/35 (100)	35/35 (100)	35/35 (100)	8/35 (23)	0/35 (0)	104/175 (60)[d]
Dry-fermented sausages (n = 2)	IMS	14/14 (100)	14/14 (100)	14/14 (100)	12/14 (86)	0/14 (0)	54/70 (77)
	PCR	14/14 (100)	14/14 (100)	8/14 (57)	4/14 (29)	0/14 (0)	40/70 (57)[e]
Filet americain (n = 2)	IMS	14/14 (100)	14/14 (100)	14/14 (100)	11/14 (79)	0/14 (0)	53/70 (76)
	PCR	14/14 (100)	14/14 (100)	14/14 (100)	11/14 (79)	0/14 (0)	53/70 (76)[d,e]
Total meat	IMS	63/63 (100)	63/63 (100)	63/63 (100)	37/63 (59)	0/63 (0)	226/315 (72)[f]
	PCR	63/63 (100)	63/63 (100)	48/63 (76)	23/63 (37)	0/63 (0)	197/315 (63)[f]

[a] Each column shows the sum of the results obtained for the seven *E. coli* O157 test strains used for spiking the sample replicates.
[b] Statistically significantly different (P = 0.027) according to the Fisher's exact test (double-sided).
[c] Statistically significantly different (P = 0.000) according to the Fisher's exact test (double-sided).
[d] Statistically significantly different (P = 0.018) according to the Fisher's exact test (double-sided).
[e] Statistically significantly different (P = 0.031) according to the Fisher's exact test (double-sided).
[f] Statistically significantly different (P = 0.017) according to the Fisher's exact test (double-sided).

One way to address these limitations is the use of effective sample processing as well as DNA extraction techniques, the area where many of the efforts have been put into (14, 16, 19). Nevertheless, none of these techniques is ideal, and in many cases a technique optimized for one matrix or microorganism is not readily adaptable for use with others. Moreover, because purification adds to the time and expense of sample preparation, as well as to the loss of target nucleic acids, a more satisfying approach to the problem of PCR inhibition would be to reduce interference rather than attempt to remove all of the offending substances. To this end, various additives have been included in the PCR mixture to minimize inhibition. Among these, BSA is widely being used (18). We also found that BSA at the concentration of 1 mg/ml in the PCR mixture significantly reduced the effects of inhibitory substances present in both feces and meats. This was confirmed by using an internal amplification control in the PCRs (results not shown). The use of an internal amplification control in the PCR can detect the level of reaction inhibition and therefore eliminates the chances of false-negative results (1).

Preliminary experiments comparing different techniques of DNA extraction showed a simple boiling method to be the most effective for animal feces and thermal lysis using Chelex-100 resin for meats. Once the DNA extraction method and PCR conditions were optimized, the recovery of the *E. coli* O157 test strains by the PCR was compared with that of the IMS method. IMS was found to be more effective than the PCR for both samples of animal feces and meat. Hence, use of PCR alone in the routine diagnosis of *E. coli* O157 in animal feces and meats might result in a lower number of positives. Furthermore, the detection efficiency of the PCR was dependent on the origin of the fecal samples. According to a previous study, sheep feces were found to possess more substances inhibitory to PCR than feces from other animals, including cattle (18). The detection efficiency of the IMS procedure was not affected by the origin of the fecal sample used for spiking. Like the results of the spiking experiments with feces, for meat samples it was observed that the detection efficiency of the IMS was not influenced by the type of meat whereas the detection efficiency of the PCR was. Based on the higher detection efficiency of the PCR for samples of *filet americain* and IMS and PCR showing the same sensitivity, *filet americain* seems to contain less PCR-inhibitory substances than raw minced beef and dry-fermented sausages.

One of the set backs of IMS is that it is sometimes insensitive due to the overgrowth of background flora, which can mimic the characteristics of target organisms (8). This can happen particularly for those samples that contain a high load of indigenous flora, for example, animal feces. But this limitation was overridden in this study by means of a

selective enrichment step preceding IMS and by using a second selective plating medium (1/2CT-CHROMagar) in addition to CT-SMAC.

The concentration at which *E. coli* O157 is shed in feces varies from animal to animal (24), where a range from 10^2 to 10^5 CFU/g was observed. An even wider range of fecal shedding of *E. coli* O157 in naturally infected cattle was found in another study (30). Animals excreting $>10^4$ CFU/g of *E. coli* O157 are normally considered as high shedding and those excreting $<10^2$ CFU/g are low shedding. In order to understand the epidemiology of *E. coli* O157 infection in cattle and to inform quantitative microbial risk assessments, detailed quantitative information on the patterns and concentration of organisms shed by cattle into the environment is required. Although animals can carry *E. coli* O157 in high numbers, only a few organisms can already give rise to human infection. This accentuates the need for enforcing zero tolerance of this organism in foods and for markedly decreasing the risk of contamination of raw meat (10, 28). Therefore, the demand of a sensitive and reliable detection and isolation technique is obvious. According to this study, both for animal feces and meat IMS can be used more successfully to detect *E. coli* O157 than PCR, being more sensitive, not influenced by the type of animal feces or meat, and resulting in the isolation of the pathogen.

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Chapter 5

Prevalence and genetic characterization of Shiga toxin-producing Escherichia coli *isolated from slaughtered animals in Bangladesh*

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Abstract

To determine the prevalence of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) in slaughter animals in Dhaka, Bangladesh, we collected rectal contents immediately after animals were slaughtered. Of the samples collected from buffalo (n = 174), cows (n = 139), and goats (n = 110), 82.2%, 72.7%, and 11.8% tested positive for stx_1 and/or stx_2, respectively. STEC could be isolated from 37.9%, 20.1%, and 10.0% of the buffalo, cows, and goats, respectively. STEC O157 strains were isolated from 14.4% of the buffalo, 7.2% of the cows, and 9.1% of the goats. More than 93% (n = 42) of the STEC O157 isolates were positive for the stx_2, *eae*, *katP*, *etpD*, and enterohemorrhagic *E. coli hly* (hly_{EHEC}) virulence genes. STEC O157 isolates were characterized by seven recognized phage types, of which types 14 (24.4%) and 31 (24.4%) were predominant. Subtyping of the 45 STEC O157 isolates by pulsed-field gel electrophoresis showed 37 distinct restriction patterns, suggesting a heterogeneous clonal diversity. In addition to STEC O157, 71 STEC non-O157 strains were isolated from 60 *stx*-positive samples from 23.6% of the buffalo, 12.9% of the cows, and 0.9% of the goats. The STEC non-O157 isolates belonged to 36 different O groups and 52 O:H serotypes. Unlike STEC O157, most of the STEC non-O157 isolates (78.9%) were positive for stx_1. Only 7.0% (n = 5) of the isolates were positive for hly_{EHEC}, and none was positive for *eae*, *katP*, and *etpD*. None of the isolates was positive for the *iha*, *toxB*, and *efa1* putative adhesion genes. However, 35.2% (n = 25), 11.3% (n = 8), 12.7% (n = 9), and 12.7% (n = 9) of the isolates were positive for the lpf_{O113}, *saa*, $lpfA_{O157/01-141}$, and $lpfA_{O157/OI-154}$ genes, respectively. The results of this study provide the first evidence that slaughtered animals like buffalo, cows, and goats in Bangladesh are reservoirs for STEC, including the potentially virulent STEC strain O157.

5.1 Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) organisms, also called verocytotoxin (VT)-producing *E. coli* (VTEC), are one of the most important groups of foodborne pathogens (2, 32). Infection can cause gastroenteritis that may be complicated by hemorrhagic colitis or the hemolytic-uremic syndrome (HUS), which is the main cause of acute renal failure in children. STEC strains causing human infections belong to a large, still-increasing number of O:H serotypes. Most outbreaks and sporadic cases of hemorrhagic colitis and HUS have been attributed to the STEC O157 strains (39). However, infections caused by some non-O157 serotypes have also been frequently associated with severe illness in humans. In some geographic areas, STEC non-O157 strains are more commonly isolated from persons with diarrhea or HUS than STEC O157 strains (33).

STEC are defined by the production of one or more types of Shiga toxin (Stx1 or Stx2 or their variants), which inhibit the protein synthesis of host cells, leading to cell death. Stx1 and Stx2 are encoded by alleles in the genome of temperate, lambdoid bacteriophages that are integrated in the *E. coli* chromosome (38). Besides the *stx* gene(s), human pathogenic STEC strains often carry the *eae* gene, encoding the adherence factor intimin, which is an outer membrane protein (32). The *eae* gene is carried by a pathogenicity island in the chromosome called the locus of enterocyte effacement (LEE), which is required for intimate attachment to the host intestinal mucosa (32). Furthermore, human pathogenic STEC strains often harbor a large plasmid encoding possible additional virulence traits such as the enterohemorrhagic *E. coli* (EHEC) hemolysin (hly_{EHEC}) gene, which acts as a pore-forming cytolysin on eukaryotic cells (36); the bifunctional catalase peroxidase (*katP*) gene (7); a secreted serine protease (*espP*) gene, which can cleave human coagulation factor V (8); and the *etpD* gene cluster, which probably encodes a type II secretion pathway (37). With more LEE-negative STEC strains being reported, investigations of adhesins encoded outside of the LEE have been carried out (40). Several proteins were proposed to be novel adhesion factors; these include ToxB (a protein identified from the large, 93-kb plasmid pO157 and required for full expression of adherence of O157:H7 strain Sakai), Saa (an autoagglutinating adhesin identified in the LEE-negative strains), Iha (an adherence-conferring protein similar to the *Vibrio cholerae* IrgA protein), Efa1 (an EHEC factor for adherence), and LPF (the long polar fimbriae protein, closely related to LPF of *Salmonella enterica* serovar Typhimurium) (40).

Domestic ruminants, mainly cattle, sheep, and goats, have been established as major natural reservoirs for STEC and play a significant role in the epidemiology of human infections (16). During the processing of the carcasses, fecal contamination or transfer of bacteria from the animal's hide to the carcass can facilitate transmission of pathogenic *E. coli* to the meat (15).

In most of the developing and underdeveloped countries, hygienic conditions are severely compromised, and living with domestic animals within the same premises is a common practice in both rural and urban areas. Although the socioeconomic status and living style of the people in developing countries support the prevalence of STEC infection, surprisingly, until recently, only a few studies have been done in this part of the world. Human infections associated with STEC strains have been described in Latin America, India, and other developing countries (24, 26). In Bangladesh, the predominant group of *E. coli* associated with childhood diarrhea is enterotoxigenic *E. coli*, accounting for approximately 20% of all diarrheal cases (34). Recently, we determined the prevalence of STEC and the organisms' characteristics among hospitalized diarrheal patients and children with diarrhea in the urban slum community of Dhaka, Bangladesh. The prevalence of STEC was 0.5% among the hospitalized patients, which was 1.9% of the cases of community patients. No STEC O157 organisms were isolated in the study (23). This result is comparable with data from other developing countries. The low prevalence of STEC compared with other enteric pathogens in diarrheal patients can be explained by different facts, the first of which is the absence of any potential reservoir for this organism. Limited studies of the ecology of STEC have been reported, particularly from developing countries. In Bangladesh, no study of the occurrence of STEC in animals had been done. Therefore, the primary objective of the present study was to determine the prevalence of STEC in feces collected from buffalo, cows, and goats slaughtered for meat production.

5.2 Materials and methods

5.2.1 Sampling

In the period from 1 January to 1 May 2006, we collected fecal samples from 174 buffalo, 139 cows, and 110 goats at a slaughterhouse in Dhaka of Mohakhali area in Bangladesh. The animals sampled were randomly selected. The origin of the animals could not be recorded due to the lack of information. However, according to the local information, animals were

received from different places across the country. Immediately after slaughter, a piece of large intestine containing fecal material (~5 cm), 1 to 1.5 cm away from the rectum was excised aseptically and kept at 4 to 8°C. The samples were transported immediately to the laboratory, where the microbiological examination was started within 8 h.

5.2.2 Isolation of STEC O157

From each sample, a 25-g portion of feces was collected from a piece of intestine (~5 cm), which was aseptically excised from the animal immediately after slaughter and added to 225 ml of modified tryptone soy broth (Oxoid Ltd., Basingstoke, United Kingdom). After homogenization in a stomacher for 1 min, the samples were incubated for 18-20 h at 37°C. STEC O157 organisms were isolated as described previously (22), by using the immunomagnetic separation (IMS) technique and presumptive isolates were confirmed by an agglutination test using *E. coli* O157 latex agglutination kit (Murex Biotech Ltd., Dartford, Kent, United Kingdom) and a PCR for the *rfb* (O-antigen-encoding) region of *E. coli* O157 (*rfb*E_{O157}) (31).

5.2.3 Isolation of STEC non-O157

The overnight enrichment cultures were screened by PCR for the presence of stx_1 and stx_2 genes. DNA was extracted from 1 ml of broth culture by thermal cell lysis using Chelex-100 resin (Bio-Rad Laboratories Ltd., Richmond, Calif.) and 5 µl of the DNA extract was used in the PCR. Multiplex PCR for stx_1 and stx_2 was carried out in a PTC-200 peltier thermal cycler (Bio-Rad) as described previously (23). Broth cultures that yielded positive PCR results for stx_1 and/or stx_2 were streaked onto tryptone bile x-glucuronide (TBX) agar (Oxoid) supplemented with cefixime (20 µg/l), cefsulodin (3 mg/l), and vancomycin (30 mg/l) (CCV-TBX). STEC colonies were identified by a colony patch technique described previously (23).

5.2.4 Serotyping

All isolates were sent to the National Institute of Public Health and the Environment (RIVM) in Bilthoven, The Netherlands, for O and H typing.

5.2.5 Phage typing

Isolates confirmed to be STEC O157 were phage typed at the laboratory of Enteric Pathogens, Central Public Health Laboratory, London, United Kingdom.

5.2.6 PCR detection of putative virulence and adhesin genes

Isolates were tested for both chromosomally (e.g., *stx*$_1$, *stx*$_2$, and *eae*)- and plasmid (e.g., *hly*$_{EHEC}$, *katP*, and *etpD*)-encoded virulence genes by PCR according to the procedure described earlier (23). The presence of eight putative adhesin genes in STEC non-O157 strains (*iha*, *toxB*, *saa*, *efa*1, *lpfA*$_{O113}$, *lpfA*$_{O157/OI-141}$, and *lpfA*$_{O157/OI-154}$) was tested using the primer sets and reaction conditions as described by Toma et al. (40).

5.2.7 PFGE

All STEC O157 and STEC non-O157 isolates that belonged to the same serotype were selected to be analyzed by PFGE. PFGE was performed following the standardized protocol developed by PulseNet for *E. coli* O157:H7 (35). Analysis of the TIFF images was carried out by the BioNumerics software (Applied Maths, Belgium) using the dice coefficient and unweighted-pair group method using average linkages to generate dendrograms with 1.0% tolerance values.

5.2.8 Statistical methods

The chi-square test with Yates' correction or, if necessary, Fisher's exact test, was used as indicated to compare the test results. Statistical testing was performed using Epi Info version 3.3.2 on a standard personal computer. Statistically, P values < 0.05 were considered significant for comparisons.

5.3 Results

5.3.1 Prevalence of STEC

Of the fecal samples collected from buffalo (n = 174), cows (n = 139), and goats (n = 110), 82.2% (n = 143), 72.7% (n = 101), and 11.8% (n = 13) tested positive for *stx*$_1$ and/or *stx*$_2$, respectively. Higher numbers of samples from buffalo (66.7%; n = 116) and cows (45.3%; n = 63) were positive for both the *stx*$_1$ and the *stx*$_2$ genes than were positive for only the *stx*$_1$ or the *stx*$_2$ gene ($P < 0.001$). Only *stx*$_1$ was positively identified in 8.6% (n = 15) buffalo, 12.9% (n = 18) cow and 3.6% (n = 4) of goat samples. Only *stx*$_2$ was positively identified in 6.9% (n = 12), 14.4% (n = 20) and 7.3% (n = 8) of buffalo, cow and goat samples, respectively. STEC could be isolated from 37.9% (n = 66), 20.1% (n = 28) and 10.0% (n = 11) of the buffalo, cow and goat samples, respectively. Significantly higher

numbers of buffalo samples were positive for STEC than cow and goat samples ($P < 0.001$). STEC O157 strains were isolated from 14.4% ($n = 25$) of the buffalo, 7.2% ($n = 10$) of the cows, and 9.1% ($n = 10$) of the goats. STEC non-O157 strains were isolated from 23.6% ($n = 41$) of the buffalo, 12.9% ($n = 18$) of the cows, and 0.9% ($n = 1$) of the goats. Sometimes more than one serotype was isolated from a single sample, so the number of samples with STEC non-O157 isolates was 41 (49 different isolates from 41 *stx*-positive samples) for buffalo and 18 (21 different isolates from 18 *stx*-positive samples) for cows.

5.3.2 Characterization of STEC O157

The results of the PCR assays for different virulence genes are presented in Table 5.1. Among 45 STEC O157 isolates, seven phage types (PT) were identified. Ten isolates from buffalo could not be characterized by any recognized PT, five of which were identified as untypeable and the remaining were reacts but not confirmed. Digestion of genomic DNA from the 45 isolates with *Xba*I and analysis by contour-clamped homogenous electric field (CHEF)-PFGE revealed 37 distinct restriction profiles (Fig. 5.1).

5.3.3 Characterization of STEC non-O157

The results of the PCR assays for different virulence and (putative) adhesin genes are presented in Table 5.1. None of the isolates was positive for putative virulence and adhesion genes, including *eae*, *katP*, *etpD*, *iha*, *tox*B, and *efa*1 (results not shown). More than 30% ($n = 15$) of the isolates from buffalo and 47% ($n = 10$) of the isolates from cows were positive for the lpf_{O113} gene. The *saa* gene was present in 8.1% ($n = 4$) and 19.0% ($n = 4$) of the isolates from buffalo and cows, respectively. Seven isolates from buffalo and two isolates from cows were positive for both the $lpfA_{O157/01\text{-}141}$ and $lpfA_{O157/OI\text{-}154}$ genes. The goat isolate was negative for all adhesin genes.

Serotyping of the STEC non-O157 isolates revealed that the isolates belonged to 36 different O-groups (grouping isolates with an untypeable O serogroup, as serogroup ONT) and 52 O:H serotypes (Table 5.2). Isolates of different serotypes were found in the same sample; at the same time, isolates of the same serotype were found in different samples of same/different origins (Table 5.2). Isolates belonging to the same serotype isolated from same/different samples were analyzed by PFGE, which showed a clonal relationship among different isolates of the same serotype (results not shown). However, polymorphism was also observed between isolates of the same serotype.

