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Shiga toxins (Verocytotoxins)

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Shiga toxins (Stxs) also called Verocytotoxins (Vtxs) and Shiga-like toxins (SLTs), are bacterial toxins produced by some members of the Enterobacteriaceae particulary *Shigella dysenteriae* and *Escherichia coli* 0157:H7 as well as *Acinetobacter* spp (Moraxellaceae), *Enterobacter cloacae* and *Aeromonas hymophilus.* The toxin is made of two moieties, the B-moiety that is responsible for its binding to cell surface receptors, and the A- moiety which enters the cytosol and inhibits protein synthesis enzymatically. Their pathological effect in humans is mainly as a result of inhibition of cellular protein synthesis. Shiga toxins are haboured mainly by ruminants, principally cattle as well as sheep, buffaloes, pigs, goats, dogs, cats and pigeons. The two major groups of the toxin, Stx1 and Stx2 are associated with mild or bloody diarrhea to hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) and also, nosocomial infections in humans. Predisposing factors to infection with Shiga toxin producing bacteria includes old age, immunosupression, malnutrition, under developed immunity in neonates, poor hygiene, lack of portable water and excreta contamination of existing traditional water sources. Transmission is through consumption of contaminated food and water, person-to-person and animal contact. High rate of antibiotic resistance amongst Stxs-producing bacteria is causing concern all over the world, therefore improved personal and food hygiene and the provision of portable drinking water appears to be the best preventive measure against the infection.

Key words: Antibiotic resistance, diarrhea, Enterobacteriaceae, Moraxellaceae, nosocomial infection, ruminants, shiga toxin, transmission.

INTRODUCTION

Shiga toxins (Stx) or Verocytotoxins (Vtxs) are produced by some strains of *Escherichia coli* of the Enterobacteriaceae family or 'coliform group. The genus *Escherichia* are group of bacteria found as commensal flora inhabiting the gut of humans and animals. The bacteria are acquired by ingestion during the first few days after birth. *E. coli*, the medically important species of the genus are motile Gram-negative bacilli with or without capsules. They grow over a wide range of temperatures (15 - 45°C) and are able to survive under adverse environmental conditions for extended periods of time, thus creating many opportunities for exposure and infection (Cameron et al., 1995; Guth et al., 2002). Most strains of the bacteria are indole positive and ferments lactose with the production of acids and gas. They can be readily cultivated under laboratory conditions on artificial media. Their colonial morphology vary from smooth colourless (on non-selective media) to pinkish or red (on MacConkey agar) with or without hemolysis on blood agar and colorless, sorbitol-negative colonies on sorbitol MacConkey agar (Koneman et al., 1997).

E. coli are widespread intestinal parasites of mammals and birds and are present wherever there is faecal contamination. Certain strains however are pathogens in

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Table 1. Diarrhoeagenic strains of *Eschecrichia coli*.

***E. coli* Strain**	**Clinical symptoms**	**Mechanism**
Enteropathogenic (EPEC)	Watery diarrhea	Pili, type III secretion
Enterohemorrhagic (EHEC	Bloody diarrhea, HUS	Shiga-like toxin
Enteroinvasive (EIEC)	Dysentry	Cellular invasion and cell-cell spread
Enterotoxigenic (ETEC)	Watery diarrhea	Colonization factors, heat-labile/-stable toxins
Enteroaggregative (EaggEC)	Watery diarrhea, persistent	Fimbriae, heat-stable toxins
Diffusely adherent (DAEC)	disease	Toxins?
Verocytotoxin producing (VTEC)	Watery diarrhea, persistent disease	Verocytotoxins (or Shiga toxins)
	Bloody diarrhea	

