Molecular epidemiology of Shiga toxin-producing *Escherichia coli* in India

S. A. Wani*, F. Pandit, I. Samanta, M. A. Bhat and A. S. Buchh

Division of Microbiology and Immunology, Faculty of Veterinary Science and Animal Husbandry, S.K. University of Agricultural Sciences and Technology of Kashmir, Shuhama (Alusteng), Srinagar 190 006, India

Shiga toxin or verocytotoxin-producing *Escherichia coli* (STEC or VTEC) is recognized as an important food-borne pathogen, responsible for sporadic cases to serious outbreaks worldwide. The morbidity and mortality associated with several recent outbreaks due to STEC have highlighted the threat this organism poses to public health. Since its discovery in 1982, the organism has been the focus of extensive research on its epidemiology, pathogenesis, diagnosis, etc. Though the most important virulent attribute of the STEC is verotoxin or Shiga toxin, other virulence factors like intimin and enterohaemolysin have also been found to contribute to the human or animal disease. The organism may cause serious human complications like bloody diarrhoea, haemolytic uraemic syndrome, haemorrhagic colitis and thrombocytopaenic perpura. Domestic animals, especially cattle and sheep, are the main reservoirs and sources of infection. Contaminated meat and meat products, dairy products, vegetables, drinking water and swimming pools have been recognized as main vehicles of spread of infection to humans. Recently, air-borne dispersion (contaminated buildings) has also been incriminated as another route of spread of this infection to humans. There have been many advances in rapid diagnosis of this infection using the current molecular biology, immunological and cytotoxic methods. The present review describes the various virulence attributes, vehicles and routes of spread of infection and recent advances in diagnosis of this infection. Besides, the research work carried out in India on the subject is also summarized.

Historical background

Konowalchuk *et al.*[1] and O'Brien *et al.*[2] were the first to independently report a toxin from several *E. coli* strains active on cultured vero cells (hence the name verocytotoxin) and HeLa cells respectively. The cytopathic effect of the cytotoxin was quite different from the non-cytopathic effect of the classical heat-labile enterotoxin of enterotoxigenic *E. coli* (ETEC). This cytotoxicity was neutralized by an immune serum specific for the Shiga toxin from *Shigella dysenteriae* (*S. dysenteriae*) type1, hence the name Shiga-like toxin[3]. The discovery of these cytotoxins by different research teams resulted in parallel nomenclature system, although they are synonyms. The proposal to use the names 'Shiga toxins' for all the cytotoxins was made by Calderwood *et al.*[4], but did not receive unanimous approval.

The organism was initially isolated in USA in 1975 from a patient during an illness with severe abdominal cramps and bloody diarrhoea[5]. Canadian investigators[6], while screening for enterotoxigenic and cytotoxic *E. coli* strains from diarrhoeal patients, identified six isolates as cytotoxic *E. coli* O157 : H7 from 1978 to 1982. These findings from sporadic cases of gastroenteritis suggested that this organism might be a human pathogen. However, the conclusive evidence was provided in 1982 when association of *E. coli* O157 : H7 was firmly established with haemorrhagic colitis in two food-associated outbreaks of an unusual gastroenteritis[5]. The association between diarrhoea in calves and STEC was confirmed in 1985 by Mohammad *et al.*[7].

Introduction

E. coli, first described by Theodor Escherich in 1885, and a member of the family Enterobacteriaceae, is present as normal flora in the lower intestine of both humans and animals; however, some strains can cause gastrointestinal illness ranging from mild to cholera-like diarrhoea and may lead to potentially fatal complications, such as haemolytic uremic syndrome (HUS). *E. coli* is a genetically and phenotypically diverse species whose strains are identified on the basis of 'O', 'H' and sometimes 'K' antigens, which together constitute the serotype.

Based on toxigenicity, *E. coli* is now classified into distinct groups, viz. ETEC, which causes diarrhoea by producing heat-labile and/or heat-stable enterotoxins. Vero cytotoxin- or Shiga toxin-producing *E. coli* (VTEC or STEC) that causes severe disease in humans, such as haemorrhagic colitis (HC) and HUS by the production of Shiga toxin (Stx1, Stx2 and their variants). Those VTEC strains that are able to induce HC and HUS are called enterohaemorrhagic *E. coli* (EHEC). Necrotoxigenic *E. coli* (NTEC) is able to elaborate two types of cytotoxic necrotizing factors (CNF1 and CNF2)[8,9]. Enteropathogenic *E. coli* (EPEC), a term more recently used as a synonym for attaching and effacing *E. coli* (AEEC), is an emerging cause of diarrhoea in humans and animals[10].

The STEC family is diverse, and strains belong to a broad range of O : H serotypes[11]. More than 200 serotypes of STEC have been identified so far and more than 160 of these have

*For correspondence. (e-mail: microlab@sancharnet.in)

been recovered from humans with HC or HUS[12]. Epidemiological evidence, however, indicates that O157 : H7 accounts for a disproportionately large number of serious infections in humans. The other important serotypes[13] include O26 : H11, O111 : H-, O145 : H-, O45 : H2 and O4 : H-. The morbidity and mortality associated with several recent outbreaks of gastrointestinal disease caused by STEC have highlighted the threat these organisms pose to public health[14]. Distribution of STEC serogroups varies with geographical location. In Canada, most HUS cases are associated with a single *E. coli* serotype O157 : H7, although multiple other STEC serotypes also cause HUS. In Austria and Germany, non-O157 is associated with 43% of STEC-positive HUS patients. In Australia, O157 : H7 is rare and O111 strains are the common STEC type[15]. In contrast, in the US more than 80% of STEC infections are by O157 strains[16].

Shiga toxins of *Escherichia coli*

STEC strains have been found to produce a family of related cytotoxins known as Shiga toxins (Stxs). Stxs have been classified into two major classes, Stx1 and Stx2 coded by stx_1 and stx_2 genes respectively. Studies in the early 1980s established that stx_1 and stx_2 were encoded on a variety of bacteriophages[17]. However, toxin-converting bacteriophages have not been isolated from *S. dysenteriae* type1 or from STEC strains associated with piglet oedema disease[11]. One study has identified an insertion sequence element adjacent to an stx_1 operon in an O111 : H-STEC[18]. There was no duplication of target sequence at the insertion site, which raised the possibility that the segment of DNA containing the toxin gene was part of a transposon. However, the direct involvement of such mobile elements in transmission of *stx* genes is yet to be demonstrated. Involvement of bacteriophages and transposons may help explain why many STEC strains readily lose their *stx* genes after subcultivation *in vitro*[19].