Table 5.1 Virulence and (putative) adhesin gene typing of Shiga toxin-producing *E. coli* O157 and non-O157 isolates from fecal samples of slaughter animals

Source (*n*)	No. of isolates positive for (%)[a]:										
	Virulence genes							Adhesin genes			
	stx$_1$	*stx*$_2$	*stx*$_1$ and *stx*$_2$	*eae*	*katP*	*etpD*	*hly*$_{EHEC}$	*Lpf*$_{O113}$	*saa*	*LpfA*$_{O157/01-141}$	*LpfA*$_{O157/OI-154}$
STEC O157 isolates											
Buffalo (25)	3 (12)	22 (88)	0 (0)	23 (92)	22 (88)	22 (88)	22 (88)	ND	ND	ND	ND
Cow (10)	0 (0)	9 (90)	1 (10)	10 (100)	10 (100)	10 (100)	10 (100)	ND	ND	ND	ND
Goat (10)	0 (0)	10 (100)	0 (0)	10 (100)	10 (100)	10 (100)	10 (100)	ND	ND	ND	ND
Total (45)	3 (6.7)	41 (91.1)	1 (2.2)	43 (95.5)	42 (93.3)	42 (93.3)	42 (93.3)	ND	ND	ND	ND
STEC non-O157 isolates											
Buffalo (49)	41 (83.7)	4 (8.2)	4 (8.2)	0 (0)	0 (0)	0 (0)	3 (6.1)	15 (30.6)	4 (8.1)	7 (14.3)	7 (14.3)
Cow (21)	15 (71.4)	4 (19.0)	2 (9.5)	0 (0)	0 (0)	0 (0)	2 (9.5)	10 (47.6)	4 (19.0)	2 (9.5)	2 (9.5)
Goat (1)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total (71)	56 (78.9)	9 (12.7)	6 (8.4)	0 (0)	0 (0)	0 (0)	5 (7.0)	25 (35.2)	8 (11.3)	9 (12.7)	9 (12.7)

[a] ND, Not done.

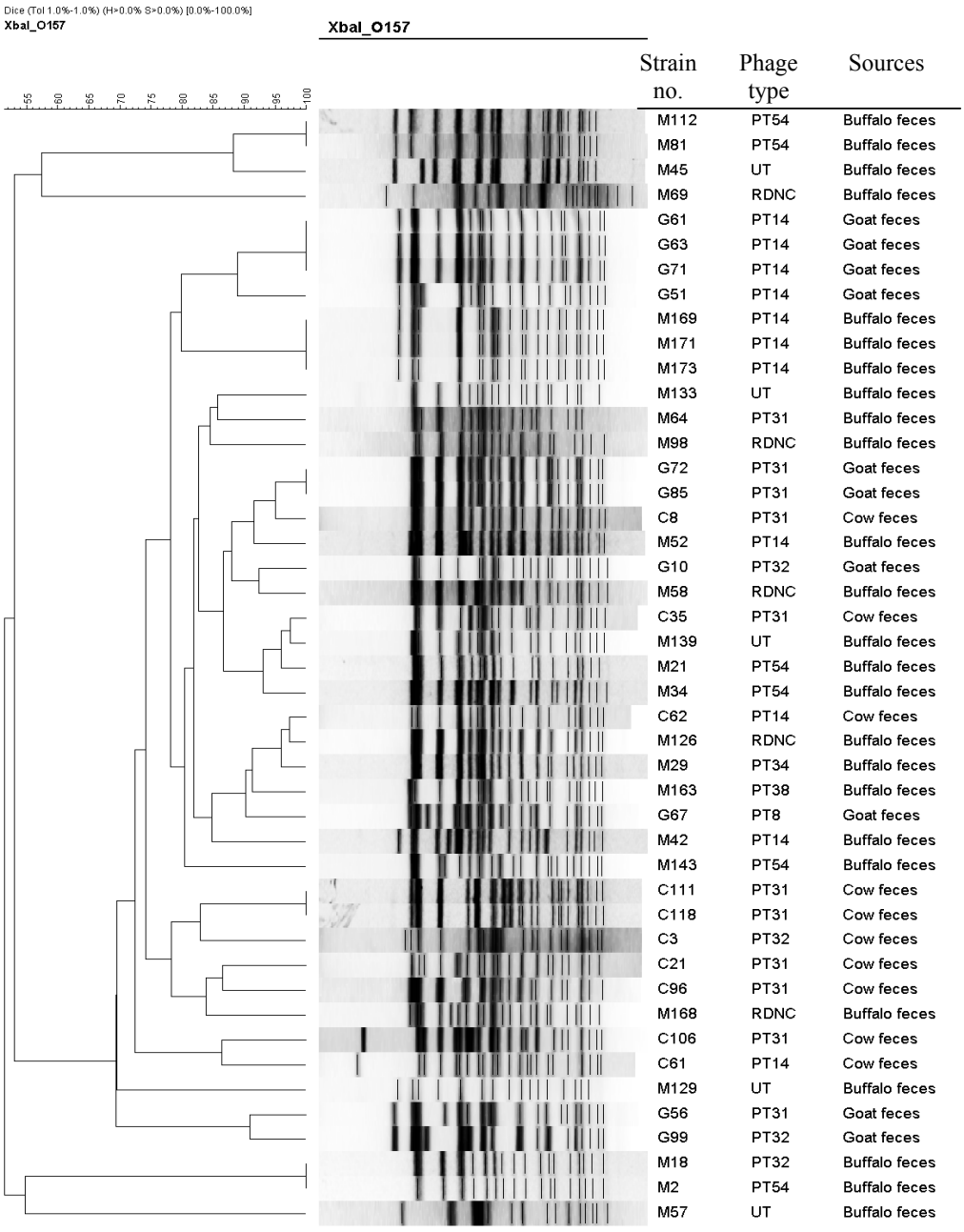

Fig. 5.1 Dendrogram generated by Bionumeric software, showing the distance calculated by the dice similarity index of PFGE *Xba*I profiles for 45 Shiga toxin-producing *E. coli* O157 isolates isolated from fecal samples of slaughter animals. The degree of similarity (%) is shown on the scale.

Three isolates of serotype O80:H19 and two isolates of serotype O125:H19 isolated from both cow and buffalo feces showed identical PFGE patterns. Likewise, three isolates of serotype O149:H8 and two isolates of serotype O141:H21 isolated from buffalo feces generated identical patterns.

5.4 Discussion

This study provides the first evidence that slaughtered animals in Bangladesh are reservoirs for STEC, including STEC O157. Around 37.9% of buffalo, 20.1% of cows and 10.0% of goats were positive for STEC. STEC O157 strains were isolated from 14.4% of buffalo, 7.2% of cows, and 9.1% of goats. The prevalence of STEC, including STEC O157, was significantly higher in buffalo feces than in cow or goat feces ($P < 0.05$). Within similar geographical locations, a few studies of the occurrence of STEC have been done, and most of the studies were done in India and Thailand (26, 28). Recently, a study was done in central Vietnam that found a prevalence of STEC of 27% in buffalo, 23% in cows, and 38.5% in goats. Although a high number of isolates were recovered and characterized based on the virulence determinants, no STEC O157 could be isolated, and little information was provided on the serotypic diversity of the isolates (41). In India, Manna et al. reported that the prevalence of STEC O157 in fecal samples from slaughtered cattle and diarrheic calves was 2.0% and 7.6%, respectively (28). STEC O157 strains have also been isolated in India from foods of cattle origin, namely, raw minced beef samples (9%; $n = 22$) (14), beef surface swabs (3.7%; $n = 27$), and milk samples (2.4%; $n = 81$) (28). In China, STEC O157:H7 was isolated from 10 to 20% of the animals in the villages, including pigs, cattle, goats and chickens (43).

The results of the investigations of the prevalence of STEC O157 in cattle are clearly influenced by the sampling and detection methods adopted. As a matter of fact, the use of specific immunoconcentration procedures for STEC O157 (21, 22) strongly enhances the sensitivity of the isolation methods. Hence, studies based on such procedures reported prevalence rates for STEC O157 that were much higher than those reported with the use of conventional methods (9). It is therefore difficult to determine whether the results reported reflect true differences in isolation rates or are the consequence of the different methodologies adopted.

Table 5.2 Characteristics of Shiga toxin-producing *E. coli* non-O157 isolates from fecal samples of slaughter animals in Bangladesh

Isolate	Origin	Presence of putative virulence and adhesin gene(s)	Serotype	Previous source of isolation[a]	Reference(s) or source
C12	Cow	stx_1	ONT:H8	Human (D), Cattle, Meat, Milk, Sheep	3, 4
C14(1)	Cow	stx_1, $lpfA_{O113}$	O89:H-	Human (D)	27
C14(2)	Cow	stx_1, $lpfA_{O113}$	O89:H38		This study
C20	Cow	stx_1, $lpfA_{O157/OI-141}$, $LpfA_{O157/OI-154}$	O80:H19		This study
C22	Cow	stx_1	ONT:H-	Human (D, HUS) Cattle, Sheep, Milk, Beef, Pigs	3, 4
C25	Cow	stx_1, $lpfA_{O113}$	O18:H-	Pigeon	13
C26	Cow	stx_1	O146:H21	Human (H, D, BD), Cattle, Sheep	18
C31	Cow	stx_2, $lpfA_{O113}$	O8:H19	Human (HUS, D), Beef, Cattle, Pork	1
C33	Cow	stx_2, $lpfA_{O113}$	O103:H21	Human (BD, D, HUS)	1
C59	Cow	stx_1	O110:H2	Cattle	20
C64	Cow	stx_1	O110:H2	Cattle	20
C91	Cow	stx_1	O91:H21	Human (D, HUS, BD), Milk, Beef, Ham, Cheese, Pork, Cattle, Sheep, Sausage	1
C94	Cow	stx_1	O57:H21		This study
C100(1)	Cow	stx_1	O103:H16	Cattle	19
C100(2)	Cow	stx_1, stx_2, hly_{EHEC}, *saa*, $lpfA_{O113}$	O174:H-	Human (D, HUS, H), Cattle	20
C100(3)	Cow	stx_1, stx_2, hly_{EHEC}, *saa*, $lpfA_{O157/OI-141}$, $LpfA_{O157/OI-154}$	O130:H9	Cattle	19
C102	Cow	stx_2, *saa*, $lpfA_{O113}$	ONT:H16	Human (D), Cattle, Sheep, Mutton	20
C103	Cow	stx_2, *saa*	O74:H-	Cattle, goat	20
C109	Cow	stx_1	ONT:H8	Human (D), Cattle, Meat, Milk, Sheep	3, 4
C124	Cow	stx_1, $lpfA_{O113}$	O125:H19	Human (D), Cattle	2, 4
C138	Cow	stx_1, $lpfA_{O113}$	O52:H-		This study
M5	Buffalo	stx_1	ONT:H41	Human (D), Bovine	20
M7	Buffalo	stx_2, $lpfA_{O113}$	ONT:H41	Human (D), Bovine	20
M10	Buffalo	stx_1	O65:H-	Cattle	11
M12	Buffalo	stx_1, $lpfA_{O157/OI-141}$, $LpfA_{O157/OI-154}$	O121:H15		This study
M15	Buffalo	stx_1, $lpfA_{O157/OI-141}$, $LpfA_{O157/OI-154}$	O132:H49		This study
M16	Buffalo	stx_1	O121:H15		This study
M17	Buffalo	stx_1	ONT:H7	Human (D, H), Cattle, Meat, Beef	20
M23(1)	Buffalo	stx_1	O103:H28		This study
M23(2)	Buffalo	stx_1, $lpfA_{O113}$	ONT:H8	Human (D), Cattle, Meat, Milk, Sheep	3, 4
M35	Buffalo	stx_1	O80:H12		This study
M37	Buffalo	stx_1	O75:H10		This study
M41	Buffalo	stx_1	O175:H15		This study

Table 5.2 Continued

Isolate	Origin	Presence of putative virulence and adhesin gene(s)	Serotype	Previous source of isolation[a]	Reference(s) or source
M45	Buffalo	*stx*$_1$	ONT:H?	Human (D), Cattle, Meat, Water, Sheep, Beef	3, 4
M55	Buffalo	*stx*$_1$, *lpfA*$_{O113}$	ONT:H49	Bovine	20
M63	Buffalo	*stx*$_1$	O158:H?		This study
M66(1)	Buffalo	*stx*$_1$	ONT:H19	Human (D), Cattle, Beef, Pork, Meat, Milk	20
M66(2)	Buffalo	*stx*$_1$, *lpfA*$_{O157/OI-141}$, *LpfA*$_{O157/OI-154}$	O35:H31		This study
M70	Buffalo	*stx*$_1$, *lpfA*$_{O113}$	ONT:H-	Human (D, HUS) Cattle, Sheep, Milk, Beef, Pigs	3, 4
M80	Buffalo	*stx*$_1$	O112:H2	Cattle, Beef	20
M82	Buffalo	*stx*$_1$, *lpfA*$_{O113}$	ONT:H16	Human (D), Cattle, Sheep, Mutton	20
M84	Buffalo	*stx*$_1$	O149:H8	Cattle	11
M85(1)	Buffalo	*stx*$_1$	O149:H8	Cattle	11
M85(2)	Buffalo	*stx*$_1$, *lpfA*$_{O113}$	O149:H8	Cattle	11
M88	Buffalo	*stx*$_1$	ONT:H2	Cattle	4
M99	Buffalo	*stx*$_1$	ONT:H41	Human (D), Cattle	20
M100(1)	Buffalo	*stx*$_1$	ONT:H14	Human (D), Cattle	20
M100(2)	Buffalo	*stx*$_1$, *lpfA*$_{O113}$	O8:H30	Beef , Sheep	1
M101(1)	Buffalo	*stx*$_1$	O19:H-		This study
M101(2)	Buffalo	*stx*$_1$	O116:H9	Cattle	12
M110	Buffalo	*stx*$_1$	O116:H9	Cattle	12
M111(1)	Buffalo	*stx*$_1$	O80:H19		This study
M111(2)	Buffalo	*stx*$_1$, *lpfA*$_{O157/OI-141}$, *LpfA*$_{O157/OI-154}$	O80:H19		This study
M117(1)	Buffalo	*stx*$_1$, *hly*$_{EHEC}$, *saa*, *lpfA*$_{O113}$	O141:H21		This study
M117(2)	Buffalo	*stx*$_1$, *hly*$_{EHEC}$, *saa*	O141:H21		This study
M117(3)	Buffalo	*stx*$_2$, *lpfA*$_{O113}$	ONT:H16	Human (D), Cattle, Sheep, Mutton	20
M118	Buffalo	*stx*$_2$, *lpfA*$_{O113}$	O73:H18	Human (D)	2
M122	Buffalo	*stx*$_1$	07:H6	Human (D), Bovine	3,4
M130	Buffalo	*stx*$_1$, *lpfA*$_{O113}$	O8:H7		This study
M135	Buffalo	*stx*$_1$, *stx*$_2$, *hly*$_{EHEC}$, *saa*, *lpfA*$_{O113}$	O76:H21	Cattle	20
M137	Buffalo	*stx*$_1$	O26:H32	Cattle	1
M140	Buffalo	*stx*$_1$	O38:H21	Human (HUS), Bovine	10, 5
M141	Buffalo	*stx*$_1$, *stx*$_2$	O41:H-		This study
M142	Buffalo	*stx*$_1$, *stx*$_2$, *lpfA*$_{O157/OI-141}$, *LpfA*$_{O157/OI-154}$	O35:H32		This study
M145	Buffalo	*stx*$_1$, *stx*$_2$, *saa*, *lpfA*$_{O113}$	076:H21	Cattle	20

Table 5.2 Continued

Isolate	Origin	Presence of putative virulence and adhesin gene(s)	Serotype	Previous source of isolation[a]	Reference(s) or source
M148	Buffalo	stx_2	ONT:H-	Human (D, HUS) Cattle, Sheep, Milk, Beef, Pigs	3
M149	Buffalo	stx_1	O39:H21	Bovine	4
M152	Buffalo	stx_1, $lpfA_{O113}$	O125:H19	Human (D), Cattle	2, 4
M153	Buffalo	stx_1, $lpfA_{O113}$	O51:H2		This study
M159	Buffalo	stx_1, $lpfA_{O157/OI-141}$, $LpfA_{O157/OI-154}$	O26:H32	Cattle	1
G19	Goat	stx_2	O32:H25	Human (D)	23

[a] HUS, hemolytic-uremic syndrome; BD, bloody diarrhea; D, nonbloody diarrhea; H, healthy human.

When buffalo samples were tested for the presence of *stx* genes by PCR, around 80% of the samples were positive. Of the cows and goats, around 73% and 12%, respectively, were positive. STEC non-O157 strains could be isolated from 23.6%, 12.9% and 0.9% of the buffalo, cow and goat samples, respectively. Failure to isolate the STEC non-O157 strains from PCR-positive fecal samples is a common problem (3). In India, viable STEC were recovered from 24 to 40% of PCR-positive samples, depending on the origin of the sample. The nonisolation of viable STEC from PCR-positive samples might be due to the presence of very low numbers of bacteria, the presence of free *stx* phages in the sample, and the loss of *stx* genes upon subcultivation of strains (23). Under routine diagnostics, there is no definitive biochemical characteristic, like sorbitol fermentation in the case of serogroup O157, which can identify STEC irrespective of the serotypes from commensal flora.

Most of the STEC O157 isolates (91.1%) were positive for the stx_2 gene only. In humans, epidemiologic data suggest that *E. coli* O157 strains that express Stx2 are more important than Stx1 in the development of HUS (17) and that strains that express Stx2 alone are more likely to be associated with the progression to HUS than are strains that produce both Stx1 and Stx2 (16). When the presence of other virulence genes was analyzed, most of the strains were found to be positive for the *eae* (95.5%), hly_{EHEC} (93.3%), *katP* (93.3%), and *etpD* (93.3%) genes (Table 5.1). Thus, STEC O157 isolates in this study were found to be carrying additional virulence factors, clearly associated with increased human pathogenicity. In our previous study of patients in Bangladesh, we found the prevalence of STEC in diarrheal patients was relatively low compared to that of other enteric pathogens, including *V. cholerae* and *Shigella* spp. (23). STEC O157 were not found in any of the patients. The higher prevalence of STEC in cattle, in tandem with a low isolation rate in diarrheal patients in Bangladesh, can be explained by the presence of the high prevalence of antibodies in the population, which prevent infection and/or clinical manifestations of STEC infection. However, further studies are needed to establish this explanation.

At least 90 PT have been reported for STEC O157 (25), but only seven of these (PT2, PT4, PT8, PT14, PT21/28, PT32, and PT54) account for the majority (>75%) of the human strains isolated in Europe and Canada (30). More than 77% (n = 35) of the STEC O157 isolates in this study were characterized by seven recognized PT. PT14 (24.4%), -31 (24.4%), and -32 (8.9%) were commonly found in all three types of animals, with differences in prevalence. Other PTs that were found in this study were PT8 for goat isolates and PT34, PT38, and PT54 for buffalo isolates. PT14 has commonly been found among STEC O157 strains from different sources, including cattle feces, meat, and human patients (25).

Furthermore, a significant association has been detected between PT14 and the presence of acute pathologies (30). PT8 is one of the PTs most frequently found among STEC O157:H7 strains from humans and bovines in many European countries, including Spain, Belgium, Finland, Germany, Italy, England, and Scotland (25). PT34 and PT54 have also been found among strains from both human and animal origin but appeared to be more frequent among strains from animal origins (30). PT32 was detected in STEC O157 strains associated with several large outbreaks, including a multistate outbreak in the United States (6) and a recent one in Ireland (29). PT31 was found in both human and non-human sources (meat and slaughterhouses) in Canada, suggesting a foodborne transmission in human cases (25).

A high degree of polymorphism was observed for STEC O157 isolates analyzed by PFGE. A total of 37 distinct profiles were obtained among 45 isolates. Strains which had identical PFGE patterns belonged to the same type of animal (Fig. 5.1). No PFGE profiles were found to be commonly generated by isolates from different types of animals (Fig. 5.1).