HUS: Haemolytic Uremic Syndrome (Source; Henderson et al., 2000)

humans and animals and cause opportunistic infections (Greenwood et al., 1992). Three general clinical syndromes associated with infection from pathogenic *E. coli* strains include; urinary tract infection, sepsis/ meningitis and enteric/diarrhoeal disease (Nataro and Kaper, 1998). Though the organisms are known to cause enteric infections and diarrhea (gastroenteritis), it was not more than two decades that some strains were identified to produce the toxins: Shiga toxins or verocytotoxins. These toxins are responsible for lethal bloody diarrhea (haemolytic colitis and haemolytic uremic syndrome) in humans (Karmali et al., 1983; Karch et al., 1999). Recently however, *Salmonella enterica* (Enterobacteriaceae), *Acinetobacter haemolyticus* and *Acinetobacter baumanni, Aeromonas hydrophila, Aeromonas cavia, Citrobacter freundii* and *Enterobacter cloacae* has also been associated with bloody diarrhea and Shiga toxin production (Paton and Paton, 2000; Pedersen et al., 2006). *A. haemolyticus* and *A. baumanni* are aerobic, non motile, catalase positive and oxidase negative Gram-negative coccobacilli that belong to the Moraxellaceae family (Lambert et al., 1993; Bergogne-berezin and Towner, 1996).

Shiga toxin producing *E. coli* (STEC) was first recognized as a human pathogen in 1982, in the USA, during two outbreaks of hemorrhagic colitis (HC) caused by some strains of the serotype O157:H7 (CDC, 1982; Riley et al., 1983; Wells et al., 1983; Karch et al., 1999). In 1983, the association of *E. coli* O157:H7 and several other STEC serotypes with sporadic cases of classical haemorrhagic uremic syndrome (HUS) was first described (Karmali et al., 1983) and subsequently confirmed in a prospective study (Karmali et al., 1985) . 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 uncomplicated watery diarrhea in some geographic areas (Caprioli and Tozzi, 1998; Griffin, 1998; Smith et al., 1998; Spika, 1998; Karch et al., 1997; Huppertz et al., 1996; Piekrard et al., 1997). STECs are rated today as one of the most important human pathogens in the developed countries (Reilly, 1998; WHO, 1999; Kaddu-Mulindwa et al., 2001).

Research on STEC and associated infections in developing countries however, is at low ebb despite the rising cases of infantile diarrhea. The understanding of these toxins, their mode of action, predisposing factors, health implications and control measures will be of importance to curtail its threat particularly in Africa.

Diarrhoeagenic strains of *Escherichia coli*

E. coli associated with diarrhoeal disease are collectively referred to as enterovirulent *E. coli*. The *E. coli* strain 0157 also known as the 'hamburger bug' is now recognized as the strain with the propensity to cause several gastrointestinal disease out breaks (Henderson et al., 2000). Seven groups have been defined based on various virulence factors including toxin production and adhesion (Table 1; Nataro and Kaper, 1998). The enteroaggregative (EAggEC), enteroinvasive (EIEC), enteropathogenic (EPEC) and enterotoxigenic (ETEC) strains are common in developing countries, with ETEC and EAggEC being the cause of most cases of the dreaded travelers' diarrhea (Lawson, 2004). VTECs or STECs are directly responsible for the hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS) developed by some patients following infection with the bacteria (Cantarelli et al., 2000). Subsets of STEC that are able to cause attaching and effacing (A/E) lesions similar to EPEC organisms are termed enterohemorrhagic *E. coli* (EHEC or typical EHEC) (Cantarelli et al., 2000). EHEC is rare in the developing countries, but is one of the emerging infectious diseases in the developed countries associated with bloody diarrhea (Lawson, 2004). Even though STEC serotype O157:H7 is the organisms most often implicated in large outbreaks, other serotypes; O111:H8, O26:H11, O103:H2, have been reported to cause a considerable number of cases of HUS in many countries (Nataro and Kaper, 1998; Hyatt et al., 2001; Safarikova and Safarik, 2001; Guth et al., 2003). There is need to investigate the presence of these serotypes and possibly newer ones that might be involved in causing diarrhea in Africa.

Table 2. Nomenclature of members of Shiga toxin (verocytotoxin) family.