A single STEC strain can produce Stx1 or Stx2, or both. Stx1 is virtually identical to the Stx of *S. dysentriae* type 1. stx_1 sequences derived from STEC strains and from three bacteriophages (H19B, 933J and H30) have been reported to be similar to that derived from *S. dysentriae*[20]. Variants of Stx1 have been described which only differ by one amino acid with no consequences on toxicity or on antigenicity. Zhang *et al.*[21] investigated the presence of an atypical Stx_{1OX3} variant among 212 STEC strains recovered from humans and renamed it as stx_{1c}. Unlike common stx_1 subtypes, stx_{1OX3} possesses 43 nucleotide mismatches compared to $stx_{1933\text{-}J}$, resulting in 12 amino acid changes[20]. In marked contrast to the expression of stx_2, expression of stx_1 in *E. coli* is regulated by host-derived signals such as the availability of iron and body temperature[22].

Stx2 is considered to be the most important virulence factor associated with human diseases. It is about 400-fold more toxic to mice than Stx1 and has also been shown to induce foetoplacental reabsorption, intrauterine haematoma, fibrin deposition and neutrophil infiltration[23]. Stx2 has approximately 55% amino acid identity to Stx1 and consists of at least 10 stx_2 gene variants, viz. stx_2, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, stx_{2vh} etc.[24]. stx_{2c} variant produced by the *E. coli* E32511 strain appears epidemiologically and clinically most important in humans. The Stx2e toxin is produced by *E. coli*-type strains E57, S1191 and 412 associated with oedema disease in weaned piglets[25]. Stx2e is 10,000 times less toxic and other Stx2 variants are 10–100 times less toxic for HeLa cells than for vero cells. The situation is the opposite for MDBK cells. These findings are related to the amount of Gb3 and Gb4 receptors on the cells[26]. stx_{2f}, a new variant of stx_2 was recently described in STEC isolates from pigeons[27].

Members of the Stx family are compound toxins (the holotoxin is approximately 70 kDa), comprising a single catalytic 32 kDa A-subunit and a multimeric B-subunit (7.7 kDa monomers) that is involved in the binding of the toxin to the specific glycolipid receptors on the surface of the target cell[28]. Biochemical cross-linking analysis suggested that the holotoxins of both Stx1 and Stx2 include five B-subunit monomers[29]. The crystallographic analysis of the Stx holotoxin demonstrated that the B-subunits form a pentameric ring, which encircles a helix at the C-terminus of the single A-subunit[30].

Pathogenesis of STEC

Multiple virulence factors contribute to the pathogenicity of STEC. Stxs inhibit the protein synthesis of host cells leading to cell death. Stxs play a major role in inducing vascular injury in the intestinal epithelium. More specifically, Stxs perturb cytokine expression patterns as a consequence of their interaction with epithelial cells[31].

The ability to adhere to intestinal epithelial cells and to colonize the human gut is undoubtedly one of the key determinants of virulence. The invasion process is dependent upon both bacterial protein synthesis and host cell microfilaments. It has been known for a decade that certain strains of STEC are capable of causing attaching and effacing (AE) lesion on enterocytes. Production of AE lesion is a multistep event initiated by adherence of bacteria to the microvilli, transduction of a signal into enterocytes, which is followed by aggregation of the cytoskeletal actin with effacement of microvilli leading to intimate attachment of the bacteria to the cell surface[32]. The formation of this AE lesion is mediated by secreted and surface-arrayed bacterial proteins encoded by a pathogenecity island called the locus for enterocyte effacement (LEE)[17]. LEE, which is inserted at 82 min in the *E. coli* chromosome, includes the *eaeA* gene that encodes intimin, a 939-amino acid (outer membrane) protein which mediates intimate attachment of the bacterium to the enterocyte. The LEE also encodes the receptor for intimin[33,34]. This receptor protein was previously referred to as Hp90, but has now been renamed translocated intimin receptor (Tir). Efficient delivery of Tir into the host

cell is dependent upon the type-III secretion system and other LEE-encoded secreted proteins. Different variants of the Tir molecule have also been described[17] and the different intimins and *tir* products are determinants of host tissue tropism[35]. Moreover, STEC strains invariably harbour a 97 kb plasmid encoding additional virulence traits such as STEC hemolysin, which acts as a pore-forming cytolysin on eukaryotic cells; the bifunctional catalase peroxidase *KatP*; a secreted serine protease (*espP*) and the *etpD* gene cluster which probably encodes a type II secretion pathway[36]. However, not all STEC strains from patients with HC and HUS harbour the LEE, indicating that intimin is not essential for human virulence. This raises the possibility that these strains produce additional yet uncharacterized virulence factors. Recently, Paton *et al.*[37] have described a gene, designated *saa* (STEC autoagglutinating adhesion), which is carried on the large plasmid of certain LEE-negative STEC strains. This gene encodes a novel outer membrane protein, which functions as an adhesin. However, no significant association was found between the *saa* gene and STEC isolated from patients with HUS and from healthy controls. Although other genes such as *iha*, *ure*C and *efa*I have been found in association with the genomes of a subset of STEC strains often referred to as EHEC, further studies are needed to determine their role in pathogenesis[12]. Pradel *et al.*[38] revealed for the first time that Twin arginine translocation system is required for the secretion of Stx and synthesis of the H7 flagellin, which are two of the major virulence factors specific to EHEC O157 : H7.

Sources and vehicles of spread of STEC

Cattle are known to be the most important reservoirs of STEC strains across the world. Other animals known to harbour STEC strains, with or without symptoms, include sheep, goats, pigs[39], buffaloes[7], horses[40], deer[41], wild boar, antelope, reindeer[42], birds[43], etc. STEC can potentially enter the human food chain most commonly through contamination with faeces or intestinal contents after slaughter as explained in Figure 1. The organism may also be transmitted from person to person through the faecal–oral route[31]. Recently, Varma *et al.*[44] have demonstrated another mode of spread of STEC in humans by investigating an outbreak of *E. coli* O157 : H7 infection in humans following exposure to a contaminated building.

Diagnosis of STEC

Currently, three main methods based on cytotoxicity, serology and gene detection in conjunction with isolation (Figure 2) are being followed for identification of STEC.