STEC non-O157 isolates have been shown to be important pathogens, despite being severely underreported, because in many laboratories, the facilities to isolate, identify, and characterize them do not exist. Where facilities do exist, STEC non-O157 isolates are found (1). In the present study, we have characterized 71 STEC non-O157 isolates. These isolates belonged to 36 O serogroups and 52 O:H serotypes (Table 5.2), of which 32 were isolated from buffalo only, 14 from cow only, 1 from goat only, and 5 from both cow and buffalo. All non-O157 serotypes in the study were compared with the serotypes isolated in previous studies from different sources. The majority of STEC isolates belonged to serotypes (42.3%; n = 22) previously found among STEC of human origin, of which 31.8% (n = 7) were of serotypes associated with severe infections, including bloody diarrhea and HUS (Table 5.2). In addition, 17 serotypes found in this study were found previously among STEC strains isolated from multiple origins, including those of human patients, cattle, and milk/meat (Table 5.2). Interestingly, 18 serotypes in this study could not be matched with the recognized STEC serotypes reported so far (Table 5.2).

When virulence genes were analyzed, 78.9% of the isolates were found to be positive for stx_1 only, 12.7% were positive for stx_2 only, and 8.4% were positive for both stx_1 and stx_2. Higher prevalence rates of stx_1 have been reported in strains from animal origin in previous studies (42), which corroborates the results of the present study. Only 7% of the isolates were found to be positive for the hly_{EHEC} gene (Table 5.1), and no isolates were positive for other virulence genes, including *eae*, *katP*, and *etpD*. STEC strains, which are positive for *eae* that is harbored by the large pathogenicity island LEE are considered to be highly virulent in

humans (40). The LEE appears to confer enhanced virulence; however, the presence of the LEE is not essential for pathogenesis, since a number of cases of severe STEC disease, including HUS, as well as occasional outbreaks, were caused by the LEE-negative strains (31). It has been suggested that additional virulence factors, including adhesions encoded outside of the LEE, may play a role in STEC pathogenesis (40). Since all non-O157 isolates in this study were negative for *eae*, the isolates were tested for the presence of several novel adhesin genes. About 30% (n = 15) of the buffalo and 47% (n = 10) of the cow isolates were positive for the lpf_{O113} gene (Table 5.1). This is in accordance with a previous study where lpf_{O113} was found to be one of the most prevalent adhesin genes among STEC non-O157 strains (40). More than 8% (n = 4) of the buffalo and 19% (n = 4) of the cow isolates possessed the *saa* gene. In the study previously referred to, it was shown that there is a correlation between the presence of *saa* and hly_{EHEC} for certain serotypes (40). In the present study, similar results were found; among eight isolates positive for *saa*, five (62.5%) were positive for hly_{EHEC}.

Although a high degree of serotypic diversity was found among STEC non-O157 isolates in this study, isolates of the same serotype were found in different samples from different animal types. In addition, more than one isolate belonging to the same serotype with different virulence profiles was isolated from the same sample. PFGE analysis was applied to find out if there was any genetic relationship among isolates of the same serotype. Identical PFGE patterns were found among isolates belonging to the same serotype but isolated from different samples, and at the same time, different PFGE patterns were found in the case of some serotypes.

In our previous study, we found a low prevalence of STEC in diarrheal patients in Bangladesh, with no cases of STEC O157 infection (23). In this study, we found a significantly higher prevalence of both STEC O157 and non-O157 serotypes in the animal population. Therefore, there must be a limiting factor existing in the transmission route of this pathogen from the reservoir to the human population or there might be natural protection against STEC infection among the general population in Bangladesh. Further studies are being carried out to confirm these assumptions.

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Chapter 6

Shiga toxin-producing Escherichia coli *in raw meat, raw milk and street vended juices in Dhaka, Bangladesh*

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Abstract

The prevalence of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) has been investigated in 210 food samples, including 87 raw meat samples collected from slaughterhouses, 20 raw milk samples from domestic cattle, and 103 fresh juice samples from street vendors in Dhaka city. More than 71% (n = 62) of the raw meat samples were positive for the *stx* gene(s); 34% (n = 21) of buffalo meats and 66% (n = 41) of beef. Approximately 10% (n = 2) of the raw milk and 8% (n = 8) of the fresh juice samples were positive for *stx*. STEC O157 were isolated from seven meat samples (two buffalo meats and five beef). No other STEC serotypes could be isolated. STEC could not be isolated from any of the *stx*-positive raw milk and juice samples. The STEC O157 isolates from raw meats were positive for the stx_2, *eae*, *katP*, *etpD* and enterohemorrhagic *E. coli hly* (hly_{EHEC}) virulence genes, and belonged to three different phage types: 8 (14.3%), 31 (42.8%), and 32 (42.8%). PFGE subtyping revealed six distinct restriction patterns suggesting a heterogeneous clonal diversity. This is the first report of the isolation of STEC O157 from raw meat samples and the presence of Shiga toxin-producing organisms in different food samples in Bangladesh.

6.1 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) organisms, also called verocytotoxin (VT)-producing *E. coli* (VTEC), are one of the most important groups of foodborne pathogens (14, 21). They are a major cause of gastroenteritis that may be complicated by hemorrhagic colitis (HC) or the hemolytic-uremic syndrome (HUS), which is the main cause of acute renal failure in children. STEC are a serologically diverse group of zoonotic pathogens. Serotype O157:H7 has been the predominate type worldwide (23). Since its identification as a pathogen in 1982, STEC O157:H7 has been the cause of a series of outbreaks in Canada, Japan, the United Kingdom, the United States and other countries (14, 21, 25). However, more than 200 STEC serotypes have been reported and more than 100 have been linked with human infection (5).

The pathogenicity of STEC strains is associated with various virulence factors. The main factor is the capacity to form two potent phage-encoded cytotoxins called Shiga toxins (Stx1, Stx2 and their variants) or verocytotoxins (VT1 and VT2) (21). Another virulence factor associated with clinical STEC isolates is the protein intimin, encoded by the *eae* gene and responsible for the intimate attachment of STEC strains to the intestinal epithelial cells and causing the formation of attaching and effacing (A/E) lesions in the intestinal mucosa (12). Enterohemolysin, also called enterohemorrhagic *E. coli* (EHEC) hemolysin has also been identified as a possible STEC virulence factor (2).

STEC represent the only pathogenic group of *E. coli* that has a definite zoonotic origin, although not all the STEC strains have been demonstrated to cause disease in humans. STEC strains rarely cause disease in animals, and ruminants are recognized as their main natural reservoir. Cattle are considered to be the major animal source of STEC that are virulent to humans, in particular STEC O157 (3). STEC O157 and other serotypes associated with human infections have also frequently been isolated from the intestinal content of other ruminant species, including sheep, goat, water buffalo, and wild ruminants. Contamination of carcasses with STEC usually occurs during slaughter and subsequent processing through fecal material originating directly or indirectly from the rectal-cecal area (6). Dairy products (milk, cheese, cream) associated with infection have included those that are unpasteurized, have had a pasteurization failure or have been contaminated post-pasteurization. Ready-to-eat foods have also been associated with infection, particularly cooked meats contaminated by raw materials during processing, in catering establishments, at retail sale, and in the home. In

developed countries, STEC O157 outbreaks associated with beef have caused concern among public health workers, clinicians, and the public, prompting major changes in clinical and laboratory practice, meat production, and food preparation (17).

Like many of the developing and underdeveloped countries, the hygienic conditions in Bangladesh are severely compromised, and living with domestic animals within the same premises is a common practice. In our previous study we found that the prevalence of STEC in slaughter animals was 24%, and 43% of these STEC isolates belonged to serogroup O157 (11). This is in contrast to our study of patients in Bangladesh where we found the prevalence of STEC was 0.5% among the hospitalized diarrheal patients, which was 1.9% of the cases of community patients. No STEC O157 organisms were isolated in the study (10). From these two studies, it can be concluded that although there is a high prevalence of STEC, including serogroup O157 in animal reservoirs, these organisms are rarely associated with human infections in Bangladesh. One of the reasons might be the absence of any food vehicles that can transmit these organisms from animal reservoirs to humans. In Bangladesh, no study of the occurrence of STEC in food sources had been done. Therefore, the objective of this study was to determine the occurrence of STEC in different types of food samples, including raw meat, raw milk and freshly prepared juices collected in Dhaka city.

6.2 Materials and methods

6.2.1 Sampling

In the period from 1 July 2006 to 31 January 2007, we collected raw meat samples from 28 buffalo, and 59 beef carcasses at slaughterhouses in Dhaka of the different areas in Bangladesh. The origin of the animals could not be recorded due to the lack of information. In the same period, we collected 20 raw milk samples from individual domestic dairy cows at different suburbs of Dhaka city. In addition, we collected 103 freshly prepared (untreated) juice samples, including mixed herbal juice (n = 49), sugar cane juice (n = 40), wood apple juice (n = 7) and some other types of juice (three milk shake, one lemon, two mix fruit, and one pineapple) from street vendors at different places of Dhaka city. Samples were transported to the laboratory maintaining the cool chain (4 to 8°C) and microbiological examination was started within 4 h.

6.2.2 Isolation of STEC O157

For meat samples, a 25-g portion, and for raw milk and juice samples, a 25-ml portion was added to 225 ml of modified tryptone soy broth (Oxoid Ltd., Basingstoke, United Kingdom). After homogenization in a stomacher for 1 min, the samples were incubated for 18-20 h at 37°C. STEC O157 strains were isolated as described previously (9), by using the immunomagnetic separation (IMS) technique and presumptive isolates were confirmed by an agglutination test using *E. coli* O157 latex agglutination kit (Murex Biotech Ltd., Dartford, Kent, United Kingdom) and a PCR for the *rfb* (O-antigen-encoding) region of *E. coli* O157 (*rfb*E_{O157}) (21).

6.2.3 Isolation procedure for STEC non-O157

The overnight enrichment cultures were screened by PCR for the presence of stx_1 and stx_2 using the primers as described earlier (10). DNA was extracted from 1 ml of broth culture by thermal cell lysis using Chelex-100 resin (Bio-Rad Laboratories Ltd., Richmond, Calif.) and 5 µl of the DNA extract was used in the PCR (18). Multiplex PCR for stx_1 and stx_2 was carried out in a PTC-200 peltier thermal cycler (Bio-Rad) using the program as described previously (10). DNA extracted from *E. coli* O157:H7 strain NCTC 12079 and *E. coli* strain ATCC 25922 were used as positive and negative controls, respectively in each PCR reaction. Broth cultures that yielded positive PCR results for stx_1 and/or stx_2 were streaked onto tryptone bile x-glucuronide (TBX) agar (Oxoid) supplemented with cefixime (20 mg/l), cefsulodin (3 mg/l) and vancomycin (30 mg/l) (CCV-TBX). Single colonies, including different morphological types were transferred from the CCV-TBX and plated onto Luria agar (Difco, BD diagnostics, USA) to create a grid pattern of 96 colonies (12×8). The plates were incubated overnight at 37°C. The isolates were subjected to PCR for the detection of stx_1 and stx_2 as described above. The number of PCR reactions was reduced to 20 by pooling the colonies per row and per column in 500 µl sterile distilled water. DNA was extracted by boiling the suspensions for 10 min and 5 µl of the supernatant was used in the PCR. The isolates with positive PCR results were identified as *E. coli* by an API 20E test (bioMérieux, Marcy l'Etoile, France).

6.2.4 Phage typing

Isolates confirmed to be STEC O157 were phage typed at the laboratory of Enteric Pathogens, Central Public Health Laboratory, London, United Kingdom.

6.2.5 PCR detection of virulence factors

PCR for detecting both chromosome (e.g., *stx*$_1$, *stx*$_2$, and *eae*)- and plasmid (e.g., *hly*$_{EHEC}$, *katP*, and *etpD*)-encoded virulence genes was performed as described earlier (10) in a total volume of 25 µl containing 2.5 µl of 10× Fast Start buffer with 1.5 mM $MgCl_2$ (Applied Biosystems, Weiterstadt, Germany), 200 µM concentration of each deoxynucleoside triphosphate, a 0.2 pmol of each primer, and 1 U of Fast start *Taq* DNA polymerase (Roche, Mannheim, Germany) using a PTC-200 peltier thermal cycler (Bio-Rad).

6.2.6 PFGE

PFGE was performed following the standardized protocol developed by PulseNet for *E. coli* O157:H7 (22). Analysis of the TIFF images was carried out by the BioNumerics software (Applied Maths, Belgium) using the dice coefficient and unweighted-pair group method using average linkages to generate dendrograms with 1.0% tolerance values.

6.3 Results

6.3.1 Prevalence of STEC

Of the 87 meat samples collected from the slaughterhouses, 62 (71%) were positive for *stx* gene(s); 21 samples of buffalo meats, and 41 samples of beef (Table 6.1). STEC O157 strains were isolated from seven samples; two samples of buffalo meats, and five samples of beef (Table 6.1). No STEC non-O157 strains were isolated. Of the 20 raw milk samples, two (10%) were positive for *stx* gene(s) (Table 6.1). Of the 103 fresh juices, eight (8%) samples were *stx*-positive; six samples of mixed herbal juices, and two samples of wood apple juices (Table 6.1). However, no STEC could be isolated from *stx*-positive positive samples of raw milk and street vended juices.

6.3.2 Characterization of STEC O157

All seven STEC O157 isolates were positive for the *stx*$_2$, *eae*, *etpD, katP* and *hly*$_{EHEC}$ genes. The strains could be grouped into three different phage types: phage type 31 (n = 4), phage type 32 (n = 2), and phage type 8 (n = 1). PFGE analysis revealed six distinct restriction profiles (Fig. 6.1).

Table 6.1 PCR detection of *stx* gene(s) in broth cultures from food samples and isolation of Shiga toxin-producing *E. coli* O157 and non-O157 by subculturing onto selective agar

Sources (n = 210)	No. of PCR positive (%)				Isolation of:		
	stx_1	stx_2	stx_{1+2}	Total	*E. coli* O157 (%)[a]	Non-O157 (%)	Total
Meat (87)	1 (1.1)	9 (10.3)	52 (59.8)	62 (71.3)	7 (8.0)	0	7 (8.0)
Buffalo (28)	0	2 (7.1)	19 (67.9)	21 (75)	2 (7.1)	0	2 (7.1)
Beef (59)	1 (1.7)	7 (11.9)	33 (55.9)	41 (69.5)	5 (8.5)	0	5 (8.5)
Raw milk (20)	1 (5)	1 (5)	0	2 (10)	0	0	0
Fresh juices (103)	2 (1.9)	4 (3.9)	2 (1.9)	8 (7.8)	0	0	0
Mixed herbal (49)	2 (4)	2 (4)	2 (4)	6 (12)	0	0	0
Sugarcane (40)	0	0	0	0	0	0	0
Wood apple (7)	0	2 (28.6)	0 (0)	2 (28.6)	0	0	0
Others (7)	0	0	0	0	0	0	0

[a] by using immunomagnetic separation.

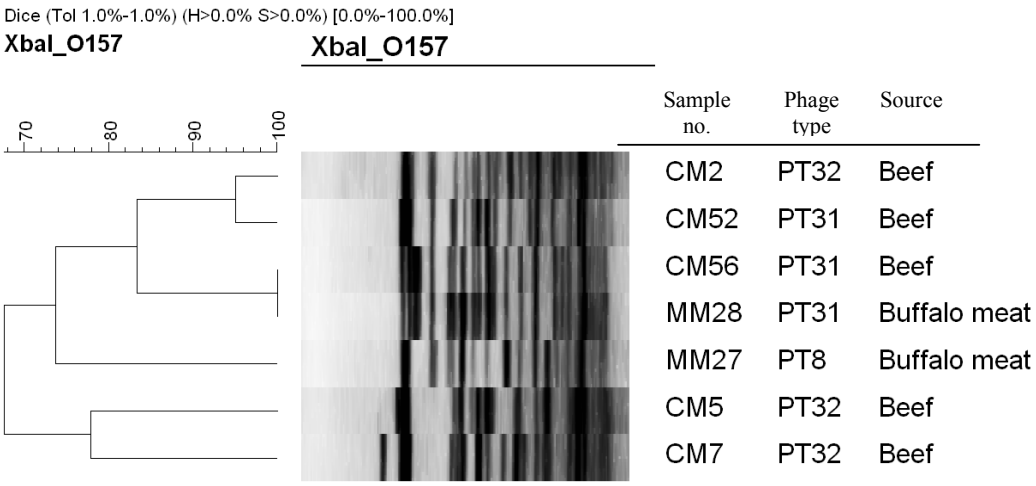

Fig. 6.1 Dendrogram generated by Bionumeric software, showing distance calculated by the dice similarity index of PFGE *Xba*I profiles for seven STEC O157 isolates isolated from raw meat samples. The degree of similarity (%) is shown on the scale.

6.4 Discussion

Outbreaks of STEC O157 infection have been reported previously from direct contact with cattle, environments contaminated with cattle manure, and contaminated recreational and drinking water (3, 6). Unpasteurized milk, apple cider, and produce items such as lettuce and sprouts that may be contaminated directly by animal manure or indirectly from

contaminated irrigation water have also been implicated in STEC O157 infection (3, 6). However, the leading food vehicle for sporadic and outbreak causes of STEC O157 is undercooked ground beef (8). According to our previous studies, there is a high prevalence of STEC including serogroup O157 in animal reservoirs while these organisms are rarely associated with human infections in Bangladesh (10, 11). In order to understand the kinetics of STEC transmission in the community and the associated risk of infection, it is very important to study the occurrence of these organisms in the food chain. Although numerous studies have been done in developed countries, there is still a paucity of reports of STEC in the food chain in developing countries. Recently in India, STEC O157 have been identified as one of the predominant serogroups from buffalo meats (7), and bovine and ovine diarrheal fecal samples (24). STEC O157 strains were also isolated from raw minced beef samples (9%; *n* = 22) (4), beef surface swabs (3.7%; *n* = 27) and milk samples (2.4%; *n* = 81) (19) in India. In this study, STEC O157 strains were isolated from 8% of the raw meat samples. During the processing of carcasses, meat can easily become contaminated with animal feces if there are unhygienic conditions, lack of good processing practices and a lack of knowledge of the slaughterhouse personnel on basic hygiene. All these factors are significantly present in the slaughterhouses in Bangladesh where the same premise is being used to slaughter the animal, to process carcasses and to sell meats to the consumers. In addition, in our previous study we found that the feces of around 10% slaughtered animals were positive for STEC O157 (11).

Although a high number of raw meat samples (71%; *n* = 62) were positive for *stx* gene(s), only STEC O157 organisms were isolated and no STEC non-O157 organisms could be isolated in the study. No STEC isolates could be recovered from raw milk and freshly prepared juice samples, while 10% of the raw milk and 8% of the juice samples were positive for *stx* gene(s) (Table 6.1). For the isolation of STEC O157 organisms, IMS followed by plating onto selective agars was performed, which has been proven to be a highly sensitive and effective method and is recommended by the International Organization for Standardization (ISO) (1). For STEC non-O157, there is no definitive isolation technique; therefore, different laboratories have been using different methods and no standardized method has yet been recommended. In this study, we used a PCR-based method that we had used successfully in our previous studies for the isolation of STEC non-O157 strains from human stool samples and animal feces (10, 11). Nevertheless, this method might not work well for the food samples that normally contain a low number of organisms. The other possible reasons for the nonisolation of viable STEC from *stx*-positive samples might be: 1)

the presence of other organisms that possess *stx* genes; 2) the presence of free bacteriophages in the sample that contain *stx* genes (16); or 3) due to the loss of *stx* genes upon subcultivation of strains (13).