Previous nomenclature	Proposed new nomenclature	
	Gene	Protein
Shiga toxin (Stx) or Verocytotoxin (Vtx)	*stx* or *vtx*	Stx or Vtx
Shiga toxin (Stx),Verotoxin 1 (VT1) or Shiga-like toxin I (SLT-I)	*stx1* or *vtx1*	Stx1 or Vtx1
ST2, VT2 or SLT-II	*stx2* or *vtx2*	Stx2 or Vtx2
ST2c, VT2c or SLT-IIc	*stx2c* or *vtx2 c*	Stx2c or Vtx2c
ST2e, VT2e or SLT-IIe	*stx2e* or *vtx2e*	Stx2e or Vtx2e

(Source; Paton and Paton, 1998).

Structure and nomenclature of members of Shiga toxin (verocytotoxin) family

Shiga toxins (Stxs) or Verocytotoxins (Vtxs) or Shiga-Like toxins (SLT) are a family of structurally and functionally related exotoxins produced by enteric pathogens (Caldenvood et al., 1996; Pikrard et al., 1997). The discovery of *E. coli* O157: H7 in 1982 as producer of Shiga toxin and causative agent of hemorrhagic colitis (HC) and HUS made it to be considered as an emerging pathogen (Dundas, 1999; Schmitt et al., 1999; O'Brien et al., 2001; Khan et al., 2003). SLTs are produced by *Shigella dysenteriae* (Enterobacteriaceae) serotype 1, the infectious agent associated with epidemic outbreaks of bacillary dysentery (Pikrard et al., 1997; Caprioli et al., 2005).

Stx belongs to a defined protein subfamily, the RNA N-glycosidases that can be classified into two antigenic groups: Shiga toxin 1 (Stx 1) or verocytotoxin 1 (Vtx 1) and Shiga toxin 2 (Stx2) or verocytotoxin 2 (Vtx 2) . Stx1 is a rather homologous group with three variants (stx1, stx1c and stx1d). The Stx2 group is more heterogeneous and comprises several subtypes (Stx2, Stx2c, Stx2d, Stx2e, Stx2f, Stx2g and activatable Stx2d) (Table 2; Caldenvood et al., 1996; Pikrard et al., 1997; Collaway, 2004; Caprioli et al., 2005; Grotiuz et al., 2006; Vu-Khac and Cornick, 2008). All the Stxs including those secreted by *S. dysenteriae* (Shiga-like toxin 1) and Stx1 secreted by *E. coli* are identical but differ only in one amino acid (Binnington et al., 2002; Leung et al., 2003). Exposure to antibiotics and other chemical agents might induce mutation in the existing strains. Continues research is required for monitoring of novel genetic strains that might require different approach to control measures.