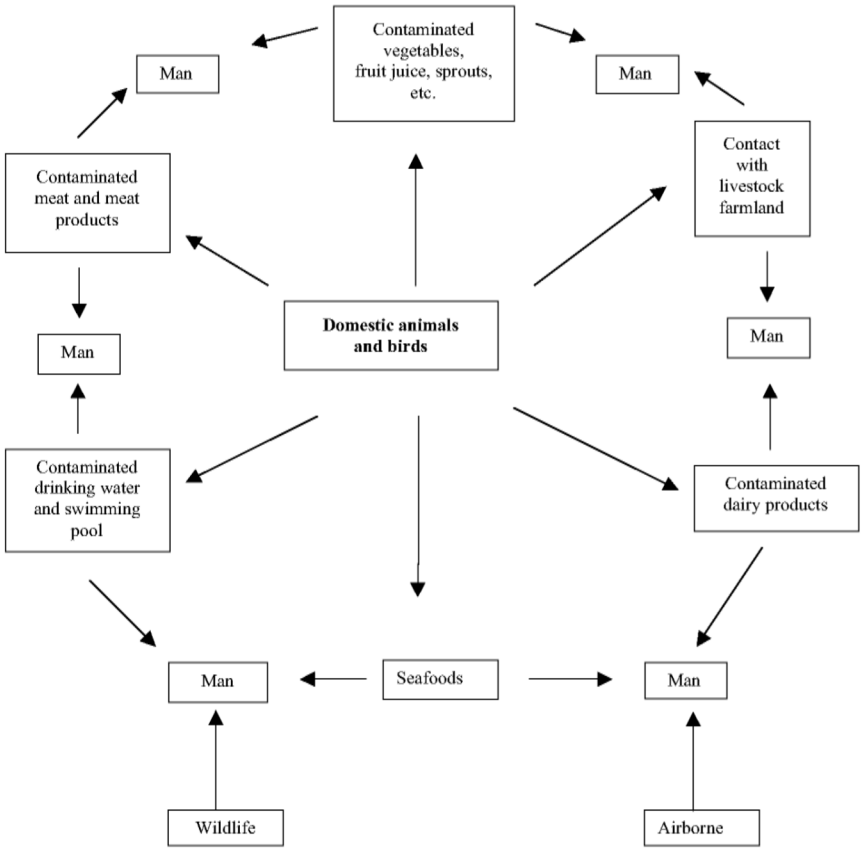

Figure 1. Sources and vehicles of spread of STEC to humans.

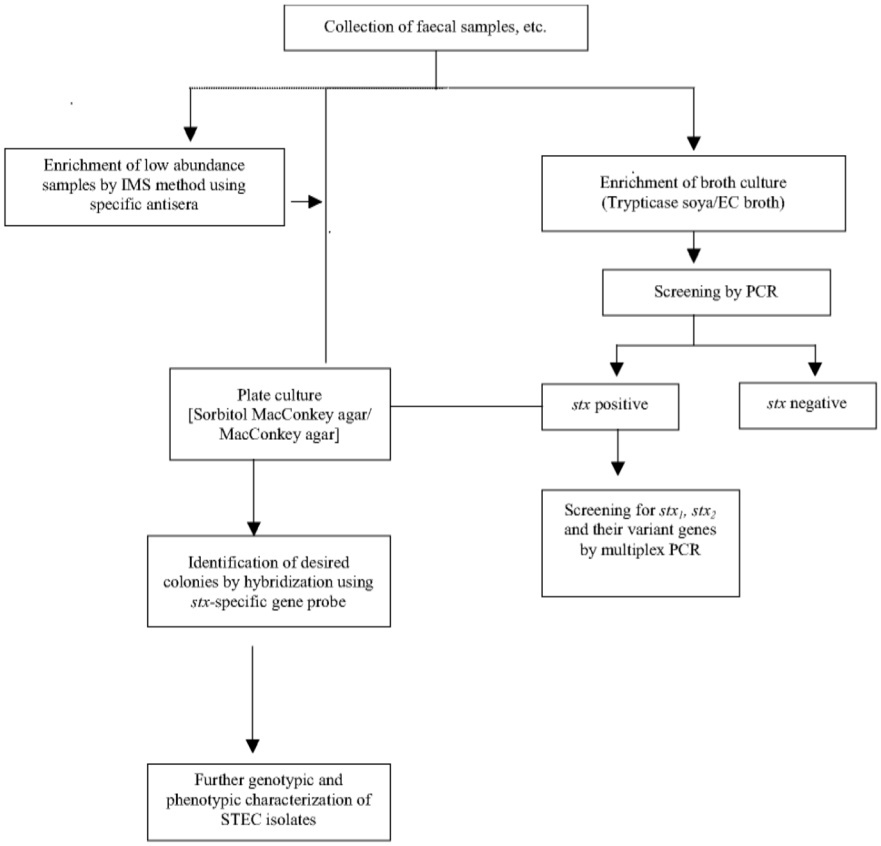

Figure 2. A simple and systematic procedure for isolation and characterization of STEC.

Cytotoxic assays

The profound cytotoxicity of Stx to vero cells remains the 'gold standard' for confirmation of putative Stx-producing isolates[17]. The assay involves treatment of vero monolayers (usually in 96-well trays) with sterile extracts or filtrates of test materials and examining cells for cytopathic effects after 48–72 h of incubation. The specificity of cell-culture cytotoxicity tests can be considerably improved by employment of Stx1- and Stx2-specific neutralizing antisera[45]. Since vero cells have a high concentration of Gb_3 and Gb_4 receptors in their plasma membranes, they can be used to detect all known Stx variants. HeLa cells have also been used, but this cell line lacks Gb_4 moiety and, therefore, is less sensitive to Stx2e[17].

Serological assays

Several immunoassays have been developed to detect Stx in culture supernatants, in bacterial extracts, or directly in faecal samples: sandwich and other ELISA techniques with immune sera, affinity-purified polyclonal antibodies, immobilized monoclonal antibodies or a Gb_3 receptor as a capture system, and reverse passive latex agglutination assay[17]. Over the years, a number of other immunological methods like colony blot and passive agglutination assays have also been developed. The major advantage of these immunological methods is ease and flexibility of use. Most of the ELISA methods involve a sandwich technique with immobilized monoclonal or affinity-purified polyclonal antibodies to the toxins as capture ligands. Reymond *et al.*[46] described a Western immunoblot assay for Stx1 antibodies, which was more specific and sensitive than either ELISA or cytotoxicity neutralization methods and proposed this as a more useful tool for seroepidemiological studies. Several commercial kits are available for STEC diagnosis.