STEC O157 strains that were isolated from raw meat samples were further characterized. In addition to *stx*, the STEC O157 isolates were found to carry other virulence genes, including *eae*, *katP*, *etpD* and hly_{EHEC} and therefore, appeared to be potential human pathogens. Phage types (PT) 31 and 32 encompassed more than 85% (n = 6) of the isolates. Interestingly, these two phage types were found to be the predominant PTs among STEC O157 strains isolated previously from animal feces (11). It has been reported from many countries that these phage types are commonly found in both human and non-human sources (meat and slaughterhouses) suggesting a foodborne transmission in human cases (15). Phage type 32 was detected in STEC O157 strains associated with the largest outbreak ever recorded in Ireland (20). One strain in this study was typed as phage type 8, which is one of the most frequently found phage types among STEC O157 strains from humans and bovine in many European countries, including Spain, Belgium, Finland, Germany, Italy, England and Scotland (15). PFGE analysis of the seven STEC O157 isolates revealed six distinct patterns, suggesting a genetically heterogeneous group. Two strains, one from beef and one from buffalo meat belonged to the same clonal type (Fig. 6.1).

To our knowledge, this is the first report of the occurrence of STEC O157 in meat samples in Bangladesh. The results of this study highlight the poor hygienic conditions of slaughterhouses in Dhaka and the lack of good processing practices. Although STEC organisms could not be isolated from any raw milk and juice samples, the presence of *stx* genes in the samples indicated that these might be contaminated with Shiga toxin-producing organisms. Street vended juices are becoming increasingly popular in Bangladesh due to rapid urbanization. These juices are commonly drunk in raw, exposing consumers to a significant risk of infection with foodborne disease. Historically, people in Bangladesh prefer to eat hot meals and extensive boiling is a common means of cooking. Therefore, raw meats contaminated with STEC does not pose a potential risk to public health unless the issue of cross-contamination of prepared food is taken into account. However, given the abundance of STEC in the animal reservoirs (11), high risk of contamination of raw meats with STEC O157 and the poor socioeconomic status of the people in Bangladesh, it is evident that STEC are present at a significant rate in the food chain but are rarely causing infections in human (10). This accentuates the need for further research on the antibody response to STEC, especially to serogroup O157 among the healthy populations in Bangladesh.

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Chapter 7

Antibody responses to Escherichia coli *O157 lipopolysaccharide among the healthy population in Bangladesh*

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Abstract

An investigation was conducted to determine the serum antibody response against *Escherichia coli* O157 lipopolysaccharide (LPS) among the healthy population in Bangladesh. Participants from different occupations, including butchers and from different locations, including rural and urban areas were included. Of the 233 serum samples, 116 (50%) had antibodies (IgG, IgA and/or IgM) to *E. coli* O157 LPS by Western blot analysis. Antibody classification showed that all 116 samples positive for polyvalent antibodies were positive for IgG and 87 were also positive for IgM. Among nine household members of butchers involved in other occupations, five showed positive antibody response. Of the 133 serum samples collected from butchers, 54 (41%) were antibody positive. No significant difference in antibody response was found between the butchers from urban (33%, $n = 14$) and rural areas (44%, $n = 40$). Among participants other than butchers and their family, 57 (63%) showed a positive antibody response, which was significantly higher than that of butchers ($P < 0.05$). The prevalence of IgM-class antibody was found to be also higher among nonbutcher participants (42%, $n = 38$) than butchers (33%, $n = 44$); however, the difference was not statistically significant. For all categories of participants, no significant association was found between the age and antibody response results. Use of water from municipal water supply system or other contaminated water sources for drinking and washing was associated with a positive antibody response to *E. coli* O157 significantly among urban population than rural population ($P < 0.001$). This study concludes that the lack of STEC O157 infection among Bangladeshi population might be attributable to the protective immunity against these pathogens acquired by the frequent exposure to the antigens.

7.1 Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are significant zoonotic pathogens that are capable of causing severe gastrointestinal disease that can lead to a number of sequelae, including hemorrhagic colitis (HC), hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (31). HUS is the most common cause of acute renal failure in children. The association between HUS and STEC infection has been well documented over the past two decades (27) and is due to the production of one or more Stx's produced by the organisms (26).

Cattle are considered as the primary reservoir of STEC and infection may be transmitted to humans through contaminated food, water or by direct animal contact (5). Rural populations have been postulated to be at greater risk for exposure to STEC by virtue of increased exposure to animals or their excreta (33). Dairy farm visits have been implicated as a source for infection in many countries and animal contacts are a risk factor for the development of HUS (17).

Most outbreaks and sporadic cases of HC and HUS have been associated with STEC O157 infection (42) and reported from developed countries. Studies conducted in developing areas of the world have shown little involvement of STEC strains as causal agents of diarrheal disease in these countries (42). Interestingly, the conditions for transmission of these strains from animals to humans exist, as do the inherent problems relating to basic sanitation (16). Furthermore, studies in developing countries showed that STEC is significantly present in animal reservoirs and in the food chain (18, 25, 43, 47). Therefore, the question that remains to be answered is why there is such a lack of contribution of STEC as a cause of major outbreaks of disease in these areas of the world. The reason is not always due to a lack of technical capacity in participating laboratories or to inadequate surveillance systems in these areas. One of the explanations would be the protective immunity against this pathogen, which has already been suggested by several studies as a probable cause of low incidence of STEC infection in developing countries (32, 46).

In general, patients infected with STEC produce serum antibodies to various components, including Stx, secreted virulence factors and lipopolysaccharide (LPS) specific to a particular serogroup (9, 22, 29, 30). Many studies have been done on the detection of antibodies to the O157 LPS antigen, which is considered the basis for routine serological testing to provide evidence of recent infection even in the absence of fecal *E. coli* O157 (12). Antibodies to *E.*

coli O157 LPS may be found in healthy persons (20). A study in Canada showed that 6.3% of apparently healthy people associated with cattle carrying fecal STEC also had fecal STEC, including the serogroup O157, and suggested that occupational exposure to cattle harboring STEC may lead to subclinical infections in humans (49). This study also found higher frequencies of antibodies to O157 LPS among residents of rural areas compared with residents of urban areas. It has been suggested that repeated and cumulative exposure to STEC O157 as in the case of dairy farm residents or rural populations provides adequate antigenic stimulation (a booster effect) to induce a systemic antibody response against this pathogen (38).

There have been numerous investigations on the systemic immune response towards STEC O157, especially in countries where its prevalence is high. Little research has been done in countries having endemic and epidemic burden of various enteric pathogens with a low incidence rate of STEC infection. No study has yet been carried out to investigate the antibody response to *E. coli* O157 in a population having occupational exposure to animals in a developing country. In our previous studies, we found that the prevalence of STEC in diarrheal patients is significantly lower in Bangladesh compared with other enteric pathogens (24). There have been no reports of HUS or HC in association with STEC O157 infection. In contrast, there is a significant prevalence of potentially virulent STEC, including those of serogroup O157 in food and food producing animals (25, M. A. Islam, A. S. Mondol, I. J. Azmi, E. de Boer, R. R. Beumer, M. H. Zwietering, A. E. Heuvelink and K. A. Talukder, Submitted for publication). This urges the necessity to test the antibody response against STEC O157 among the healthy population, especially those who are in frequent contact with animals compared to those who are not.

Therefore, the aim of this study was to determine the antibody response against *E. coli* O157 LPS among the healthy Bangladeshi population, including people who are working in slaughterhouses located in both urban and rural areas.

7.2 Materials and methods

7.2.1 Human sera

Sera were obtained from a total of 233 blood samples collected from different groups of people, including butchers (people who work in the slaughterhouses) from both urban (n = 42) and rural areas (n = 91), household members of butchers (n = 9), and people with other

occupations (n = 91), including businessmen, students, service holders and housewives. Five ml of blood sample from each person were collected using a sterile syringe and kept at room temperature for 2 h for separation of the blood cells and serum. Serum was collected from the upper layer after centrifuging at 3,000 rpm for 15 min. Informed consent for participation in this research was obtained from participants or their guardians. A standard questionnaire was completed by all participants at the time of blood sampling and provided information on age, sex, blood group, occupation, any intestinal problem from two weeks before sample collection, characteristics of stool and contact with animals. Sera from healthy volunteers from Mexico known to contain antibodies to *E. coli* O157 were used as positive controls, and sera from anonymous blood donors were used as negative controls for Western blot analysis. Sera were stored at –20°C until tested.

7.2.2 LPS prepared from whole bacteria

LPS was prepared by digesting whole bacteria with proteinase K (9) from a reference STEC O157:H7 strain (NCTC 12079), which is positive for *stx*$_1$, *stx*$_2$ and *eae* genes. Bacteria were grown overnight on TSA plates and the cells were suspended in SDS-PAGE sample buffer in pre-weighted eppendorf tubes. Cell concentration was adjusted to 1 mg per 30 µl prior to incubation at 100°C for 10 min. After cooling, samples were mixed with an equal volume of SDS-PAGE buffer containing 100 µg proteinase K (Sigma Chemical Co., St. Louis, Mo) per 30 µl prior to incubation at 60°C for 1 h.

7.2.3 Purification of LPS

LPS was prepared from bacterial outer membranes based on the hot-phenol method of Westphal and Jann (48). Briefly, STEC O157:H7 reference strain (NCTC 12079) was grown on TSA plates and the cells were harvested from overnight cultures and suspended in 5 ml 25 mM Tris-HCl (pH 7.4) containing 1 mM EDTA-Na_2. Bacterial cells were disrupted by sonication and cell suspensions were poured into a pre-cooled volume of 20 ml of the same buffer and centrifuged to sediment any residual whole bacteria (5,000× g, 30 min, 4°C). Supernatants, containing bacterial envelopes were transferred into new tubes and centrifuged (45,000× g, 1 h, 4°C). Pellets were re-suspended in 1 ml of 25 mM Tris-HCl (pH 7.4) and then added to 19 ml of the same buffer. The approximate protein concentration in the suspension was determined by measuring the OD (at 280 nm). The protein was mixed with Sarkosyl (20 µl/mg) and incubated for 30 min at room temperature. Then, outer membranes

were sedimented by centrifugation (45,000× g, 1 h, 4°C). After removing the supernatant, the pellets were re-suspended in 1 ml of 25 mM Tris-HCl (pH 7.4) and added to an ice-cold volume of 29 ml of the same buffer and centrifuged as before. After centrifugation, the outer membrane pellets were re-suspended in 200 µl of the same buffer and stored at –20°C.

7.2.3.1 Large-scale LPS purification

This method is a modification of the hot-phenol procedure of Westphal and Jann (48). For each outer membrane preparation, a 5 ml-volume of 80% aqueous phenol was dispensed into a centrifuge tube and kept in a 68°C water bath to equilibrate. The outer membrane preparation was suspended in 5 ml of deionized water and then added to the pre-heated phenol solutions and the two solutions were mixed with a Pasteur pipette to form a uniform 'milky' emulsion. Then, it was incubated at 68°C for 15 min followed by centrifugation at 68°C (3,000× g for 45 min). After centrifugation, the upper aqueous phase was harvested and transferred to a clean glass container. The remaining phenol phase was mixed with 5 ml deionized water, incubated at 68°C for 15 min and centrifuged (as above). The aqueous phase was removed and mixed with the initial aqueous phase fraction prior to dialysis (three times) against 5 L deionized water at 4°C. The aqueous fraction was lyophilized and the LPS was weighed. The LPS was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) alongside LPS prepared from whole bacteria (mentioned above). Gels were stained with silver to show LPS profiles (45) and to show that the purified LPS was representative of whole-cell LPS.

7.2.4 SDS-PAGE and Western blotting

SDS-PAGE of O157 LPS was carried out as described earlier (28). Samples (LPS) were loaded (10 µg/lane) onto gels comprising a 4.5% acrylamide stacking gel and a 12.5% acrylamide separation gel. Electrophoresis was performed using the mini-protean III system (Bio-Rad Laboratories Ltd., Richmond, Calif.) with a current of 12 mA for first 12-15 min then 15 mA for 1 h.

Western blotting analysis of the O157 LPS with the serum samples was performed by the method described previously (44). Briefly, profiles were transferred onto nitrocellulose membrane paper (NCP) by electroblotting (with a constant current of 80 V for 2 h). Membranes were cut into strips and blocked with PBS containing 3% skim milk by incubating at room temperature for 30 min. Strips were reacted with 30 µl of human sera in 5 ml of PBS containing 3% of skim milk and incubated at room temperature for 2 h. Thereafter, the strips were washed with PBS+Tween 20 three times for 10 min each. Then the strips were

incubated for 1 h with goat anti-human polyvalent antibodies (IgG, IgA and/or IgM) conjugated with alkaline phosphatase (Zymed Laboratories, San Francisco, Calif.) with a dilution of 1:5,000 in blocking buffer, and then washed with PBS+Tween 20 as before. Antigen-antibody complexes formed on the strips were detected by adding enzyme substrate buffer containing 90 µl nitroblue tetrazolium (Sigma; 75 mg/ml in 70% (v/v) aqueous dimethyl formamide) and 70 µl of aqueous 5-bromo-4-chloro-3-indolylphosphate (Sigma; 50 mg/ml). In case of a positive result for polyvalent antibodies, the same procedure was followed to classify the antibodies to *E. coli* O157 using immunoglobulins to human IgG and human IgM.

7.2.5 Statistical methods

The chi-square test with Yates correction or if necessary Fisher's exact test, were used as indicated to compare the test results. Statistical testing was performed using Epi Info version 3.3.2 and SPSS version 12.0 (SPSS, Inc., Chicago, III.) on a standard personal computer. Statistically, P values < 0.05 were considered significant for comparisons.

7.3 Results

7.3.1 Antibody responses to STEC O157 LPS

Of the 233 serum samples, 116 (50%) were shown to contain antibodies to the LPS of *E. coli* O157 when tested with a human polyvalent antibody-conjugate by Western blot analysis; examples are given in Fig. 7.1. Among 133 serum samples collected from butchers working in both urban and rural areas, 54 (41%) were antibody positive (Table 7.1). Median age of the butchers was 26 years with a standard deviation of 9.8 years. No significant difference in antibody response was found between the butchers from urban (33%; n = 14) and rural areas (44%; n = 40) (Table 7.1). Of the nine household members of butchers not being involved in slaughterhouse practices five (56%) showed a positive antibody response to O157 LPS. Statistically no significant difference was found in antibody response between butchers and their household members. In addition to butchers and their household members, serum samples from 91 participants who do not have regular contact (or occupational contact) with animals were analyzed for antibodies against *E. coli* O157 LPS. The median age of the participants was 25 years with a standard deviation of 11.8 years. Of these nonbutcher participants, 57 (63%) showed a positive response to *E. coli* O157 LPS. No significant

difference in antibody response was found between the participants from urban (61%; $n = 46$) and rural areas (73%; $n = 11$) (Table 7.1). Interestingly, more female participants were positive (77%; 23 out of 30) than male (56%; 34 out of 61) ($P = 0.05$).

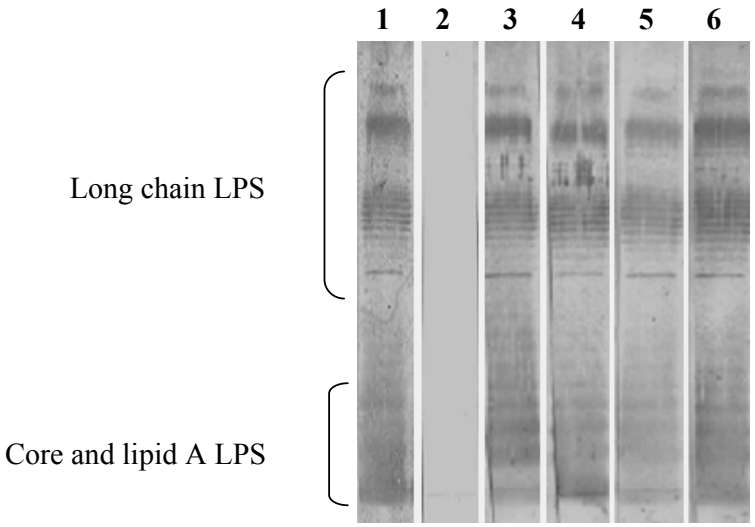

Fig. 7.1 Examples of immune responses of human serum samples to *E. coli* O157 LPS by Western blotting of *E. coli* O157 LPS with human serum samples. Lanes: 1, positive control; 2 negative control; 3-6, human serum samples.

All 116 samples positive for polyvalent antibodies were further tested separately for the presence of IgG and IgM-class antibodies by Western blotting. All 116 samples had IgG-class antibodies and 87 also had IgM-class antibodies (Table 7.1).

Table 7.1 Responses of human serum samples to *E. coli* O157 LPS determined by Western blot analysis

Source of serum samples (*n*)	No. of samples positive for antibodies to *E. coli* O157 LPS (%)		
	Polyvalent antibodies[a]	IgG	IgM
Butchers (133):	54 (41)	54 (41)	44(33)
Rural butchers (91)	40 (44)	40(44)	32(35)
Urban butchers (42)	14 (33)	14 (33)	12(29)
Household members of butchers (9)	5 (56)	5 (56)	5(56)
Other occupations (91):	57 (63)	57 (63)	38(42)
Urban residents (76)	46 (61)	46 (61)	32(42)
Rural residents (15)	11 (73)	11 (73)	6(40)

[a] Antibodies belonging to the IgG, IgA and/or IgM-class.

Overall, the presence of antibodies (IgG and IgM-class) was not significantly different between the rural and urban population (Table 7.2). A significantly higher number of participants from the nonbutcher population was positive for IgG-class antibody in comparison to the butcher population ($P < 0.001$) (Table 7.2) and no statistically significant difference was observed in case of the presence of IgM-class antibodies (Table 7.2).

Table 7.2 Comparison of IgG and IgM-class antibody response by location and occupation of the participants

Location and occupation of participants	Antibody response (%)			
	IgG +	*P*	IgM +	*P*
Location				
Rural area (*n* = 111)	54 (49)	0.740	41 (37)	0.903
Urban area (*n* = 122)	62 (51)		46 (38)	
Occupation				
Butcher (*n* = 133)	54 (41)	< 0.001	44 (33)	0.185
Nonbutcher (*n* = 91)	57 (63)		38 (42)	

For all categories of participants, presence of serum antibodies to *E. coli* O157 was not significantly associated with the age of the participants (data not shown). Similarly, antibody response was not associated with blood groups of the participants (Table 7.3). Significantly higher numbers of participants from urban areas were antibody positive than the participants from rural areas as compared by their practice of using contaminated water for drinking and washing ($P < 0.001$) (Table 7.3). However, drinking of contaminated water was not significantly associated with the positive antibody response within the same population in rural or urban areas (Table 7.4).

Table 7.3 Characteristics of participants showing positive antibody response to *E. coli* O157 LPS

Variables[a]	Location of the participants (%)		*P*	Occupation of the participants (%)		*P*
	Urban (*n* = 60)	Rural (*n* = 51)		Butcher (*n* = 54)	Nonbutcher (*n* = 57)	
ABO typing						
A	11 (18)	14 (27)	0.251	14 (26)	11 (19)	0.403
B	19 (32)	20 (39)	0.406	17 (32)	22 (39)	0.432
O	22 (37)	14 (27)	0.301	16 (30)	20 (35)	0.539
Drinking MW/contaminated water	27 (45)	2 (4)	< 0.001	11 (20)	17 (30)	0.251
Washing with MW/contaminated water	58 (97)	34 (67)	< 0.001	35 (65)	50 (88)	0.004

[a] MW, water from municipal water supply system.

Table 7.4 Comparison of IgG-class antibody response and water-use patterns for drinking and washing by location and occupation of participants

Water-use pattern[a]	Location						Occupation					
	No. of rural participants (%):		*P*	No. of urban participants (%):		*P*	No. of butcher participants (%):		*P*	No. of nonbutcher participants (%):		*P*
	IgG +	IgG -		IgG +	IgG -		IgG +	IgG -		IgG +	IgG -	
Drinking												
MW/contaminated water	2 (1.8)	3 (2.7)	0.692	27 (22.1)	29 (23.8)	0.595	11 (8.3)	19 (14.3)	0.728	17 (18.7)	11 (12.0)	0.914
Tube-well/boiled water	52 (46.8)	54 (48.6)		35 (28.7)	31 (25.4)		41 (30.8)	61(45.8)		39 (42.8)	24 (26.4)	
Bathing and washing												
MW/contaminated water	32 (28.8)	36 (32.4)	0.767	57 (46.7)	48 (39.3)	0.018	33 (24.8)	50 (37.6)	0.840	55 (60.4)	33 (36.3)	0.328
Tube-well water	19 (17.1)	24 (21.6)		4 (3.3)	13 (10.6)		19 (14.3)	31 (23.3)		1 (1.0)	2 (2.2)	

[a] MW, water from municipal water supply system.

Table 7.5 Comparison of the water-use patterns of the participants for drinking and bathing/washing by their location and occupation

Location and occupation of participants	Water-use pattern[a]				
	Drinking water (%)		*P*	Bathing and washing water (%)		*P*
	MW/contaminated water	Tube-well/boiled water		MW/contaminated water	Tube-well water	
Location						
Rural area (*n* = 111)	5 (4.5)	106 (95)	< 0.001	68 (61)	43 (39)	< 0.001
Urban area (*n* = 122)	56 (46)	66 (54)		105 (86)	17 (14)	
Occupation						
Butcher (*n* = 133)	31 (23)	102 (77)	0.213	83 (62)	50 (38)	< 0.001
Nonbutcher (*n* = 91)	28 (31)	63 (69)		88 (97)	3 (3.3)	

[a] MW, water from municipal water supply system.

Water-use pattern for bathing and washing had no significant effect on antibody response among rural population but in urban areas a significantly higher number of people who used MW for bathing and washing were positive for antibodies than people who used tube-well water (Table 7.4). No significant difference in antibody response was observed between the butcher and nonbutcher populations by their practice of using contaminated water for drinking (Table 7.4). However, significantly higher numbers of nonbutcher participants were antibody positive than butcher participants who used contaminated water for bathing and washing (Table 7.3).

Very few numbers of participants complained on intestinal problems such as diarrhea and dysentery during the last two weeks before sampling: five butchers, and eight other occupations. No statistically significant association was observed between the occurrence of diarrhea and dysentery and antibody response in the case of butchers (data not shown).

7.4 Discussion

The complete (smooth) LPS structure of *E. coli* and related species may be divided into three distinct regions: (1) the hydrophobic lipid A moiety, responsible for the endotoxic properties of LPS; (2) the core oligosaccharide; and (3) the O-polysaccharide antigen which is easily accessible to the host immune system and leads to the generation of O-specific immune responses, thus the term O-antigen is commonly used. Detection of O-antigen specific antibodies has been used widely for routine diagnosis of STEC O157 infection when stool samples from patients are culture-negative (12). Any subclinical infection among the healthy population due to *E. coli* O157 may also be identified by serodiagnosis (detection of O-antigen specific IgM antibodies) in conjunction with culture identification.

Strains of *E. coli* express LPS with one of five core structures, termed R1, R2, R3, R4 and K-12 and many, but not all, strains of STEC, including O157, express an R3 LPS-core (3). Patients infected with STEC O157 may produce serum antibodies binding to epitopes on the R3 LPS-core (3). This binding to the R3 LPS-core is not always serogroup specific. It has been demonstrated that an R3 LPS-core is widely distributed within the species *E. coli* and is not always associated with a specific pathotype (15). In addition to different STEC serotypes, R3 LPS-core is also found in enteroaggregative (EAEC) and enteropathogenic *E. coli* (EPEC) (15). Thus, based on binding with only the R3 LPS-core, it cannot be concluded that antibodies to *E. coli* O157 are present in serum samples collected from individuals suspected

with STEC O157 infection. Several studies showed that serum samples from STEC O157-infected HUS patients bind specifically to the long chain LPS region of STEC O157 in Western blotting and do not show nonspecific binding with other LPS antigens (13, 21).

In this study, we determined the serum samples positive for antibodies to *E. coli* O157 only when we detected reaction against both core components and long chain LPS (Fig. 7.1). We analyzed all the samples by Western blotting and did not apply ELISA or micro-agglutination assays. It has been suggested by previous studies that such assays should always be confirmed by Western blotting (13, 14).

Of the 233 sera, 116 (50%) reacted to both core and long chain LPS epitopes of STEC O157 when tested with anti-human polyvalent antibodies (IgG, IgA and/or IgM). Classification of antibody response to *E. coli* O157 LPS revealed that all samples gave a positive response with the polyvalent antibody (50%; $n = 116$), were positive for IgG, which is indicative of repeated exposure to the antigen. Around 37% ($n = 87$) of the samples were also positive for IgM-class antibodies, which is indicative of recent exposure to the antigen. Significantly higher numbers (63%) of participants other than butchers were positive for IgG-class antibodies than butchers (41%) ($P < 0.001$) (Table 7.2). However, no statistically significant difference was observed between butcher and nonbutcher populations with respect to the presence of IgM-class antibodies (Table 7.2).

In this study, we found no statistically significant association between the antibody response and age of the participants. Similar results were observed in studies conducted among rural Wisconsin population in the United States (6) and among healthy people in Thailand (46). In these studies, O157 LPS antibodies were not associated with increasing age, sex, manure contact, or sheep contact.

Our data suggest that people in Bangladesh are frequently exposed to an antigen or antigens that produce antibodies to the *E. coli* O157 LPS antigen. However, occupational exposure to animals is not significantly associated with the positive antibody response against *E. coli* O157 among the Bangladeshi population. High seropositivity may be due to cross-reacting antibodies of many other intestinal pathogens that are very common in Bangladesh in addition to *E. coli* O157. It has been shown that the O157 LPS antigen can be expressed by different organisms, including nonpathogenic *E. coli* (39), *Citrobacter freundii* (7), *Escherichia hermanii* (36), *Yersinia entercolitica* O9 (10), *Salmonella* O:30 (35) and *Brucella abortus* (36). It has also been reported that the serum of patients with HUS associated with *E. coli* O157 reacted with strains of *Vibrio cholerae* O1 Inaba. In another study with serum from individuals vaccinated against *V. cholerae* O1 (11), the sera were seen

to react strongly against O157 LPS. *V. cholerae* O1 is endemic in Bangladesh, occurring twice yearly in epidemic form (1). It has been identified as the leading cause of cholera in Bangladesh (40). Contaminated water is the main source of transmission of *V. cholerae* in regions where clean drinking water is not available to local populations (40). According to previous study, *V. cholerae* are native to both marine and fresh water environments in Bangladesh indicating that people are repeatedly exposed to the organisms either directly or indirectly (40).

Cross-reacting antibodies were also described in situations where enteric pathogens sharing common epitopes are endemically present (34). For example, infections with EPEC in early childhood confer cross-reacting protective immunity against STEC types that share common antigens (such as LPS and intimin) with classical EPEC strains (4). There is a huge burden of EPEC infection in Bangladesh and children below five years of age are most susceptible (2). EPEC infections occur rarely in adults and this can be explained by repeated exposure in early childhood conferring to protection later in life (4).

It would have been interesting if we could analyze the serum samples for antibodies against cross-reacting antigens. Nevertheless, antibodies to the *E. coli* O157 LPS antigen possibly represent exposure to *E. coli* O157, especially as examples exist of asymptomatic carriage of *E. coli* O157 inducing an antibody response to O157 LPS (8, 49). This can be further explained by our previous studies, where we found that more than 82% of slaughtered cattle in Dhaka were positive for *stx* genes (25). More than 10% of the cattle were positive for STEC O157 and 14% for STEC non-O157 (25). Raw meats in the slaughterhouses were also highly contaminated with STEC O157 (M. A. Islam, A. S. Mondol, I. J. Azmi, E. de Boer, R. R. Beumer, M. H. Zwietering, A. E. Heuvelink and K. A. Talukder, Submitted for publication). Therefore, people who buy raw meats from the slaughterhouses and those who prepare the meat for cooking at home are also exposed to the organism. In Bangladesh, women are mainly responsible for preparing the food at home, and therefore, woman are more exposed than man within the same population who do not have direct contact with animals. This has been reflected by the data of the present study where we found more female (77%) were antibody positive than male (56%) among participants other than butchers (P = 0.05).

STEC infection occurs in humans through acquisition of the bacteria via consumption of contaminated food and/or drink. Raw or undercooked foods (ground meat or vegetables) from animal origin have been recognized as the most important vehicle for transmission of STEC O157 (19). However, people in Bangladesh are not accustomed to eating raw or

undercooked meat. Thus, direct contamination of raw foods with bacterial pathogens, including STEC O157 does not pose a significant threat to human health. Nevertheless, lack of knowledge on good hygienic practices and unsanitary situations in food preparation can cause cross-contamination of food and water. Moreover, people living in rural and semi urban areas in Bangladesh are directly exposed to cattle feces regularly as cattle farming are still a domestic practice in Bangladesh. Although urban residents are not exposed to animals directly, they live in a contaminated environment where many enteric pathogens are considered to be endemic. Besides, due to lack of proper drainage systems in city abattoirs large amount of feces are daily exposed to the environment, including water bodies.

Dhaka, the capital city of Bangladesh is one of the mega cities in the world where 11.3 million people are living within 227.8 km^2 of metropolitan area and 80% of the total population is dependent on municipal water supply systems. It has been found that the municipal water at initial points were acceptable for drinking according to WHO guidelines in microbiological point of view but water collected from the points of use (consumer taps) was contaminated with total coliform and fecal coliform and were unacceptable for drinking (M. S. Islam, International Centre for Diarrhoeal Disease Research, Bangladesh, unpublished data). The municipal piped water might be contaminated with sewage and depleting groundwater through leaky joints of inadequately maintained pipes facilitated by low pressure, intermittent delivery systems (41). Only 20% of people in Dhaka are connected to sewer and most of the rest use septic tanks that discharge into drains or the river. Storm water drains are blocked and malfunctioning and most water runs through natural channels into rivers. In a previous study it has been recommended that interventions to secure a high-quality municipal water supply for Dhaka's population are critical for preventing the spread of enteric pathogens through piped water sources (37).

In this study, we found that a significantly higher number of participants from urban areas was antibody positive than the participants from rural areas when compared by their practice of using contaminated water for drinking and washing ($P < 0.001$) (Table 7.3). This may be due to the fact that a significantly higher number of people in urban areas used MW/contaminated water for drinking and washing than the people in rural areas (Table 7.5). In fact, the only source of drinking water in rural Bangladesh is tube-well and water from this source is considered to be free of fecal contamination. In addition, the majority of the urban population (86%) is dependent on MW for bathing and washing, while a significant percentage (39%) of the rural population used tube-well water (Table 7.5). This was further explained by analyzing the data separately from rural and urban areas where we found a

significant association between the use of MW/contaminated water and positive antibody response among urban population but not among rural population (Table 7.4).

Drinking of MW/contaminated water had no significant association with positive antibody response between the butcher and nonbutcher population. However, significantly higher numbers of nonbutcher participants were antibody positive than butcher participants who used contaminated water for bathing and washing (Table 7.3). This may be due to the inclusion of higher number of nonbutcher residents from urban areas (n = 76) than from rural areas (n = 15) in the study. When tested separately, antibody response was not significantly associated with water-use pattern for drinking and washing/bathing within butcher or nonbutcher population (Table 7.4).

Although there is no report of contamination of surface water with STEC O157 in Bangladesh, it would be interesting to test the common sources of water for this pathogen. Recently, in India STEC O157:H7 has been isolated from the river Ganges, which is regularly used for personal hygiene, laundry, and utensil washing (23).

The results of the study are in concordance with the previous studies done in other developing countries, including Thailand and Mexico where high levels of serum antibodies against *E. coli* O157 LPS have been reported in healthy people (in Thailand: 23% positive for IgG and 12% for IgM; in Mexico: 20% positive for IgG) (32, 46).

The results of the present study suggest that Bangladeshi population, irrespective of the extent of exposure to animals are being exposed to the antigens that are capable of stimulating the production of antibodies against STEC O157. According to our previous study, prevalence of STEC infection among diarrheal patients is low (0.5%) compared with other enteric pathogens and there has been no report of HUS cases associated with STEC O157 infection in Bangladesh (24). The ubiquitous presence of antibodies to STEC O157 LPS among the mass population in Bangladesh can be the reason why there is a lack of STEC O157 infection, including HUS or HC in this country compared with other enteric infections.

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Chapter 8

General discussion and future perspective

8.1 Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) were first recognized as a human pathogens in 1982, in the United States, when strains of serotype O157:H7 caused two outbreaks of hemorrhagic colitis (74). In 1983, the association of *E. coli* O157:H7 and of several other STEC serotypes with sporadic cases of the classical hemolytic-uremic syndrome (HUS) was first described (43) and subsequently confirmed in a prospective study (44). Since then, epidemiological studies from different parts of the world established STEC as the major cause of bloody diarrhea and HUS in temperate climates and as an important cause of uncomplicated watery diarrhea in some geographic areas (85).

Over the last decade, remarkable changes in the epidemiology of human infection and in the transmission routes of STEC have occurred. Although serotype O157:H7 is predominantly associated with human infections, more than 200 other STEC serotypes have been identified of which over 100 have been associated with disease in humans (23). Ruminants are considered as the most important reservoir for STEC. There are many routes by which STEC organisms can be transmitted from ruminants to humans, but still foodborne transmission remains the most important cause of infection resulting from fecal contamination of the food.

The majority of studies have been done in the industrialized countries where STEC are considered as a major problem. There is still a gap of knowledge on the epidemiology of STEC in human infections, their sources and the transmission routes in developing countries. It is only in recent years that laboratories in developing countries have started to find out the importance of these pathogens as part of the spectrum of diarrheal diseases.

Bangladesh is an endemic zone for diarrheal diseases. Apart from a few sporadic efforts to find out the presence of STEC in diarrheal patients, no systematic study had been done on the epidemiology and ecology of STEC in Bangladesh. Therefore, the primary focus of this thesis was to gain insight in both the epidemiology of STEC infection in humans and the occurrence of STEC in the human food chain in Bangladesh. Because the use of appropriate techniques for detection and isolation is a prerequisite for revealing good estimates of prevalence rates, we also evaluated three commonly used methods for the detection and isolation of STEC O157 from animal feces and raw meats.

8.2 STEC in diarrheal patients

Acute infectious diarrhea is the second most common cause of death in children living in developing countries, surpassed only by acute respiratory diseases accounting for approximately 20% of all childhood deaths (48). Despite the use of oral rehydration therapy, deaths due to diarrhea in children aged less than five years are still estimated to be about two million per year (16). Besides mortality, the long-term effects of diarrheal illness on childhood health are extremely serious and include malnutrition and growth faltering (15). Aetiology of diarrhea is variable according to geographic and climatic conditions, host factors, and socioeconomic situations. The major aetiologic agents of acute diarrhea are diarrheagenic *Escherichia coli,* rotavirus, *Vibrio cholerae*, and *Shigella* spp. (36, 48). All are known to be endemic in essentially all developing countries. A study done recently on the incidence of aetiology-specific diarrhea in a birth cohort (n = 252) in rural Bangladesh has shown that enterotoxigenic *Escherichia coli* (ETEC), enteroadherent *E. coli* (EAEC), *Shigella* spp., *Campylobacter jejuni*, *Giardia* spp., and rotavirus were the most commonly isolated pathogens (32). A similar but more comprehensive study done in a hospital setting in Bangladesh found that like studies conducted in other developing countries, the major pathogens of childhood diarrhea were rotavirus, *C. jejuni*, ETEC, enteropathogenic *Escherichia coli* (EPEC), *Shigella* spp., and *V. cholerae*. Among six pathotypes of diarrheagenic *E. coli*, ETEC was found to be the most prevalent in both of these studies. However, a study done previously in the same hospital setting found that in children (up to five years of age) with diarrhea, enteropathogenic *E. coli* (EPEC) were the most prevalent (15.5%), followed by ETEC (12%), enteroaggregative *E. coli* (EAggEC) (9.5%) and diffusely-adherent *E. coli* (DAEC) (8.2%) (2). STEC were not detected in any of the children with diarrhea but were detected in five children without diarrhea; however, these isolates were not characterized further. These studies illustrate that the aetiology of diarrheal disease and the prevalence of diarrheagenic pathogens can change over time in an endemic setting for diarrhea like Bangladesh. These changes may be attributable for example to the emergence of new pathogens or climate change. But changes in detected prevalences can also be the result of improved surveillance systems and advances in microbiological detection techniques.

As far as STEC concern, none of the previous studies done in Bangladesh identified STEC as diarrheal pathogens. The reasons might be: 1) the lack of proper surveillance for STEC; 2) lack of technical capacity for identification of STEC; 3) STEC is not present; 4)

STEC is present but relatively few infections occur due to acquired immunity in the population; or 5) consumer habits intervene the transmission of STEC from sources to humans (cooking, hygiene).

In chapter 2, we described the prevalence and characteristics of STEC in patients with diarrhea in a hospital and in a community clinic in Bangladesh. A total of 570 stool samples collected from patients with diarrhea were tested for the presence of *stx* genes. Of these patients, 410 patients were hospitalized due to severe diarrhea and the remaining 160 patients attended the community clinic in an urban slum of Dhaka city. The prevalence of *stx* in stool samples was 2.2% (n = 9) among hospitalized patients and 7% (n = 11) among patients in the community. STEC strains could be isolated from 0.5% (n = 2) and 1.9% (n = 3) of the samples from hospitalized and community patients, respectively. No statistically significant association between the age of the patients and the presence of *stx* genes was observed. All *stx*-positive patients were clinically diagnosed as having uncomplicated diarrhea. Seven of the nine patients who were hospitalized and *stx*-positive were primarily diagnosed with a *V. cholerae* O1 infection. Therefore, the role of STEC in causing the diarrheal infection is still to be speculated. The five STEC isolates belonged to five different serotypes; no STEC O157 strains were isolated. All five isolates were positive in the Vero cell cytotoxicity test. In addition to the *stx*, one isolate from a hospitalized patient and one from a community patient were positive for the *eae* gene. All three isolates from the community patients and one isolate from the hospitalized patients were positive for the hly_{EHEC} gene. It has been suggested by previous studies that STEC having both the *eae* and hly_{EHEC} genes are more virulent and causing more often severe human infection than strains carrying only the *stx* genes (25, 68, 79). However, no significant difference in clinical symptoms was observed for patients from whom these more virulent STEC were isolated (n = 2).

The findings of this study corroborated with previous studies done in other South Asian countries, including India, Thailand and Vietnam where similar prevalences of STEC were found in patients with diarrhea (47, 52, 63). Based on the results of this study, it can be concluded that STEC are not a significant problem compared with other enteric pathogens in Bangladesh. However, this study generates some interesting questions concerning the absence of STEC infection in humans in Bangladesh where many enteric infections are considered to be endemic. These are: 1) is there any potential reservoir for STEC in Bangladesh?; 2) does there any intervention exist in the transmission route of STEC from their reservoirs to human patients?; 3) are the strains in animal reservoirs or in foods, if present, virulent enough to cause human infection?; or 4) is there a high prevalence of

antibodies against STEC, including serogroup O157 among the Bangladeshi population? These issues have been discussed in detail in the following parts of the thesis.