DNA-based assays

The DNA-based assays are colony hybridization and polymerase chain reaction (PCR) assays. The colony hybridization assay with polynucleotidic probes was the first one to be used[47]. Non-radioactive labels such as digoxigenin and biotin have been used for detection of STECs without loss of sensitivity or specificity[48]. PCR-based techniques are used to determine if there is STEC in a sample at all, without having to purify the organisms or isolate separate colonies. Crude lysates or DNA extracts from single colonies, mixed broth

cultures, colony sweeps, or even direct extracts of faeces or foods can be used as templates for PCR. *stx*-specific PCR products are usually detected by ethidium bromide staining after separation of the reaction mix by agarose gel electrophoresis. A simple multiplex-PCR assay to detect stx_1, stx_2 and their variants was initially developed by Paton and Paton[49]. There are certain disadvantages of this PCR-based diagnostic method. (1) The examination of faecal samples by direct PCR can be a problem because substances inhibitory to PCR could be present in faeces or number of target organisms present in the test sample could be small, resulting in false negative or unclear results. (2) False positive results can be obtained if cryptic target gene sequences are present (free *stx* encoding phages or defective *stx* genes in bacteria)[50]. Subsequently, a rapid and specific multiplex PCR, which detected *rfbO157* gene (involved in the biosynthesis of O157 *E. coli* antigen) and main virulence genes (stx_1, stx_2 and *eaeA*) of STEC in the faeces were developed by Osek[51]. Direct PCR (DPCR) has also been described as a simple and rapid approach for the detection of STEC because the untreated environmental sample is used directly as a template in PCR, eliminating the steps of cell recovery or DNA extraction[52]. The ability of DPCR to detect dead cells or free DNA could be advantageous in some situations. The recent development of real-time PCR has added newer dimension of quantitation to the conventional PCR assays[53]. The technique has been applied to detect a number of pathogens, including STEC with the use of modified probes or oligonucleotides that are coupled with reporter and quencher dyes at the 5′ and 3′ ends respectively. In an intact probe, the quencher dyes suppress the flourescence emission of the reporter dye. The continuous measurement of incremental fluorescence of each PCR cycle provides an accurate estimate of the number of cells of a bacterial pathogen present in contaminated food and faecal samples even up to levels of 1–10 CFU/g of food or faeces[54].

The optimization of the reverse transcriptase PCR designed to detect viable STEC as recently developed should become a useful tool[55]. Ge *et al.*[56] combined PCR with ELISA to develop a sensitive and specific detection method for STEC in foods. The principle of the method is incorporation during the PCR amplification process, of digoxigenic-labelled dutp and a biotin-labelled primer specific for the *VT* genes. The labelled PCR products, bound to streptavidin-coated wells of a microtitre tray through the biotin, are then detected by an ELISA technique[57].

Isolation of STEC

Isolation of STEC strains must be considered the definitive diagnostic procedure. It permits additional characterization of STEC like O : H serotyping, antimicrobial drug resistance, phage typing, restriction fragment length polymorphism and amplification-based DNA typing.

Non-O157 STEC serogroups do not have any special biochemical feature, which can differentiate them from commensal *E. coli* strains during isolation. Thus their isolation involves the standard protocol for isolation of *E. coli*. The selective media for isolation of O157 : H7 and O157 : H^- STEC is Sorbitol–MacConkey agar (SMA). Most of these strains are unable to ferment sorbitol, which distinguishes them from majority of faecal *E. coli* belonging to other serotypes. SMA plates are inoculated with faecal specimens and examined after incubation for the presence of colourless sorbitol negative colonies. Individual colonies can be confirmed by slide or tube agglutination test using O157 and H7 antisera. Chapman *et al.*[58] improved the isolation rate of O157 by supplementing SMA with cefixime, to inhibit *Proteus* spp. and rhamnose to allow its fermentation by sorbitol negative non-O157 *E. coli* strains. Broth enrichment serves two purposes: inhibitors of PCR in the samples are diluted and bacterial growth increases the number of copies of the target sequence[17].

To avoid the problem of false negative results from low abundance specimens, Immunomagnetic separation (IMS) techniques have been developed to assist in the isolation of STEC (principally O157). The procedure involves coating magnetic beads with anti-LPS antibody and mixing them with broth cultures or suspensions of faeces or suspect food homogenates[17]. The beads and bound bacteria are then trapped in a magnetic field, the unbound suspension is decanted, and the beads are washed. After additional binding and washing cycles, the beads are plated and the resultant colonies are checked for reactivity with the appropriate O antiserum and, more importantly, for stx production. The principal drawback of IMS is, of course, its serogroup specificity. However, it is an extremely valuable enrichment technique, particularly under circumstances where STEC strains of known serogroups are being deliberately targetted, for example, when testing foods/water samples suspected of being the source of an outbreak[59]. The most comprehensive investigation of the efficacy of IMS for isolation of *E. coli* O157 is that of Karch *et al.*[60].

Indian perspective

In India, there is paucity of information on STEC. It has not been identified as a significant etiologic agent of diarrhoea for humans in India. However, isolation of O157 and non-O157 serogroups of *E. coli* that exhibited the cytotoxic activity in vero cells has been reported from human patients with diarrhoea in India, but these strains have not been well characterized, and their origin is uncertain[61]. Similarly, there are not many reports of isolation of STEC from various animal species in India. Pal *et al.*[62] were first to report the isolation of STEC from non-diarrhoeic animal sources in India. These workers collected faecal samples from 67 healthy cattle in a semi-urban community near Calcutta (now Kolkata) and examined these for STEC by multiplex PCR and culturing on SMA. STEC was isolated from the faeces of seven (10.5%) animals. The eight strains isolated belonged

to eight serotypes, viz. O146 : H1, O149 : HNT, ONT : H19, O88 : HN, ONT : H2, O82 : H8 and O28ac : H21. Bead enzyme-linked immunosorbent assay showed that three strains produced Shiga toxin 1, one produced Shiga toxin 2 and four produced both. Subsequently, Chattopadhyay *et al.*[63] isolated and characterized STEC strains from animal, human and food products. A total of 876 samples (330 animal, 184 human, 362 food samples) were screened for the presence of STEC by conventional as well as PCR techniques. Seventeen STEC strains (12, 1 and 4 from animal, human and food samples respectively) were isolated. The isolation rate was higher in diarrhoeic animals (6.02%), followed by diarrhoeic handler (3.12%) and raw beef (1.78%) samples. Though the STEC strains were isolated from different sources of animal and human origin, they showed a uniform antibiogram. This suggested a zoonotic association.

Kumar *et al.*[64] investigated the occurrence of STEC in fresh fish, shellfish and meat sold in open markets in Mangalore, India. Two out of 60 fish samples and two out of the 48 clam samples were positive for *stx* and *hlyA* genes by PCR. STEC strains belonged to non- O157 serogroups. They concluded that the seafood could be a vehicle for transmission of STEC even in tropical countries.