8.3 Detection and isolation methods for STEC O157

The low prevalence of STEC in patients with diarrhea compared to other diarrhea causing pathogens prompted us to estimate the prevalence of STEC in food and food producing animals in Bangladesh. Unlike the clinical samples, it is very difficult to isolate and identify STEC from food samples because of the presence of very low numbers of these organisms in samples with an abundance of background microflora. Although many studies have been done on the development of detection and isolation methods for STEC O157, only a few data are available on the comparison of different methods. Besides, the growing number of methods and the variety of commercially available test-kits make it difficult for a diagnostic laboratory to choose the most satisfying assay system if no evaluation data on these tests are publicly available (10). As a consequence, the prices of the tests remain an important criterion, which can be applied to all commercially available assay systems. The use of test systems that are not evaluated for the specificity and sensitivity with types of samples that the laboratory is mainly concerned might result in a risk of obtaining false-positive and/or false-negative results (10).

Traditional culture methods for the detection of STEC O157 are often inefficient due to the large numbers of other flora that either overgrow or mimic STEC O157. The sensitivity of the conventional culture method can significantly be increased by the application of an immunoconcentration step, which will result in less false-negatives. A commonly used method, which is also implemented in the international standard for the detection of STEC O157 in food and feeding stuffs (5), is immunomagnetic separation (IMS). Prior to plating onto selective isolation media, STEC O157 cells present in the enrichment culture are selectively concentrated by magnetic beads with O157-specific antibodies covalently bound onto their surface. The IMS procedure can be performed both manually and automatically.

In chapter 3, we described the evaluation of two commercially available test systems for STEC O157, both based on immunoconcentration of these bacteria: a manual IMS method using Dynabeads anti-*E. coli* O157 and the Vitek Immunodiagnostic Assay System (VIDAS) Immunoconcentration *E. coli* O157 (ICE) kit. Additionally, we compared two selective isolation media for STEC O157; sorbitol–MacConkey agar with cefixime (0.05 mg/l) and

tellurite (2.5 mg/l) (CT-SMAC), and CHROMagar O157 with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) (1/2CT-CHROMagar). A total of 997 samples comprised of animal feces and raw meats have been examined by both the Dynabeads anti-*E. coli* O157 method and the VIDAS ICE method. By using the Dynabeads anti-*E. coli* O157 method systematically more fecal samples were identified as being positive (Binomial test, $P < 0.05$). However, for meat samples, no systematic difference was found between the methods (Binomial test, $P \geq 0.05$). The relative sensitivity, specificity, and accuracy of the VIDAS ICE method in comparison with the Dynabeads anti-*E. coli* O157 method was 63.0%, 99.3%, and 96.7%, respectively, for fecal samples and 55.6%, 99.1%, and 98.1%, respectively, for meat samples. When the results obtained with CT-SMAC and 1/2CT-CHROMagar were analyzed separately, a systematic difference between the two immunoconcentration systems was observed only in case of fecal samples examined in combination with 1/2CT-CHROMagar (Binomial test, $P < 0.05$). The relative accuracy of the VIDAS ICE method compared with the Dynabeads anti-*E. coli* O157 method was 97.8% when CT-SMAC was used as the final isolation medium and 96.7% when 1/2CT-CHROMagar was used. By using the VIDAS ICE method, the entire immunoconcentration process is performed automatically within 40 min, for a maximum of 30 samples (including controls) in one run. The concentration of 30 samples with the Dynabeads anti-*E. coli* O157 procedure takes about 100 min. For the safety of the user and a saving of time, in case of meat samples it is recommended to prefer the VIDAS ICE method to the manual Dynabeads anti-*E. coli* O157 procedure. Although the performance of the Dynabeads anti-*E. coli* O157 procedure was statistically significantly better than the VIDAS ICE method for animal feces, the latter method can be a good choice in case of prevalence studies at the farm level, when more than one sample from the same origin (same farm, same herd etc.) is examined. One of the major drawbacks of the VIDAS ICE method is the high price. The cost per sample (materials and reagents) is almost three fold higher in case of the VIDAS ICE method compared with that of the Dynabeads anti-*E. coli* O157 method.

Comparison of the performance of CT-SMAC and 1/2CT-CHROMagar revealed that for animal feces, systematically more samples were found positive when 1/2CT-CHROMagar was used as the plating medium (McNemar test, $P < 0.05$), irrespective of the method of immunoconcentration carried out. For meat samples, the use of 1/2CT-CHROMagar also resulted in more positives than CT-SMAC. However, this difference between the two plating media was statistically significant only in combination with the Dynabeads anti-*E. coli* O157 (Binomial test, $P < 0.05$) but not with the VIDAS ICE method (Binomial test, $P \geq 0.05$). Therefore, it is recommended to use 1/2CT-CHROMagar as a standard isolation medium in

addition to CT-SMAC, following immunoconcentration with either the Dynabeads anti-*E. coli* O157 or the VIDAS ICE kit.

Although IMS offers significant advantages in the detection and isolation of STEC O157, the successful application of this culture-based method is still dependent on the expression of the physiological characteristics and/or metabolic activities of the bacterium (78). PCR-based methods are considered as a good alternative because the presence of small amounts of species- or strain-specific DNA is being detected rather than unique aspects of target organisms to be in a specific physiological state. For STEC O157, Abdulmawjood et al. (1) developed a diagnostic PCR assay based on the amplification of sequences for the *rfb* (O-antigen-encoding) region of *E. coli* O157 (*rfb*E_{O157}) gene. The diagnostic application of this PCR assay was subsequently validated by means of a multicenter validation study (1). In chapter 4, we described the evaluation of the performance of the IMS-based method using Dynabeads anti-*E. coli* O157 and the *rfb*E_{O157} PCR assay by testing series of animal feces and raw meats artificially contaminated with strains of STEC O157 isolated from different sources. IMS is laborious and time-consuming but ends up with the isolation of the pathogen. On the other hand, PCR is fast and less laborious but it can only be used for screening purposes, so a further culture step is required to isolate the organism. Although IMS is considered as the official standard method for detection and isolation of STEC O157 from food and animal feeding stuffs (5), no standardized protocol has yet been described for animal feces.

The sensitivity of PCR detection systems depends on the choice of reaction conditions and interactions with the particular sample matrix (78). PCR is inhibited by numerous compounds often found in feces and food (14), providing a practical hindrance to the molecular-based detection of small numbers of contaminating pathogens (4, 66). One way to address these limitations is the use of effective sample processing as well as DNA extraction techniques. However, in many cases a technique optimized for one matrix or microorganism is not readily adaptable for use with others. Moreover, because purification adds to the time and expense of sample preparation, as well as to the loss of target nucleic acids, a more satisfying approach to the problem of PCR inhibition would be to reduce interference rather than attempt to remove all of the offending substances. To this end, various additives have been included in the PCR mixture to minimize inhibition. Among these, bovine serum albumin (BSA) is widely being used (49). We also found that BSA at a concentration of 1 mg/ml in the PCR mixture significantly reduced the effects of inhibitory substances present in both feces and meats. This was confirmed by using an internal amplification control in the

PCRs. The use of an internal amplification control in the PCR can detect the level of reaction inhibition and therefore eliminates the chances of false-negative results (1).

IMS was found to be more effective than PCR for both animal feces and meat samples. Hence, use of only PCR in the routine diagnosis of STEC O157 in animal feces and meats might result in a lower number of positives. Furthermore, the detection efficiency of the PCR method was dependent on the origin of the fecal samples. The detection efficiency of the IMS method was not affected by the origin of the fecal and meat sample used for spiking. However, it is sometimes insensitive due to the overgrowth of background flora, which can mimic the characteristics of target organisms (20). This can happen particularly for those samples that contain a high load of indigenous flora, for example, animal feces. Detection of *stx* genes by PCR does not necessarily indicate that a sample has live, infectious bacteria because dead cells or free DNA would also be detected. In contrast, culture-based methods may result in false-negatives as STEC O157 organisms have been reported to enter a viable, nonculturable state under certain environmental conditions (92). Viable, nonculturable STEC O157, which may be potentially infectious, would not be detected by culture methods but would be detected by PCR. Therefore, the best approach to detect and isolate STEC O157 is to use both PCR and an IMS-based culture method, simultaneously.

8.4 STEC in the food chain

STEC have been shown to be present in the intestinal tract of many domestic and wild animals, with carriage rates being especially high in ruminants (28). Transmission of the organisms generally by a fecal route can lead to contamination of foods derived from these animals as well as other foods. It has been shown many years ago that *E. coli* isolated from the feces of animals at slaughter can be isolated throughout the food chain (82, 83). Most of the large outbreaks of STEC infection across the world have been associated with contaminated foods. Due to the very small infectious dose, a few organisms (< 100 CFU) present in the food can cause infection (69). The following two chapters described the results of surveys on the occurrence of STEC in rectal contents of slaughtered animals, raw meats, unpasteurized milk and fresh juices.

8.4.1 STEC in slaughtered animals

In order to get insight in the ecology of STEC in the food chain, the first step is to investigate the prevalence of these organisms in animal reservoirs. Numerous studies have been done in developed countries, where STEC are considered as emerging foodborne pathogens. However, there is still a paucity of information from the developing parts of the world and in Bangladesh no study had been done. In chapter 5, we described the results of investigation of fecal samples for STEC collected from buffalo (n = 174), cows (n = 139) and goats (n = 110) in a slaughterhouse located in Dhaka. We focused on these animal species, since ruminants are considered as the primary reservoirs for STEC. In order to avoid cross-contamination, a piece of large intestine containing fecal material (~5 cm), 1-1.5 cm away from the rectum was excised aseptically. The highest prevalence of *stx* genes was found in buffalo (82.2%), followed by cows (72.6%) and goats (11.8%). STEC could be isolated from 37.9%, 20.1% and 10.0% of the buffalo, cow and goat samples, respectively. STEC O157 strains were isolated from 14.4% of the buffalo, 7.2% of the cows, and 9.1% of the goats. STEC non-O157 strains were isolated from 23.6% of the buffalo, 12.9% of the cows, and 0.9% of the goats. From the majority of the samples positive for *stx* genes, no STEC could be isolated, indicating the absence of viable cells in the sample or the limitations of suitable culture media that are capable of isolating all STEC non-O157 serogroups. Most of the STEC O157 (91%) strains were positive for the stx_2 gene only. In toxin-profiling studies of clinical STEC O157:H7 isolates, Ostroff et al. (67) demonstrated that patients infected with isolates carrying only stx_2 were 6.8 times more likely to develop severe disease than those infected with isolates carrying stx_1 or both stx_1 and stx_2. Another study showed that the carriage of stx_2 by an isolate increased its association with HC and HUS fivefold (13). Subtyping of the *stx* genes has a great importance in characterizing STEC isolates for their virulence potential to cause human infections. It has been found that STEC isolates carrying certain *stx* subtypes are more commonly associated with human infections (71). Therefore, it would be interesting to characterize the STEC O157 strains isolated in the present study by *stx* subtyping. Most of the STEC O157 strains in the study were also found to be positive for other virulence genes that are associated with increased human pathogenicity, including the *eae* (96%), hly_{EHEC} (93%), *katP* (93%), and *etpD* (93%). Phage typing results gave an additional indication that these isolates can be potentially pathogenic for humans. More than 77% (n = 35) of the STEC O157 isolates could be classified into seven phage types of which phage type 14 (24.4%) and 31(24.4%) were predominant. According to previous studies, phage type 14 is commonly found among STEC O157 strains from different sources, including human patients with HUS

(46, 60). Phage type 31 has been found in both human and nonhuman sources (meat and slaughterhouses) in Canada (46).

The STEC non-O157 isolates belonged to 36 O serogroups and 52 O:H serotypes, of which 32 were isolated from buffalo only, 14 from cows only, one from a goat and five from both cows and buffalo. The majority (42.3%, *n* = 22) of the STEC non-O157 isolates belonged to serotypes previously isolated from human patients, of which 32% (*n* = 7) were of serotypes associated with severe infections, including bloody diarrhea and HUS. In addition, 17 serotypes in this study were previously isolated from multiple origins, including human patients, cattle, milk or meat. Interestingly, 18 serotypes in this study belonged to serotypes not previously recognized as STEC serotypes.

Unlike the STEC O157 isolates, the majority (79%) of the STEC non-O157 isolates were positive for stx_1 only. Only 7% of the isolates were positive for the hly_{EHEC} gene and no isolates were positive for other virulence genes, including *eae*, *katP*, and *etpD*. Although these virulence genes are essentially important for pathogenesis, also STEC isolates lacking these genes have been reported to cause human infection. Many studies have been done on the role of the functional intimin encoded by the *eae* gene in the pathogenesis of STEC in human infection (69). The *eae* gene located on a chromosomal pathogenicity island, termed the locus of enterocyte effacement (LEE) is expressed by most, if not all, STEC O157 as well as STEC non-O157 isolates commonly isolated from humans (51). Animal isolates of STEC non-O157 carry the *eae* gene less frequently and this may in part explain the reduced incidence of these isolates in human disease. It has been suggested that the presence of the LEE is not always essential for pathogenesis, as a number of cases of severe STEC disease, including HUS, as well as occasional outbreaks were caused by LEE-negative strains (45, 70). Since all STEC non-O157 strains in this study were negative for *eae*, the isolates were tested for the presence of several novel adhesin genes, including *toxB*, *saa*, *sfp*, *iha*, *efa1* and *Lpf*. About 30% of the buffalo and 45% of the cow isolates were positive for the Lpf_{O113} gene. This is in accordance with a previous study where Lpf_{O113} was found as one of the most prevalent adhesin genes among STEC non-O157 strains (86). More than 8% (*n* = 4) of the buffalo and 18% (*n* = 4) of the cow isolates possessed the *saa* gene. Interestingly, 62.5% of the *saa*-positive isolates were also positive for hly_{EHEC}, which corroborated with a previous study remarking that there is a correlation between the presence of *saa* and hly_{EHEC} for certain serotypes (86).

Further characterization of STEC strains that belonged to the same serogroup (including STEC O157) by PFGE analysis revealed that there was no clonal relationship among the

strains. This was expected since animals delivered to the slaughterhouses normally arrive from different locations throughout Bangladesh. Animals within the same herd might have higher chances of carrying the same STEC type due to similar feeding habit (or regime), grazing conditions, and horizontal transfer of the organisms. However, even in a defined ecosystem such as a farm, the repertoire of *E. coli* strains is subject to continuous amendment and may contain several distinct DNA subtypes of a particular serogroup such as STEC O157 (31). New *E. coli* strains can be introduced by movement of cattle and other livestock into a farm, or possibly by animal feeds. Similarly water may play a key role in disseminating STEC due to high volume of daily intake. Water troughs may serve as a long-term reservoir where STEC O157 can persist at least several months, and especially in warm weather it may even multiply in this environment (53). Based on this study, it can be concluded that ruminants, such as buffalo, cows and goats serve as reservoirs for STEC in Bangladesh and can be potential sources of human infection.

8.4.2 STEC in foods

Contaminated food continues to be the principal vehicle for transmission of STEC O157 and other STEC to humans. The high prevalence of STEC, including those of serogroup O157 in slaughtered animals in Bangladesh prompted us to examine food samples, especially foods that are at risk of direct fecal contamination. A large variety of foods, including those associated with outbreaks (e.g., beef, fresh produce, unpasteurized milk and juices), have been the focus of intensive studies in the past few years to assess the prevalence of STEC (O157) and to identify effective intervention and inactivation treatments for these pathogens (26). One of the major routes of STEC transmission is via consuming undercooked ground beef. A recent review on the global testing of beef showed wide ranges of prevalence rates of STEC O157 (from 0.01% to 54.2%) and STEC non-O157 (from 1.7% to 62.5%) (37).

Milk and milk products have also been implicated in outbreaks of STEC infection (91, 94, 95). These outbreaks are generally caused by improperly pasteurized products, post-pasteurization contamination, or by raw milk and raw milk products. According to a recent review, prevalence of STEC in raw milk ranges from 0.75 to 16.2% (38). Foods from other origins can become contaminated passively by contaminated foods of animal origin. Produce can also become contaminated with STEC via manure applied to the fields, the use of contaminated irrigation or process water, poor worker hygiene, and poor equipment sanitation (11). Salad, lettuce, juice, melon and sprouts are the most frequently implicated food items in produce-associated outbreaks (84).

In chapter 6, we investigated the occurrence of STEC in three different categories of food, including raw meats (*n* = 87), raw milk (*n* = 20) and fresh juices (*n* = 103). More than 71% (*n* = 62) of the raw meat samples, 10% (*n* = 2) of the raw milk samples and 7.8% (*n* = 8) of the juice samples were positive for *stx* genes. Although high numbers of samples were positive for *stx*, only STEC O157 strains could be isolated from 8% (*n* = 7) of the raw meat samples. All isolates were positive for stx_2 and other virulence genes, including the *eae*, *etpD, katP* and hly_{EHEC}. The isolates belonged to three phage types (PT), including PT8 (14.3%), PT31 (42.8%) and PT32 (42.8%), and were genetically heterogeneous by PFGE analysis.

In Bangladesh, slaughterhouses are part of a big (open) market and no special premises are used for slaughtering the animals. Animals are eviscerated at the same place where carcasses are processed. Intestinal contents are dumped on the floors of the slaughterhouse after evisceration. Contamination of carcasses with feces is very common during processing. In most of the slaughterhouses, there is a shortage of water supply and lack of proper drainage systems. The high number of *stx*-positive raw meat samples in this study are therefore corresponding to the high prevalence of STEC in animal feces (chapter 6).

Dairy cattle farming are still a domestic practice in Bangladesh. Although there is a number of milk processing industries, which supply pasteurized milk all over the country, yet people in the rural areas and many city dwellers prefer to collect raw milk from small suppliers for their daily intake. Industries also collect bulk amount of milk from these small suppliers through cooperatives. Livelihood of many of these small suppliers is based on one or two dairy cows. People are used to milk the cows by hands. Milk can become contaminated with STEC during the milking process as a result of fecal contamination of the (exterior of the) udder. Personal hygiene and good hygienic practices during the milking process are very important to avoid contamination, which are often compromised due to lack of knowledge. Although no viable STEC were isolated from raw milk samples in this study, 10% of the samples were positive for *stx* genes.

For both raw meat and unpasteurized milk, bacterial contamination at the production level does not pose a significant threat to public health in Bangladesh. This is due to the food habits; people in Bangladesh are not accustomed to eat/drink raw or undercooked meat or milk. Normally meats are cooked with spices at boiling temperature at least for half an hour before it is eaten. Raw milk and even pasteurized milk are boiled before drinking. This cooking practice widely exists all over the country irrespective of the location, religion and social status. Therefore, raw meat or raw milk contaminated with STEC will not cause any human infection unless cross-contamination occurs to other foods especially the processed or

cooked ones. Nevertheless, people who are working at the slaughterhouses, doing domestic farming, or taking care of the animals might be at the risk of STEC infection and be the sources for person-to-person transmission.

Juice samples tested in this study were collected from street vendors from different areas within Dhaka city. People are used to drink these juices in raw. These vendors were selected because they operated under perceived high-risk conditions with respect to juice preparation, holding and serving practices and exhibited a noticeable lack of personal hygiene. Vendors usually congregate in overcrowded areas where there are high numbers of potential customers. Such areas usually provide limited access to basic sanitary facilities such as running water, garbage disposal and clean toilets. Such conditions have given rise to many concerns regarding the sanitary standards of street vending operations, especially because consumers are concerned about the price of food rather than its safety and hygiene in many cases. Although no viable STEC were isolated from juice samples, around 8% of the samples were positive for *stx* genes.

In this study, no STEC organisms were isolated from the raw milk and juice samples although a significant percentage of samples were positive for *stx* genes. The nonisolation of viable STEC might be due to: 1) the presence of a very low number of organisms in the samples, which could not be isolated by the methods used in the study; 2) the presence of only *stx* DNA or dead cells containing *stx* genes in the samples that gave positive results in the PCR; or 3) the presence of other organisms in the samples that carried the *stx* genes.

Based on this study it can be concluded that raw meats are readily contaminated with STEC O157 in slaughterhouses in Bangladesh. This highlights the poor hygienic conditions of slaughterhouses and the lack of good processing practices. Although the organisms have not been isolated, the detection of the *stx* genes in raw meats, raw milk and raw juices indicated the presence of STEC or other Shiga toxin-producing organisms in the samples. Therefore, the risk of human infection due to STEC cannot be ruled out if preventive measures such as thorough cooking of the food prior consumption or hygienic practices during food preparation are not taken into account.

8.5 Antibody responses to *E. coli* O157

Given the high prevalence of STEC O157 in animal reservoirs, and raw meats in the slaughterhouses, it can be stated that these organisms are present in the human food chain but

are not associated with any severe human infections in Bangladesh. According to our studies, it is clear that the conditions for transmission of these organisms from animals to humans exist, as do the inherent problems relating to basic sanitation. Therefore, the explanation that fits into this situation is the high prevalence of antibodies among human population in Bangladesh, which has also been suggested by other studies as a probable cause of the lack of STEC O157 infection in developing countries (62, 89).

The majority of enteric infections give rise to serotype-specific antibodies where LPS plays the key role. It has been suggested that the presence of anti-LPS antibodies might play a protective role in cases of STEC infections without extra-intestinal complications such as HUS (56). The protective role that these antibodies play is suggested in populations having frequent contact with cattle, such as dairy farm workers, who have higher serum titers of antibodies to *E. coli* O157 and a lower risk of symptomatic infection than does the general population (30, 73). However, in other studies, O157 LPS antibodies among the healthy population were found not to be associated with manure contact, and contact with animal (7, 89).

In chapter 7, we described the antibody responses against *E. coli* O157 LPS among healthy Bangladeshi population, including the people who are working in the slaughterhouses (butchers). A total of 233 sera were tested for antibodies against *E. coli* O157 LPS by Western blot analysis of which 116 (50%) were positive for polyvalent antibodies (IgG, IgA and/or IgM). Positive antibody response was found among 41% of the butcher participants and 63% of the participants with other professions ($P < 0.05$).

Further classification of the antibody response showed that all samples positive for polyvalent antibodies (50%; $n = 116$) were positive for IgG-class antibodies, of which 75% ($n = 87$) were positive for IgM-class antibodies. Overall, 37% ($n = 87$) of the samples were positive for IgM-class antibodies and no statistically significant difference was observed in IgM-class antibody response between the butcher and nonbutcher populations. These results suggest that Bangladeshi population, irrespective of their living standards and the extent of exposure to animals are being exposed to the antigens that are capable of stimulating the production of antibodies against STEC O157. Exposure to the antigens might be attributed either to the infection with STEC O157 or to other organisms present in the environment that express the O157 LPS antigens.

In our previous study, we found that more than 82% of the cattle, including buffalo, cows and goats in the slaughterhouses in Dhaka were positive for *stx* genes (40). More than 10% of the cattle were positive for STEC O157 and 14% for STEC non-O157 isolates (40). In

addition, 8% of the raw meats sampled in the slaughterhouses were positive for STEC O157 (chapter 6). Therefore, people can be easily exposed to the organisms either directly through animal contact or indirectly by consuming contaminated food or contaminated drinking and bathing water. Antibodies to *E. coli* O157 LPS can also be expressed due to infection with other organisms. According to previous studies, O157 LPS antigen can be expressed by different organisms, including nonpathogenic *E. coli*, *Citrobacter freundii, Escherichia hermanii, Yersinia enterocolitica* O9, *Salmonella* O:30, and *Brucella abortus*. Cross-reacting antibodies were also described in situations where enteric pathogens sharing common epitopes are endemically present. For example, infections with enteropathogenic *E. coli* (EPEC) and *Vibrio cholerae* O1 serotype Inaba were shown to stimulate antibody production that cross-reacts with STEC O157 (12, 19). It has been suggested that repeated exposure to EPEC in early childhood might confer to cross protection against STEC infections later in life (12). There is a huge burden of EPEC infection in Bangladesh and children below 5 years of age are the most susceptible (3). Both EPEC and *V. cholerae* O1 are endemically present in Bangladesh. *V. cholerae* O1, the leading cause of cholera is ubiquitously present in the surface water in Bangladesh, where it can be present throughout the year. Therefore, people are likely to be exposed to these organisms via drinking contaminated water or by using it for household practices.

This study demonstrated the association between the antibody response and demographic characteristics of the participants, including age, sex, geographical location (urban and rural), ABO blood grouping, occupational contact with animals and water-use patterns for drinking and washing. Statistically no significant association was observed between the antibody response and age and blood groups of the participants. Similarly, no statistically significant difference in antibody response was found between the participants from urban (61%; $n = 46$) and rural areas (73%; $n = 11$). A significantly higher number of participants from the nonbutcher population was positive for IgG-class antibodies than from the butcher population ($P < 0.001$) while no statistically significant difference was observed in case of the presence of IgM-class antibodies. This result indicated that the occupational contact with animals alone was not significantly associated with a positive antibody response against *E. coli* O157 LPS among Bangladeshi population. This was explained by the repeated exposure of the population to the contaminated environment through the use of contaminated water for drinking and bathing/washing. It has been found that more than 45% of the people in urban areas (Dhaka city) drink piped water supplied by a municipal water supply system (MW) without any prior treatment. In addition, a significant proportion of urban population (86%) is

dependent on the MW for bathing and washing. In previous studies, it has been found that the municipal water in Dhaka city at initial points was acceptable for drinking according to WHO guidelines from a microbiological point of view, but water collected from the points of use (consumer taps) was contaminated with total coliform (TC) and fecal coliform (FC) and was unacceptable for drinking (M. S. Islam, International Centre for Diarrhoeal Disease Research, Bangladesh, unpublished data).

In this study, a significantly higher number of people in urban areas were positive for antibodies than in rural areas as compared by their practice of using MW/contaminated water for drinking and bathing/washing ($P < 0.001$). This might be due to the fact that most of the people in rural areas (95%) drink tube-well water, which is considered to be safe for drinking. Besides, a significant percentage of people in rural areas (39%) use tube well water for bathing and for washing the household utensils. Water-use pattern for bathing and washing had no significant effect on antibody response among the rural population but in urban areas a significantly higher number of people who used MW for bathing and washing were positive for antibodies than people who used tube-well water ($P < 0.05$). In a previous study, it has been shown that bathing and washing utensils in tube-well water has a statistically significant protective effect against cholera in rural Bangladesh (76). However, in another study it has been shown that around 39% of tube-well water samples at the supply points do not meet the Bangladesh and WHO standards of FC (35). In this situation, contaminated water remains an important route of transmission of diarrheal pathogens to human populations both in urban and rural Bangladesh. A large number of people use unsafe water sources for personal and domestic needs due to the shortage of pure drinking water supply, lack of awareness and poor understanding about the link between use of contaminated surface water and disease. Although there is no report on contamination of surface water with STEC O157 in Bangladesh, it would be interesting to test the common sources of water for these pathogens.

This study concluded that the people in Bangladesh, irrespective of their location and the extent of animal exposure are positive for antibodies to *E. coli* O157, which might be attributable to the regular exposure to common sources of pathogens, including contaminated water. This finding explains the results of our previous study, which showed a relatively low prevalence of STEC compared to other enteric pathogens with no cases of STEC O157 infection among diarrheal patients in Bangladesh (chapter 2). The ubiquitous presence of antibodies to STEC O157 among the mass population in Bangladesh can be the reason why there is a lack of STEC O157 infection in this country.

8.6 Future perspectives

8.6.1 STEC epidemiology: situation of Bangladesh in a global perspective

In order to get a global perspective, we compared our findings of the prevalence of STEC in humans, animals and foods with similar data available in published reports from other countries, both from developed and developing parts of the world (Tables 8.1 and 8.2). We did not include the reports of STEC-associated HUS in the tables, since there are no such reports available in Bangladesh. In case of STEC infection in humans, it is not possible to compare the rates of infection directly between countries because of the variation in study design and microbiological methods applied. In the United States and European countries, there are active ongoing surveillance systems for monitoring of the prevalence of STEC in humans and other sources while the published reports from the developing countries are mostly based on the small-scale surveys. In order to make a fair comparison, we included only those data from published reports, which are comparable with our data. A major difference was observed in the prevalence of STEC O157-associated human infections. In our study we did not find any STEC O157 infection among diarrheal patients, which is correlated with similar studies done in other South Asian countries including India, Thailand and Vietnam. In contrast, although the infection rate is not very high, there is a prevalence of STEC O157 in patients with enteric infections or diarrhea in the United States and in European countries.

In case of animal reservoirs, there is a relatively high prevalence of STEC O157 in Bangladesh compared with that in other developing countries and this might be due to the methodologies applied for the isolation of the organisms. Nevertheless, the prevalence of STEC O157 in animals in Bangladesh is comparable with that in the developed countries. The prevalence of STEC non-O157 in animals was found higher than the prevalence of STEC O157 in Bangladesh and in other developing countries, which is comparable with the findings in developed countries. The occurrence of STEC has been studied in different types of foods depending on the risk of contamination with the organisms and a majority of these studies have been done with raw meats and meat products. In Bangladesh, we found that around 8% of the raw meats were contaminated with STEC O157, which is relatively higher than that found in most of the European countries. In our study we could not isolate any STEC non-O157 from food samples, although a high percentage of the samples were positive for *stx*

genes. There is still a gap of sufficient information on the prevalence of STEC non-O157 in foods due to the lack of appropriate methods of isolation for these organisms.

Table 8.1 A comparative analysis of the reported occurrences of Shiga toxin-producing *E. coli* O157 in humans, animals and food vehicles from developed and developing countries

Countries	Occurrence of STEC O157[a]				Ref.
	Humans		Animal feces[b]	Food[b]	
	Infection rates per 100,000 of the population	(%) in diarrheal patients			
Denmark	0.49	NR	3.08%, young bulls/steers	NR	6, 64
Ireland	2.77	4.42% (185/4185)	0.9%, cattle	1.5%, lamb carcass	17, 57, 54
Netherlands	0.34	0.12% (5/4069)	10.6%, adult cattle 0.5%, veal calves 3.8%, ewes 4.1%, lambs	0.2%, fresh meat (bovine) 0.1%, minced meat 1.1%, raw minced beef	24, 33, 34, 87
Scotland	3.25	NR	11.2%, beef cattle	0.24%, raw beef	6, 21, 65
UK	1.81	NR	4.7%, cattle 0.7%, sheep 0.3%, pigs	1.1%, raw beef 2.9%, lamb meat 4.1%, lamb sausages 3.7%, lamb burgers	6, 59
USA	1.20	3.04% (545/17883)	29%, cattle	10.9%, beef carcasses at preevisceration 0.7%, ground beef 1.1%, fresh ground beef 1.5%, sprouts	18, 29, 75, 77
Bangladesh	NR	0% (0/570)	14.4%, buffalo 7.2%, cows 9.1%, goats	8.0%, raw meats (beef and buffalo meat)	This study
India	NR	0% (0/1525)	3.3%, cattle 0.5%, goats 1.4%, sheep	0.9%, raw meats 1.8%, raw milk 8.4%, seafood 1.6%, water	47, 81
Thailand	NR	0% (0/493), 0% (0/580)	1.8%, bovine	3.4%, under-cooked beef 4.2%, retail beef	42, 88, 90
Vietnam	NR	0% (0/400)	2%, cow	NR	61

[a] NR, No report.

[b] The occurrences of STEC O157 in food and animal feces (individual animals at slaughter) are presented by the percentage of samples positive for the organisms among the total number of samples tested.

Table 8.2 A comparative analysis of the reported occurrences of Shiga toxin-producing *E. coli* non-O157 in humans, animals and food vehicles from developed and developing countries

Country	Occurrence of STEC non-O157[a]				Ref.
	Humans		Animal feces[b]	Food[b]	
	Infection rates per 100,000 of the population	(%) in diarrheal patients			
Denmark	2.03	NR	NR	NR	6
Ireland	0.43	0.52% (22/4185)	5.4%, cattle (at farm)	NR	17, 80
Netherlands	NR	0.61% (25/4069)	NR	NR	87
Scotland	0.21	NR	13%, beef cattle (same herd)	NR	6, 41
USA	0.57	1.45% (260/17883)	19.4%, beef cattle	2.4%, fresh ground beef 4.5%, sprout	18, 39, 77
Bangladesh	NR	0.5% (2/410), (hospital survey) 1.9% (3/160), (community survey)	23.6%, buffalo (at slaughter) 12.9%, cows (at slaughter) 0.9%, goats (at slaughter)	0% (0/20), raw milk 0% (0/103), fresh juice 0% (0/87), raw meat	This study
India	NR	0.72% (11/1525)	18%, cows 9.7%, calves 6%, lambs	3.6%, raw beef 3.3%, sea fish	47, 50, 93
Thailand	NR	0.95% (2/211)	11-84%, cattle feces	9%, market beef 8-28%, fresh beef	52, 90
Vietnam	NR	1.25% (5/400)	9.5%, cows	NR	63

[a] NR, No report.
[b] Occurrences of STEC non-O157 in food and animal feces (individual animal) are presented by the percentage of samples positive for the organisms among total number of samples tested.

In our study we explained the lack of STEC O157 infection in humans in Bangladesh by the high prevalence of antibodies against these organisms among the mass population. The low prevalence of STEC O157 infection in humans in other developing countries was also found to be associated with the high prevalence of antibodies against these organisms (62, 89). People in developing countries are frequently exposed to STEC O157 and other cross-reacting organisms via the contaminated environment, which may cause asymptomatic infections and elevate the antibody response (chapter 7). Based on the assumption that people are asymptomatically infected with STEC O157, it is recommended to study further on the virulence and/or pathogenic properties of STEC O157 strains isolated from animal and food sources for their potential to cause symptomatic infections in humans.

8.6.2 Selective and differential culture media for the isolation of STEC non-O157 serotypes

Worldwide the number of human illness outbreaks due to STEC non-O157 infection has increased dramatically in the past decade. Many efforts have been carried out to develop a suitable culture medium that can detect and isolate STEC organisms irrespective of their serotype. The major problem in detecting STEC non-O157 is that apart from producing Stx(s), they do not differ significantly in their biochemical characteristics from typical commensal *E. coli*. The only exception is the possible decreased ability to ferment carbohydrate-like substances (9). However, this characteristic has not been used in developing a medium for the detection of STEC in a manner similar to that of selecting most of the STEC O157 by using SMAC agar (58). Except for sorbitol, which is an excellent marker of sorbitol-negative O157 strains, there are no other single carbohydrate sources, which enable a quick and clear discrimination between serotypes. Durso et al. (22) reported that commensal strains were capable of utilizing a broader range of carbon sources than pathogenic STEC strains. Thus, it is the desire of microbiologists in this field to be able to select STEC non-O157 by using a specialized medium.

Recently, Possé et al. (72) has described two novel selective differential media for the isolation of STEC serotypes from food and feces. The first one was for the isolation of STEC non-O157 serotypes (O26, O103, O111 and O145) and the second one was for both sorbitol-positive and -negative STEC O157 strains. These media were developed based on a chromogenic compound to signal β-D-galactosidase activity and one or more fermentative carbon sources. The chromogenic marker and carbohydrates were combined with a pH indicator and several inhibitory components, which resulted in a color-based separation of these serotypes. This study also showed that the consecutive use of a serotype-dependent choice of confirmation media (dulcitol, L-rhamnose, D-arabinose, D-raffinose, and D-arabinose) resulted in a very low incidence of false-positive isolates when comparing clinical STEC strains with a collection of commensal *E. coli* strains (72).

8.6.3 Surveillance of STEC-associated HUS in Bangladesh

In our previous study, we estimated the prevalence of STEC infection among diarrheal patients in Bangladesh (chapter 2). However, it is also very important to estimate the prevalence of STEC infection among HUS patients in the country. At present, there are no data available on the burden of HUS and therefore the burden of STEC-associated HUS in Bangladesh is not known. In order to estimate the total burden of HUS in Bangladesh, it is

recommended to perform a prospective study involving all tertiary-level hospitals and some randomly selected secondary-level hospitals. All HUS cases, as defined according to the criteria described earlier (8), admitted to these hospitals should be included in the study. Clinical history of the patients should be recorded and stool and serum samples be collected and send to the laboratory for analysis. Stool samples should be tested for the presence of Stx, *stx* genes and STEC organisms including serogroup O157. The presence of other enteric pathogens in the stool, including *Shigella* spp., should also be tested. The serum samples should be tested for the presence of antibodies against Stx and LPS of STEC O157 and other predominant serogroups of STEC in order to detect any preceding infections. Serum samples that we collected previously from the healthy population in Bangladesh can be used as controls.

8.6.4 Cross reactivity of STEC O157 specific sera with other enteric pathogens

The LPS of *E. coli* O157 has been reported to cross-react with O-antigens of *Y. enterocolitica* O9, *C. freundii*, *E. hermannii*, and *B. abortus*. It has also been reported that the serum of patients with HUS associated with *E. coli* O157 reacted with strains of *V. cholerae* O1 Inaba. Similarly, cross-reactivities between the *E. coli* O157 serogroup and the *E. coli* O7 and O116 serogroups have been described (27). It has also been described that infection with EPEC in the early childhood may confer protection against STEC infection later in life (12). In our study, we found a high prevalence of antibodies against *E. coli* O157 LPS among the healthy population in Bangladesh (chapter 7). However, we did not test the serum samples for antibodies against these cross-reacting antigens. Therefore, it would be interesting to test the serum samples collected from all groups of the people for the cross-reacting antibodies. This will potentially generate some interesting findings to explain more clearly the lack of STEC O157 infection in humans in a situation where many other enteric pathogens are endemic.

8.6.5 Occurrence of STEC in surface water in Bangladesh

Both urban and rural water supplies throughout Bangladesh are frequently contaminated with human fecal organisms (55). Those who can afford it buy bottled water (of dubious quality), and the majority are left to drink the available contaminated water. The use of contaminated water remains an important route of transmission of diarrheal pathogens to human population in Bangladesh. In our study, we found a high prevalence of antibodies against STEC O157 LPS among healthy population in Bangladesh, which might be attributable to the regular exposure to common sources of pathogens, including contaminated

water (chapter 7). There is no report of contamination of surface water with STEC in Bangladesh. Therefore, it is recommended to test the water samples from commonly used sources in both rural and urban areas of Bangladesh for STEC including serogroup O157 and other organisms that express O157 antigens.

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Summary

Summary

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are one of the most important groups of foodborne pathogens. Infection with STEC in humans can lead to mild diarrhea, bloody diarrhea or the hemolytic-uremic syndrome (HUS). Domestic ruminants appear to be important reservoirs for STEC and play a significant role in the epidemiology of human infections. STEC infections are reported from different parts of the world, being found in 75 to 100% of episodes of sporadic HUS in Europe, North America, Canada, and Latin America especially in Argentina. Still STEC infections are a much greater problem in developed countries than in developing countries. Bangladesh is an endemic zone for diarrheal diseases caused by different enteric pathogens, however, no systematic study has yet been done here on the epidemiology of STEC infection in humans. The main objective of the research described in this thesis was to gain a detailed insight in the epidemiology of STEC infection in humans in Bangladesh and to find out the reservoirs and transmission routes of these organisms.