Again in Kolkata, Khan *et al.*[65] investigated the prevalence of STEC in hospitalized patients with diarrhoea, as well as in healthy cattle and raw beef samples collected from the city's abattoir. Multiplex PCR using primers specific for stx_1 and stx_2 genes detected STEC in 18% of cow stool samples, 50% of raw beef samples and 1.4% of bloody and 0.6% of watery stool samples from hospitalized patients. Various virulence genes in the STEC isolates indicated that stx_1 allele predominated. Plasmid-borne markers, namely *hly*A, *Kat*P, *esp*P and *etp*D were also identified. However, they concluded that STEC is not an important cause of diarrhoea in Kolkata, but its presence in cattle and beef samples suggests that this enteropathogen may become a major public health problem in the future.

The first systematic study on strains of STEC in India was carried out by Khan *et al.*[36]. In this study antibiotic resistance, virulence gene and other molecular profiles of STEC strains isolated from human stool samples, cow stool samples and beef samples over a period of two years in Kolkata, were determined. Resistance to one or more antibiotics was observed in 49.2% of the STEC strains, with some strains exhibiting multidrug resistance. The dominant combinations of virulence genes present in the strains studied were stx_1 and stx_2 (44.5% of strains) and stx_2 and *hlyA* (19% of strains). Only 6.4% of the STEC strains harboured *eae*. The diversity of STEC strains from various sources was assessed by random amplification of polymorphic DNA (RAPD). STEC strains that gave identical or nearly similar DNA fingerprints in RAPD–PCR and possessed similar virulence genotypes were further characterized by pulsed-field gel electrophoresis (PFGE). Identical RAPD and PFGE profiles were observed in four sets of strains, with each set comprising two strains. There was no match in the RAPD and PFGE profiles between strains of STEC isolated from cows and those isolated from humans. It appears that the clones present in bovine sources are not transmitted to humans in Kolkata, although these strains showed evolutionary relatedness. May be for this reason, STEC has still not become a major problem in India.

Wani *et al.*[66] reported for the first time the isolation and characterization of STEC serogroups associated with diarrhoea in calves and lambs in India. Many STEC and EPEC isolates belonged to serogroups known for certain life-threatening diseases in humans. About 249 bovine and 60 ovine *E. coli* strains were recovered from faecal samples of 391 calves and 101 lambs. Then 130 bovine and 15 ovine strains were subjected to multiplex PCR for detection of stx_1, stx_2, *eaeA* and EHEC *hlyA* genes. STEC strains belonging to different serogroups were detected in 9.73% of calves and 6% of lambs studied. Majority of the STEC isolates in this study did not belong to those serogroups which had been identified earlier to be mainly associated with diarrhoea and enteritis in cattle and sheep outside India. One of the most important serogroups, O157 known to cause certain life-threatening infections in humans, was isolated from both bovine and ovine faecal samples. Similarly, Wani *et al.*[67] reported an outbreak of bloody diarrhoea in 1–16-week-old crossbred calves in an organized dairy farm in Kashmir. Seven out of ten were affected. Affected calves were diarrhoeic for 4–5 days and voided blood tinged malodourous faeces. O116 was recovered from five calves with diarrhoea. The virulence gene profile revealed stx_1, *eaeA* and *hlyA* genes. In another study, association of STEC O4 : NM serotype with an outbreak of diarrhoea in 4–7-week-old calves was also demonstrated by Wani *et al.*[68]. Nine calves with diarrhoea revealed the presence of O4 : NM serotype. Six *E. coli* O4 : NM strains carried *eaeA* and EHEC *hlyA* genes and three possessed stx_1 in addition to *eaeA* and EHEC *hlyA* genes. Recently, we screened 426 *E. coli* strains isolated from 500 chicken and 25 free-flying pigeons. None of these strains possessed Shiga toxin genes. However, six and seven *E. coli* isolates revealed the presence of *eaeA* and *hlyA* genes respectively[69]. Likewise 136 *E. coli* isolates from rabbits with and without diarrhoea did not reveal the presence of STEC. However, *eaeA* gene was found in three isolates while another isolate showed the presence of *hlyA* gene (unpublished data).

Again, Kumar *et al.*[70] characterized STEC strains isolated from seafood and beef in Mangalore by bead-ELISA, vero cell cytotoxicity assay, PCR and colony hybridization for the detection of stx_1 and stx_2 genes. Four strains from seafood, six from beef and one from a clinical case of bloody diarrhoea were found to carry Shiga toxins. The seafood isolated carried stx_1 and or stx_2 in combination or alone, while the beef isolates produced stx_1 alone. stx_1 gene of beef STEC was found to be stx_{1c} subtype. Interestingly, though all STEC strains were negative for *eae* gene, two STEC strains isolated from seafood and one from a patient with bloody diarrhoea carried STEC autoagglutinating adhesin

gene, recently identified as the gene encoding a novel auto-agglutinating adhesin.

In India, there seems to be scanty literature on antimicrobial resistance pattern of STEC.

Chattopadhyay *et al.*[63] studied the antibiotic sensitivity pattern of STEC strains from animal, human and food products and reported that STEC strains were uniformly sensitive to common antibiotics except tetracycline, cephalexin, dicloxacillin, erythromycin and lincomycin. Similarly, Khan *et al.*[36] also examined antimicrobial resistance pattern of 63 STEC isolates from 19 human stool samples, 40 cow stool samples and four beef samples in Kolkata using 15 antimicrobials. Resistance was observed most commonly to ampicillin (25.4%), tetracycline (23.8%) and streptomycin (14.3%), and less frequently to cephalothin (1.1%), cotrimoxazole (9.5%), nalidixic acid (6.4%) and neomycin (3.2%). The authors reported that more than one-third of these strains (35%) showed reduced susceptibility to different antimicrobial agents, but were not completely resistant to any of the antibiotics. About 14.3% of the isolates were sensitive to all antimicrobials used in the study. Fourteen strains showed multidrug resistance and there was no common resitance pattern among the strains.

Conclusion

The present review is an attempt to summarize information available on pathogenesis, epidemiology, advances in rapid detection and diagnosis of STEC with particular reference to India. From current knowledge, it is clear that pathogenesis of STEC is multifactorial and involves several virulence attributes of the organism. Apart from various food materials other modes of spread like drinking water, swimming in contaminated pools and airborne dispersion have also been recognized as sources of STEC infection. Rapid and sensitive methods for detection of STEC are now in force; especially there has been advance in PCR technology, which has increased the speed and has made it possible to quantitate the number of STEC organisms present in a suspected sample. STEC has not been yet recognized as major human pathogen in India. This seems to be due to lack of investigations and under-reporting of the cases to hospitals or laboratories.