To estimate the prevalence of STEC in humans, we tested stool samples collected from patients with diarrhea attending a hospital (n = 410) and a community clinic (n = 160) in Dhaka, Bangladesh (chapter 2). Around 2% of the patients with diarrhea in the hospital and 7% of the patients in the community clinic were positive for *stx* genes. STEC strains were isolated from 0.5% (n = 2) of the patients in the hospital and 1.9% (n = 3) of the patients in the community. All five isolates were positive in the Vero cell assay. Two isolates were positive for the *eae* gene and four were positive for the hly_{EHEC} gene. The five isolates belonged to different serotypes and no case of STEC O157 infection was identified.

The relatively low prevalence of STEC among patients with diarrhea compared to other diarrhea-causing pathogens prompted us to estimate the prevalence of STEC in animal reservoirs and in the food chain in Bangladesh. Unlike clinical samples, it is difficult to isolate STEC from samples of animal faeces and food because of the presence of very low numbers of STEC in an abundance of competitive microflora. To identify an effective technique for isolation of STEC O157, we evaluated the efficiency of various methods (chapter 3 and chapter 4). Immunomagnetic separation (IMS) using Dynabeads anti-*E. coli* O157 proved to be more efficient than the Vitek Immunodiagnostic Assay System (VIDAS) Immuno-Concentration *E. coli* O157 (ICE) kit (VIDAS ICE) in case of animal feces and the use of CHROMagar O157 with cefixime (0.025 mg/l) and tellurite (1.25 mg/l) as plating media resulted in more positive samples than sorbitol-MacConkey agar with cefixime (0.05

mg/l) and tellurite (2.5 mg/l). Comparison of IMS with PCR for the *rfb*E_{O157} gene showed that IMS was more sensitive than PCR to detect STEC O157. In order to estimate the prevalence of these organisms in animal feces and foods we used both IMS and PCR techniques (chapter 5 and chapter 6).

Of the rectal contents collected from buffalo, cows, and goats, 82.2%, 72.7%, and 11.8% tested positive for stx_1 and/or stx_2, respectively. We isolated STEC O157 strains from 14.4% of the buffalo, 7.2% of the cows, and 9.1% of the goats. We isolated STEC non-O157 strains from 23.6% of the buffalo, 12.9% of the cows, and 0.9% of the goats. The majority of the STEC O157 isolates possessed virulence genes that are associated with increased human pathogenicity, including stx_2, *eae*, *katP*, *etpD* and hly_{EHEC}. The STEC non-O157 isolates belonged to 36 O serogroups and 52 O:H serotypes. In case of foods, we found that more than 71% (n = 62) of raw meats, 10% (n = 2) of raw cow's milk and 8% (n = 8) of fresh juice samples were positive for *stx* genes. We isolated STEC O157 strains from 8% (n = 7) of the meat samples and no other STEC could be isolated from the remaining *stx*-positive food samples. Subtyping of the STEC O157 isolates (n = 52) from animal feces and raw meats by pulsed-field gel electrophoresis revealed 42 distinct restriction patterns suggesting heterogeneous clonal diversity.

In order to explain the lack of STEC O157 infection in humans, we investigated the presence of antibodies against O157 lipopolysaccharide (LPS) among the healthy population in Bangladesh (chapter 7). We collected serum samples from different groups of people living in urban and rural areas, comprising butchers, household members of butchers, and people with other occupations. We found that around 50% (n = 116) of the samples were positive for antibodies (IgG, IgA and/or IgM) to *E. coli* O157 LPS. By doing separate analysis, we found that all 116 samples positive for polyvalent antibodies were positive for IgG-class antibodies and 87 were also positive for IgM-class antibodies. Of the 133 serum samples collected from butchers, 54 (41%) were antibody-positive. Among nine household members of butchers involved in other occupations, five showed a positive antibody response. Among participants other than butchers and their families, 57 (63%) showed a positive antibody response, which was statistically significantly higher than that of butchers ($P < 0.05$). We did not find any statistically significant difference in the prevalence of antibody response between the urban and rural populations. The high prevalence of antibody response might be due to the frequent exposure to STEC O157 present in the food chain or other organisms present in the environment that share common epitopes of O157 LPS. Like many other enteric pathogens, exposure of the Bangladeshi population to STEC O157 or

other cross-reacting organisms might be attributable to the use of contaminated water for drinking and washing/bathing. We found a statistically significantly higher number of participants from urban areas were positive for antibodies than participants from rural areas as compared by their practice of drinking contaminated water and using it for bathing/washing. We further confirmed this result by doing separate analysis of the results obtained for both urban and rural areas, where we found a significant association between the use of municipal water and positive antibody response to the *E. coli* O157 LPS among urban populations but not among rural populations. Overall results of this study concluded that the lack of STEC O157 infection among Bangladeshi population might be attributable to the protective immunity against this pathogen acquired by the frequent exposure to the antigens.

Finally, an extensive discussion concerning chapters 2 to 7 is described in chapter 8, which also includes suggestions for future research. The overall occurrences of STEC in diarrheal patients, animal reservoirs and foods in Bangladesh were compared with similar data available in other countries from both developed and developing parts of the world in order to get a global perspective. A major difference between our findings and findings from developed countries was observed in the prevalence of STEC O157-associated human infections. Unlike developed countries, no case of STEC O157 infection was found among diarrheal patients in Bangladesh. We concluded that the lack of STEC O157 infection could be due to the high prevalence of antibodies against these organisms among the mass population in Bangladesh.

Samenvatting

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Shigatoxine (Stx) producerende *Escherichia coli* (STEC) is één van de belangrijkere voedselpathogenen. STEC-infecties kunnen bij de mens milde tot bloederige diarree veroorzaken, maar ook soms resulteren in het hemolytisch uremisch syndroom (HUS) dat verantwoordelijk is voor nierfalen. Landbouwhuisdieren, vooral runderen en andere herkauwers, zijn een belangrijk reservoir voor STEC en spelen een essentiële rol in de epidemiologie van humane infecties. Rapportage van STEC-infecties komt wereldwijd voor en in Europa, Noord-Amerika, Canada en Latijns Amerika (vooral in Argentinië) is deze pathogeen verantwoordelijk voor 75-100% van de sporadische HUS-gevallen. STEC is een veel groter probleem in ontwikkelde landen dan in ontwikkelingslanden. Alhoewel Bangladesh een endemisch gebied is voor diverse enteropathogenen die diarree veroorzaken, is er tot op heden nooit systematisch onderzoek verricht naar de epidemiologie van humane STEC-infecties. Het belangrijkste doel van het onderzoek dat in dit proefschrift is beschreven, was het verkrijgen van een gedetailleerd inzicht in de epidemiologie van die infecties in de Bengaalse populatie.

Om het voorkomen van STEC te schatten, zijn faecesmonsters onderzocht van ziekenhuispatiënten van het ICDDR,B ziekenhuis (n = 410) en van patiënten afkomstig uit een locale kliniek in een achterstandswijk (n = 160), beide gevestigd in Dhaka, Bangladesh (hoofdstuk 2). Faecesmonsters van ongeveer 2% van de ziekenhuispatiënten en 7% van de patiënten uit de kliniek waren positief voor het *stx*-gen. Uit faeces van twee ziekenhuispatiënten (0.5%) en drie personen uit de kliniek (1.9%) werden STEC-stammen geïsoleerd. Alle vijf stammen waren positief in de Vero-cell test. Twee isolaten waren positief voor het *eae*-gen en vier waren positief voor het hly_{EHEC}-gen. De vijf isolaten behoorden tot verschillende serotypen, maar er werd geen STEC O157 infectie waargenomen.

De relatieve lage prevalentie van STEC bij patiënten met diarree in vergelijking met andere pathogenen die diarree veroorzaken, stimuleerde ons een schatting te doen naar het voorkomen van STEC in dierlijke reservoirs en in de Bengaalse voedselketen. Omdat in diermonsters en voedsel slechts lage aantallen STEC voorkomen tussen hoge aantallen van de begeleidende microflora, is het veel moeilijker hieruit STEC te isoleren dan uit klinische monsters. Verschillende methoden voor het aantonen van STEC O157 werden met elkaar vergeleken om de meest effectieve methode te kunnen vinden (hoofdstuk 3 en 4). Het bleek

dat voor het onderzoek van dierlijke faeces de immunomagnetische scheiding (IMS) met Dynabeads anti-*E. coli* O157 efficiënter was dan de VIDAS-ICE methode. Door hier 1/2CT-CHROMagar als isolatiemedium te gebruiken, werden meer verdachte kolonies verkregen dan met CT-SMAC. Een vergelijking van IMS met PCR voor het aantonen van het *rfb*E_{O157}-gen toonde aan dat IMS een gevoeliger methode was dan PCR voor het aantonen van STEC O157. Om het voorkomen van deze pathogenen in dierlijke faeces en levensmiddelen te schatten, werd gebruik gemaakt van beide technieken, IMS en PCR (hoofdstuk 5 en 6)

Het stx_1- en/of het stx_2-gen werd aangetoond in rectale monsters verkregen van buffels (82.2%), koeien (72.7%) en geiten (11.8%). Uit die monsters konden ook STEC-stammen geïsoleerd worden: buffels (14.4%), koeien (7.2%) en geiten (9.1%). Verder werden STEC-non O157 stammen geïsoleerd: uit buffels 23.6%, uit koeien 12.9% en 0.9% uit geiten. Het grootste deel van de STEC O157 isolaten bezat virulentiegenen die geassocieerd zijn met verhoogde humane pathogeniteit, waaronder stx_2, *eae*, *katP*, e*tpD*, en hly_{EHEC}. De STEC non O157 isolaten behoorden tot 36 O-serogroepen en 52 O:H-serotypen. Van de onderzochte levensmiddelen waren vooral vleesmonsters positief voor het *stx*-gen: 62 monsters van rauw vlees (71%), 2 monsters rauwe koemelk (10%) en 8 monsters vers sap (8%). Uit zeven vleesmonsters (8%) konden STEC O157 stammen worden geïsoleerd, maar niet uit de resterende *stx*-positieve monsters. Met behulp van pulsed-field gel electroforese konden 52 STEC O157 isolaten afkomstig uit faeces en rauw vlees onderscheiden worden in 42 duidelijke restrictiepatronen. Dit duidt op een heterogene klonale diversiteit.

Om te kunnen verklaren waarom er nauwelijks humane STEC-infecties voorkomen in Bangladesh, werd bloed van gezonde mensen getest op de aanwezigheid van antilichamen tegen O157 LPS (hoofdstuk 7). Van verschillende groepen mensen, zowel uit de stad als van het platteland, waaronder slagers, gezinsleden van slagers, en andere beroepsgroepen, werden serummonsters afgenomen en onderzocht. Circa 50% van de monsters (n = 116) waren positief voor antilichamen tegen *E. coli* O157 LPS (IgG, IgA en/of IgM). Uit verder onderzoek bleek dat alle 116 monsters die positief waren voor polyvalente antilichamen, ook positief waren tegen IgG, en 87 van de monsters waren bovendien positief tegen IgM. Van de 133 serummonsters afgenomen bij slagers, waren er 54 (41%) positief tegen antilichamen. Van de 9 gezinsleden met een ander beroep, hadden er vijf een positieve respons tegen de antilichamen. Bij de andere personen testten er 57 (63%) positief op de antilichamen, en dat was statistisch significant hoger dan bij de slagers ($P < 0.05$). Er werd geen significant verschil gevonden in antilichaamrespons bij slagers tussen stads- en plattelandsbevolking. Deze hoge antibody-respons kan het gevolg zijn van het veelvuldig in contact komen met

STEC O157 in de voedselketen, maar het kan ook veroorzaakt zijn door andere micro-organismen, die gemeenschappelijke epitopen hebben met O157 LPS. Zoals voor veel andere pathogenen geldt, kan de Bengaalse bevolking blootgesteld zijn aan STEC O157 (of aan andere, kruisreagerende micro-organismen) door contact met bad- of drinkwater dat met dierlijke uitwerpselen besmet is. Het drinken van, of wassen met besmet water in aanmerking genomen, waren er significant meer stadsbewoners positief voor antilichamen dan bewoners van het platteland. Dit werd ook nog eens bevestigd door een andere analyse van de data, waar een significant verband werd gevonden tussen het gebruik van water uit publieke bronnen en een positieve antilichaamrespons voor *E. coli* LPS bij stadsbewoners, maar niet bij de plattelandsbevolking. Uit alle resultaten van dit deel van het onderzoek bleek dat het geringe aantal STEC-infecties in de Bengaalse bevolking toegeschreven zou kunnen worden aan de immuniteit tegen deze pathogeen, die verkregen wordt door veelvuldige blootstelling aan antigenen.

In hoofdstuk 8 worden alle resultaten uit de hoofdstukken 2 tot en met 7 uitvoerig bediscussieerd. In dit hoofdstuk worden ook aanbevelingen gedaan voor verder onderzoek. Om het algemeen voorkomen van STEC in Bangladesh bij diarreepatiënten, in dierlijke reservoirs en in levensmiddelen in een globaal perspectief te kunnen plaatsen, werden de resultaten van dit onderzoek vergeleken met gegevens over deze pathogeen uit andere landen, zowel gelegen in ontwikkelde als in minder ontwikkelde delen van de wereld. Een belangrijk verschil tussen de resultaten van dit onderzoek en resultaten uit ontwikkelde landen was het voorkomen van STEC O157-geassocieerde humane infecties. In tegenstelling tot in ontwikkelde landen, werd er in Bangladesh geen STEC O157 infectie gevonden bij diarreepatiënten. De reden hiervoor kan te danken zijn aan de hoge titer antilichamen in de Bengaalse bevolking tegen deze pathogeen.

Curriculum vitae

Mohammad Aminul Islam was born on October 26th, 1976, in Faridpur, Bangladesh. After completing primary, secondary and higher secondary education from Dhaka, Bangladesh, he enrolled at the department of Microbiology, University of Dhaka and completed his BSc. (honors) in 2000 and MSc. in 2001. He conducted his MSc. thesis research on "phenotypic and genotypic characterization of *Shigella flexneri* type 4 isolated from diarrheal patients in Bangladesh" at the Enteric Microbiology Laboratory, International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). After completing his MSc. he joined the same laboratory at ICDDR,B as a research officer and worked on the molecular epidemiology of *Shigella* spp., among diarrheal patients in Bangladesh. In 2003, he was awarded a grant by the Netherlands Organization for Scientific Research (NWO-WOTRO) to work on a PhD research proposal. He started his PhD in the beginning of 2004 at the Food Microbiology Laboratory of Food and Consumer Product Safety Authority (VWA), Zutphen, the Netherlands in collaboration with the Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, the Netherlands and ICDDR,B, Bangladesh.

List of publications

1. **Islam, M. A., Md. M. Rahaman, A. E. Heuvelink, E. de Boer, M. H. Zwietering, H. Chart, A. Navarro, A. Cravioto, and K. A. Talukder.** Antibody responses to *Escherichia coli* O157 lipopolysaccharide among the healthy population in Bangladesh. Submitted.
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3. **Islam, M. A., A. S. Mondol, E. de Boer, R. R. Beumer, M. H. Zwietering, K. A. Talukder, and A. E. Heuvelink.** 2008. Prevalence and genetic characterization of Shiga toxin-producing *Escherichia coli* isolated from slaughtered animals in Bangladesh. Appl. Environ. Microbiol. **74:**5414-5421.
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11. **Talukder, K. A., B. K. Khajanchi, D. K. Dutta, Z. Islam, M. A. Islam, M. S. Iqbal, G. B. Nair, and D. A. Sack.** 2005. An unusual cluster of dysentery due to *Shigella dysenteriae* type 4 in Dhaka, Bangladesh. J. Med. Microbiol. **54:**511-513.
12. **Talukder, K. A., B. K. Khajanchi, M. A. Islam, D. K. Dutta, Z. Islam, A. Safa, G. Y. Khan, K. Alam, M. A. Hossain, S. Malla, S. K. Niyogi, M. Rahman, H. Watanabe, G. Balakrish Nair, and D. A. Sack.** 2004. Genetic relatedness of ciprofloxacin-resistant *Shigella dysenteriae* type 1 strains isolated in south Asia. J. Antimicrob. Chemother. **54:**730-734.
13. **Talukder, K. A., M. A. Islam, B. K. Khajanchi, D. K. Dutta, Z. Islam, A. Safa, K. Alam, A. Hossain, G. B. Nair, and D. A. Sack.** 2003. Temporal shifts in the

dominance of serotypes of *Shigella dysenteriae* from 1999 to 2002 in Dhaka, Bangladesh. J. Clin. Microbiol. **41:**5053-5058.

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15. **Talukder, K. A., M. A. Islam, D. K. Dutta, F. Hassan, A. Safa, G. B. Nair, and D. A. Sack.** 2002. Phenotypic and genotypic characterization of serologically atypical strains of *Shigella flexneri* type 4 isolated in Dhaka, Bangladesh. J. Clin. Microbiol. **40:**2490-2497.

Overview of completed training activities

Completed training activities

Discipline specific activities:	Credit points
Courses	
• Introductory course on epidemiology and biostatistics, ICDDR,B, Bangladesh, 2007	5.6
• VLAG course Management of Microbiological Hazards in Foods, Wageningen, The Netherlands, 2006	1.4
Conferences/meetings/training	
• Training on molecular typing techniques, Institut Pasteur, France, 2007	5.6
• 41st international basic training workshop Bionumerics and GelcomparII, Sint-Martens-Latem, Belgium, 2007	0.6
• 14th International Workshop on Campylobacter, Helicobacter and related organisms (CHRO), Rotterdam, The Netherlands, 2007	1.2
• Training on food microbiology, VWA, Zutphen, The Netherlands, 2004	5.6
• 11th Annual Scientific Conference (11th ASCON), ICDDR,B, Bangladesh, 2007	0.6
• Symposium Sectie Levensmiddelenmicrobiologie van de NVvM, Wageningen, The Netherlands, 2006	0.3
• 6th International Symposium on Shiga toxin (verocytotoxin)-producing *Escherichia coli* infections, Melbourne, Australia, 2006	1.4
• Enteropathogenic *Escherichia coli* (EPEC) pathogenesis workshop, Melbourne, Australia, 2006	0.3
• 11th Asian Conference on Diarrheal Diseases and Nutrition (ASCODD), Bangkok, Thailand, 2006	0.9
• 8th Commonwealth Congress on Diarrhea and Malnutrition, ICDDR,B, Bangladesh, 2006	0.9
• PulseNet Asia Pacific PFGE workshop, Hong Kong, 2006	1.4
General courses:	
• Career options in Microbiology: Bangladesh perspective, 2007	0.3
• Conference on promotion of biotechnology in Bangladesh: national and international perspectives, 2007	0.9
• Belgium-Netherlands-ICDDR,B symposium, ICDDR,B, Bangladesh, 2006	0.3
• Gender Awareness Workshop at ICDDR,B, Bangladesh, 2006	0.3
Optional courses and activities:	
• Preparation of PhD research proposal, 2003	6.0
• PhD study trip, South Africa, 2005	2.0
• Weekly participation, presentation and brainstorming in Journal club (Young Scientists), ICDDR,B, Bangladesh, 2006-2007	2.0
• Section talk Food Microbiology group, Wageningen, 2004-2007	2.0
• Section talk Enteric Microbiology group, ICDDR,B, 2004-2007	2.0

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