At present, a major challenge in the area of STEC research is to design widely accepted, rapid methods for detection, elimination or reduction of the STEC load in food materials and other sources. Immunization of ruminants like cattle and sheep has shown promise for reducing the shedding of STEC strains in their faecal materials[71,72]. This needs to be evaluated and advanced further before it can be applied on a large scale in the field. Further ruminant shedding of STEC is influenced by dietary factors and fasting regimes imposed during transport to slaughterhouse[73]. More work in this direction will answer its actual implications on STEC shedding. Irradiation of food materials like ground beef as approved by the Food and Drug Administration in USA will efficiently diminish the STEC load in food materials[74]. Similarly, development of cost-effective candidate vaccines for human use would be helpful for efficient management of STEC infection. Before this can happen, there must be at least an effective treatment strategy that will reduce the progression of infection to HUS. An efficient reporting system based on public awareness will determine the epidemiology and importance of STEC infection in India for efficient management of the problem.

1. Konowalchuk, J., Speirs, J. I. and Stavric, S., Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immunol.*, 1977, **18**, 775–779.
2. O'Brien, A. D., Thompson, M. R., Cantey, J. R. and Formal, S. B., Production of a *Shigella dysenteriae*-like toxin by pathogenic *Escherichia coli*. 77th Annual Meeting of the American Society for Microbiology, American Society for Microbiology, Washington, DC, USA, Abstr. B-103, 1977.
3. O'Brien, A. D., LaVeck, G. D., Thompson, M. R. and Formal, S. B., Production of a *Shigella dysenteriae*-like toxin by pathogenic *Escherichia coli*. *J. Infect. Dis.*, 1982, **146**, 763–769.
4. Calderwood, S. B. *et al.*, Proposed new nomenclature for SLT (VT) family. *ASM News*, 1996, **62**, 118–119.
5. Riley, L. W. *et al.*, Haemorrhagic colitis associated with a rare *Escherichia coli* serotypes. *N. Engl. J. Med.*, 1983, **308**, 681–685.
6. Johnson, W. M., Lior, H. and Bezanson, G. S., Cytotoxic *Escherichia coli* O157 : H7 associated with haemorrhagic colitis in Canada. *Lancet*, 1983, **1**, 8314–8315.
7. Mohammad, A., Peiris, J. S. M., Wijewanta, E. A., Mahalingam, S. and Gunasekara, G., Role of verocytotoxigenic *Escherichia coli* in cattle and buffalo calf diarrhoea. *FEMS Microbiol. Lett.*, 1985, **26**, 281–283.
8. Orden, J. A., Ruiz-Santa-Quiteria, J. A., Cid, D., Garcia, S., Sanz, R. and de la Fuente, R., Prevalence and characteristic of necrotoxigenic *Escherichia coli* (NTEC) strains isolated from diarrhoeic dairy calves. *Vet. Microbiol.*, 1999, **66**, 265–273.
9. Staats, J. J., Chengappa, M. M., DeBey, M. C., Fickbohm, B. and Oberst, R. D., Detection of *Escherichia coli* Shiga toxin (stx) and enterotoxin (estA and elt) genes in fecal samples from non-diarrheic and diarrheic greyhounds. *Vet. Microbiol.*, 2003, **94**, 303–312.
10. Beutin, L. *et al.*, Hep-2 cell adherence, actin aggregation and intimin types of attaching and effacing *Escherichia coli* strains isolated from healthy infants in Germany and Australia. *Infect. Immunol.*, 2003, **71**, 3995–4002.
11. Karmali, M. A., Infection by verotoxin-producing *Escherichia coli* *Clin. Microbiol. Rev.*, 1989, **2**, 15–38.
12. Brett, K. N., Ramachandran, V., Hornitzky, M. A., Bettelheim, K. A., Walker, M. J. and Djordjevic, S. P., Stx_{1c} Is the most common Shiga toxin 1 subtype among Shiga-toxin producing *Escherichia coli* isolates from sheep but not among isolates from cattle. *J. Clin. Microbiol.*, 2003, **41**, 926–936.
13. Beutin, L., Aleksic, S., Zimmermann, S. and Gleier, K., Virulence factors and phenotypical traits of verotoxigenic strains of *Escherichia coli* isolated from human patients in Germany. *Med. Microbiol. Immunol.*, 1994, **183**, 13–21.
14. Ahmed, S. and Cowden, J., An outbreak of *E. coli* O157 in central Scotland, 3rd International Conference and Workshop on Shiga Toxin (verotoxin) Producing *Escherichia coli* Infections, Lois Joy Galler Foundation for Haemolytic Uraemic Syndrome Inc., Melville, NY, USA, Abstr. V191/I, 1997, p. 22.
15. Elliott, E. J. *et al.*, Nationwide study of haemolytic-uraemic syndrome: clinical, microbiological, and epidemiological features. *Arch. Dis. Child.*, 2001, **85**,125–131.
16. Ochoa, T. J. and Cleary, T. G., Epidemiology and spectrum of disease of *Escherichia coli* O157. *Curr. Opin. Infect. Dis.*, 2003, **16**, 259–263.

17. Paton, J. C. and Paton, A. W., Pathogenesis and diagnosis of Shiga-toxin producing *Escherichia coli* infections. *Clin. Microbiol. Rev.*, 1998, **11**, 450–479.
18. Paton, A. W., Paton, J. C., Goldwater, P. N., Heuzenroeder, M. W. and Manning, P. A., Sequence of a variant Shiga-like toxin type I operon of *Escherichia coli* O111 : H-. *Gene*, 1993, **129**, 87–92.
19. Karch, H., Meyer, T., Russmann, H. and Hessemann, J., Frequent loss of Shiga-like toxin genes in clinical isolates of *Escherichia coli* upon subcultivation. *Infect. Immunol.*, 1992, **60**, 3464–3467.
20. Paton, A. W., Beutin, L. and Paton, J. C., Heterogeneity of the amino acid sequences of *Escherichia coli* Shiga-like toxin type-1 operons. *Gene*, 1995, **153**, 71–74.
21. Zhang, W., Bielaszewska, M., Kuczius, T. and Karch, H., Identification, characterization and distribution of Shiga toxin1 gene variant (stx_{1c}) in *Escherichia coli* strains isolated from humans. *J. Clin. Microbiol.*, 2002, **40**, 1441–1446.
22. Calderwood, S. B. and Mekalanos, J. J., Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. *J. Bacteriol.*, 1987, **169**, 4759–4764.
23. Tesh, V. L. *et al.*, Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immunol.*, 1993, **61**, 3392–3402.
24. Ramachandran, V., Hornitzky, M. A., Bettelheim, K. A., Walker, M. J. and Djordjevic, S. P., The common ovine higa toxin-2 containing *Escherichia coli* serotypes and human isolates of the same serotypes possess a Stx2d toxin type. *J. Clin. Microbiol.*, 2001, **39**, 1932–1937.
25. Gyles, C. L., De Grandis, S. A., MacKenzie, C. and Brunton, J. L., Cloning and nucleotide sequence analysis of the genes determining verocytotoxin production in a porcine edema disease isolate of *Escherichia coli. Microb. Pathogen*, 1988, **5**, 419–426.
26. Mainil, J., Shiga/verocytotoxins and Shiga/verotoxigenic *Escherichia coli* in animals. *Vet. Res.*, 1999, **30**, 235–257.
27. Schmidt, H., Scheef, J., Morabito, S., Caprioli, A., Wieler, L. H. and Karch, H., A new Shiga toxin 2 variant (stx2f) from *Escherichia coli* from pigeons. *J. Clin. Microbiol.*, 2000, **66**, 1205–1208.
28. O'Brien, A. D. and Holmes, R. K., Shiga and Shiga-like toxin. *Microbiol. Rev.*, 1987, **1**, 206–220.
29. Donohue-Rolfe, A., Keusch, G. T., Edson, C., Thorley-Lawson, D. and Jacewicz, M., Pathogenesis of *Shigella* diarrhoea. IX. Simplified high yield purification of *Shigella* toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies. *J. Exp. Med.*, 1984, **160**, 1767–1781.
30. Fraser, M. E., Chernaia, M. M., Kozlov, Y. V. and James, M. N., Crystal structure of the holotoxin from *Shigella dysentery* at 2.5A resolution. *Nature Struct. Biol.*, 1994, **1**, 59–64.
31. Yamasaki, C. *et al.*, Induction of cytokines in a human colon epithelial cell line by Shiga toxin 1 (Stx1) and Stx2 but not by non-toxic mutant Stx1 which lacks *N*-glycosidase activity. *FEBS Lett.*, 1999, **442**, 231–234.
32. Nataro, J. P. and Kaper, J. B., Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.*, 1998, **11**, 142–201.
33. Donnenberg, M., Kaper, J. B. and Finlay, B. B., Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *Trends Microbiol.*, 1997, **5**, 109–114.
34. Cookson, A. L. and Woodward, M., The role of intimin in the adherence of enterohaemorrhagic *Escherichia coli* (EHEC) O157 : H7 to HEp-2 tissue culture cells and to bovine gut explant tissues. *Int. J. Med. Microbiol.*, 2003, **292**, 547–553.
35. Fitzhenry, R. J. *et al.*, Tissue tropism of enteropathogenic *Escherichia coli* strains belonging to the O55 serogroup. *Infect. Immunol.*, 2002, **70**, 4362–4368.
36. Khan, A. *et al.*, Antibiotic resistance, virulence gene, and molecular profiles of Shiga toxin- producing *Escherichia coli* isolates from diverse sources in Calcutta, India. *J. Clin. Microbiol.*, 2002, **40**, 2009–2015.
37. Paton, A. W., Srimanote, P., Woodrow, M. C. and Paton, J. C., Characterization of *saa*, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infect. Immunol.*, 2001, **69**, 6999–7009.
38. Pradel, N., Ye, C., Livrelli, V., Xu, J., Joly, B. and Wu Long-Fei, Contribution of twin arginine translocation system to the virulence of enterohaemoorhagic des *Escherichia coli* O157 : H7. *Infect. Immunol.*, 2003, **71**, 4908–4916.
39. Beutin, L., Geiger, D., Steinruck, H., Zimmermann, S. and Scheutz, F., Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J. Clin. Microbiol.*, 1993, **31**, 2483–2488.
40. Peeters, J. E., *Escherichia coli* infection in rabbits, cats, dogs, goats and horses. In *Escherichia coli in Domestic Animals and Humans* (ed. Gyles, C. L.), CAB International, Wallingford, UK, 1994, pp. 261–284.
41. Kenee, W. E. *et al.*, A prolonged outbreak of *Escherichia coli* O157 : H7 infections caused by commercially distributed raw milk. *J. Infect. Dis.*, 1997, **176**, 815–818.
42. Pierard, D., Van Damme, L., Stevens, D., Moriau, L. and Lauwers, S., Detection of verocytotoxin-producing *Escherichia coli* in meat in Belgium. In *Recent Advances in Verocytotoxin-Producing Escherichia coli Infections* (eds Karmali, M. A. and Goglio, A. G.), Elsevier Science, Amsterdam, The Netherlands, 1994, pp. 77–80.
43. Wallace, J. S., Cheasty, T. and Jones, K., Isolation of verocytotoxin-producing *Escherichia coli* O157 from birds. *J. Appl. Microbiol.*, 1997, **82**, 399–404.
44. Varma, J. K. *et al.*, An outbreak of *Escherichia coli* O157 infection following exposure to a contaminated building. *JAMA*, 2003, **290**, 2709–2712.
45. Karmali, M. A., Petric, M. and Bielaszewska, M., Evaluation of a microplate latex agglutination method (verotox-F-assay) for detecting and characterizing verotoxins (Shiga toxins) in *Escherichia coli*. *J. Clin. Microbiol.*, 1999, **37**, 396–399.
46. Reymond, D., Karmali, M. A., Clarke, I., Winkler, M. and Petric, M., Comparison of the Western blot assay with the neutralizing-antibody and enzyme-linked immunosorbent assays for measuring antibody to verocytotoxin1. *J. Clin. Microbiol.*, 1997, **35**, 609–613.
47. Newland, J. W. and Neill, R. J., DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. *J. Clin. Microbiol.*, 1988, **26**, 1292–1297.
48. Thomas, A., Smith, H. R., Willshaw, G. A. and Rowe, B., Non-radioactively labelled polynucleotide and oligonucleotide DNA probes for selectively detecting *Escherichia coli* strains producing vero cytotoxins VT1, VT2, and VT2 variant. *Mol. Cell Probes*, 1991, **5**, 129–135.
49. Paton, A. W. and Paton, J. C., Detection and characterization of Shiga toxigenic *Escherichia coli* using multiplex-PCR assay for *stx1*, *stx2*, *eaeA*, and enterohaemorrhagic *E. coli* (*hlyA*), *rfbO111* and *rfbO157*. *J. Clin. Microbiol.*, 1998, **36**, 598–602.
50. Beutin, I., Horbach, I., Zimmermann, S. and Gleier, K., Vergleich verschiedener diagnostischer Methoden zum Nachweis von Verotoxin (Shiga-toxin) bilenden *Escherichia coli* Stammen (VTEC) aus klinischen Stuhlproben. *J. Lab. Med.*, 1997, **21**, 537–546.
51. Osek, J., Rapid and specific identification of Shiga toxin producing *Escherichia coli* in faeces by multiplex PCR. *Lett. Appl. Microbiol.*, 2002, **34**, 304–310.
52. Fode-Vaughan, K. A., Maki, J. S., Benson, J. A. and Collins, M. L. P., Direct PCR detection of *Escherichia coli* O157 : H7. *Lett. Appl. Microbiol.*, 2003, **37**, 239–243.
53. Bellin, T., Pulz, M., Matussek, A., Hempen, H. G. and Gunzer, F., Rapid detection of enterohemorrhagic *Escherichia coli* by real-time PCR with fluorescent hybridization probes. *J. Clin. Microbiol.*, 2001, **39**, 370–374.
54. Sharma, V. K., Detection and quantitation of enterohaemorrhagic *Escherichia coli* O157, O111, and O26 in beef and bovine faeces

by real-time polymerase chain reaction. *J. Food Prot.*, 2002, **65**, 1371–1380.

55. Mcingvale, S. C., Elhanafi, D. and Drake, M. A., Optimization of reverse transcriptase PCR to detect viable Shiga-toxin producing *Escherichia coli*. *Appl. Environ. Microbiol.*, 2002, **68**, 799–806.
56. Ge, B. L., Zhao, S. H., Hall, R. and Meng, J. H., A PCR–ELISA for detecting Shiga toxin producing *Escherichia coli*. *Microb. Infect.*, 2002, **4**, 285–290.
57. Bettelheim, K. A. and Beutin, L., Rapid laboratory identification and characterization of verocytotoxigenic (Shiga toxin-producing) *Escherichia coli* (VTEC/STEC). *J. Appl. Microbiol.*, 2003, **95**, 205–217.
58. Chapman, P. A., Siddons, C. A., Zadik, P. M. and Jewes, L., An improved selective medium for the isolation of *Escherichia coli* O157. *J. Med. Microbiol.*, 1991, **35**, 107–110.
59. Paton, A. W. *et al.*, Molecular microbiological investigation of an outbreak of hemolytic uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. *J. Clin. Microbiol.*, 1996, **34**, 1622–1627.
60. Karch, H., Janetzki-Mittman, C., Aleksic, S. and Datz, M., Isolation of enterohaemorrhagic *Escherichia coli* O157 strains from patients with hemolytic uremic syndrome by using immunomagnetic separation, DNA-based methods, and direct culture. *J. Clin. Microbiol.*, 1996, **34**, 516–519.
61. Gupta, S., Sini, N. K., Kaur, P. and Sood, D. K., Verocytopathic activity of *Escherichia coli* O157 and other 'O' serogroups isolated from patients of diarrhoea. *Indian J. Med. Res.*, 1992, **95**, 71–76.
62. Pal, A. *et al.*, Shiga-toxin producing *Escherichia coli* from healthy cattle in a semi-urban community in Calcutta, India. *Indian J. Med. Res.*, 1999, **110**, 83–85.
63. Chattopadhyay, U. K., Dutta, S., Deb, A. and Pal, D., Verotoxin producing *Escherichia coli* – an environment-induced emerging zoonosis in and around Calcutta. *Int. J. Environ. Health Res.*, 2001, **1**, 107–112.
64. Kumar, S. H., Otta, S. K., Karunasagar, I. and Karunasagar, I., Detection of Shiga-toxigenic *Escherichia coli* (STEC) in fresh seafood and meat marketed in Mangalore, India by PCR. *Lett. Appl. Microbiol.*, 2001, **33**, 334–338.
65. Khan, A. *et al.*, Prevalence and genetic profiling of virulence determinants of non-O157 Shiga toxin-producing *Escherichia coli* isolated from cattle, beef, and humans, Calcutta, India. *Emerg. Infect. Dis.*, 2002, **8**, 54–62.
66. Wani, S. A., Bhat, M. A., Samanta, I., NishiKawa, Y. and Buchh, A. S., Isolation and characterization of Shiga toxin-producing *Esherichia coli* (STEC) and enteropathogenic *Esherichia coli* (EPEC) from calves and lambs with diarrhoea in India. *Lett. Appl. Microbiol.*, 2003, **37**, 121–126.
67. Wani, S. A., Bhat, M. A., Samanta, I., Nishikawa, Y. and Buchh, A. S., *Escherichia coli* O116 associated with an outbreak of calf diarrhoea. *Vet. Rec.*, 2004, **154**, 506–508.
68. Wani, S. A., Bhat, M. A., Samanta, I., Nishikawa, Y. and Buchh, A. S., *Escherichia coli* O4 : NM associated with an outbreak of calf diarrhoea. *Vet. J.*, 2005 (in press).
69. Wani, S. A., Samanta, I., Bhat, M. A. and Nishikawa, Y., Investigation of Shiga toxin-producing *Escherichia coli* in avian species in India. *Lett. Appl. Microbiol.*, 2004, **39**, 389–394.
70. Kumar, H. S., Karunasgar, I., Karunasagar, I., Teizou, T., Shima, K. and Yamasaki, S., Characterization of Shiga toxin-producing *Escherichia coli* (STEC) isolated from seafood and beef. *FEMS Microbiol. Lett.*, 2004, **233**, 173–178.
71. Dean-Nystrom, E. A., Gansheroff, L. J., Mills, M., Moon, H. W. and O'Brien, A. D., Vaccination of pregnant dams with intimin$_{O157}$ protects suckling piglets from *Escherichia coli* O157: H7 infection. *Infect. Immunol.*, 2002, **70**, 2414–1418.
72. Potter, A. A. *et al.*, Decreased shedding of *Escherichia coli* O157 : H7 by cattle following vaccination with type III secreted proteins. *Vaccine*, 2004, **22**, 362–369.
73. Leitch, E. C. M., Duncon, S. H., Stanley, K. N. and Stewart, C. S., Dietary effects on the microbiological safety of food. *Proc. Nutr. Soc.*, 2001, **60**, 247–255.
74. Khan, A. *et al.*, Shiga toxin-producing *Escherichia coli* infection: current progress and future challenges. *Indian J. Med. Res.*, 2003, **118**, 1–24.

ACKNOWLEDGEMENTS. The work was supported in part by Indian Council of Agricultural Rsearch, New Delhi. We thank Prof. Anwar Alam, Vice Chancellor, SKUAST-K for support.

Received 3 March 2004; revised accepted 24 August 2004