Evolution of terms: STEC EHEC and VTEC

Shiga toxin (Stx) was first named after Kioshi Shiga (Shiga, 1898) who initially described the toxin as the agent of epidemic bacterial dysentery. In 1972, it was reported that Stx alone caused fluid accumulation and enteritis in ligated rabbit intestinal segments, the discovery which later lead to the purification of the toxin (Keusch et al., 1972; Olsnes and Eiklid, 1980; Khan et al., 2003). Ten years after discovery, O'Brien et al. (1983) established that certain strains of *E. coli* produce a cytotoxin that can be neutralized by anti Stx, an observation that explains the original Shiga-like toxin nomenclature. The *E. coli* strains that produce the Shiga-like toxin (SLT) were named as Shiga toxin producing *E. coli* (STEC) showing that one of the cytotoxins produced by these organisms is essentially identical at the genetic and protein levels to the Stx produced by *S. dysenteriae* 1. Furthermore, Konowalchuk et al. (1977) reported that certain diarrhoeagenic *E. coli* strains produce a cytotoxin that can kill the cultured Vero cells (of the green monkey kidney) *in vitro*, hence the name verocytotoxin (Vtx). The *E. coli* strains that produced this type of toxin became known as verotoxigenic *E. coli* or verotoxin producing *E. coli* (VTEC) (Chapman, 1995) . It was subsequently shown that Shiga-like toxin and Vero cytotoxin was the same toxin produced by *E. coli* O157: H7 strains. Thus, in 1983, the paths of researches on Stxs and Vtxs merged. Further research unraveled that some *E. coli* strains are capable of producing gastrointestinal lesions and hence are referred to as enterohaemorrhagic *E. coli* (EHEC). Thus, EHEC denotes a subset of STEC which are considered to be pathogens, whereas, not all STEC strains are believed to be pathogenic (Griffin and Tauxe, 1991; Nataro et al., 1998; Khan et al., 2003). Though Stxs, Vtxs and SLT were considered to be the same toxin, it was later realized that EHEC could produce more than one antigenically distinct Stxs. Thus, a new nomenclature was adopted; Shiga toxin 1 (ST1) or Verocytotoxin 1 (VT1) and Shiga toxin 2 (ST2) or Verocytotoxin 2 (VT2). ST1 is equivalent to VT1 and SLT-1; and ST2 is synonymous with VT2 and SLT-II. SLT-I is identical to ShT and is not neutralized by antibody to SLT-II. STEC has also been classified based on the host sources; STEC from the animals as ST1(a), VT1(a) or SLT-I(a) and ST2 (a), VT2(a), or SLT-II(a) and those from human sources as ST1(h), VT1(h), or SLT-I(h) and ST2 (h), VT2 (h), or SLT-II (h), Figure 1; Agbodaze, 1999).

Structure of Shiga toxins (verocytotoxins)

Structurally, all verotoxins have, in principle the AB_5 toxin structure (including Cholera and Pertusis toxins: Figure 2;

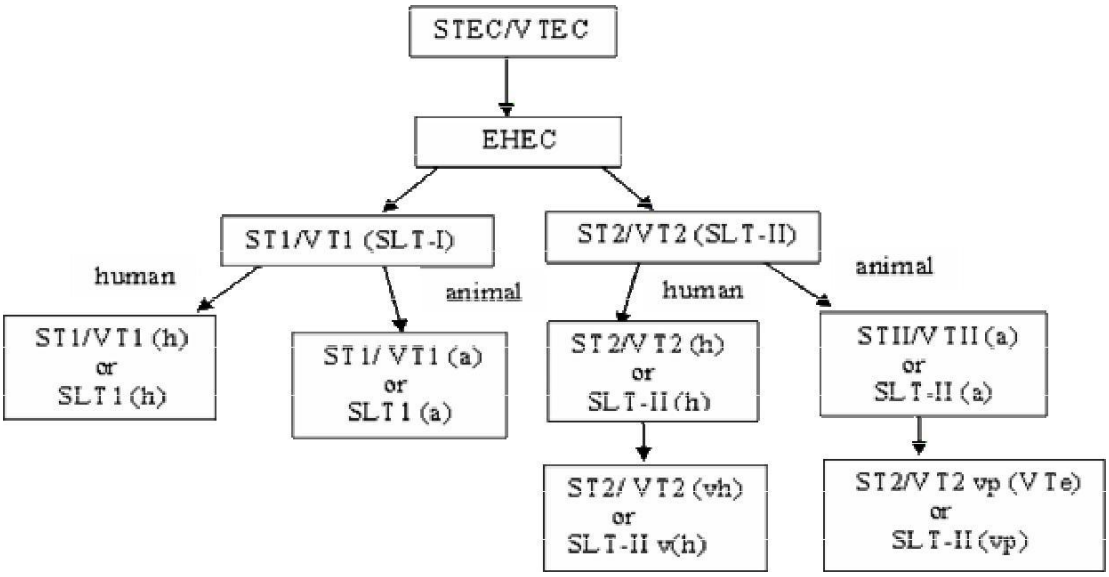

Figure 1. Classification of Shiga toxin producing *Escherichia coli* (STEC) according to host source.

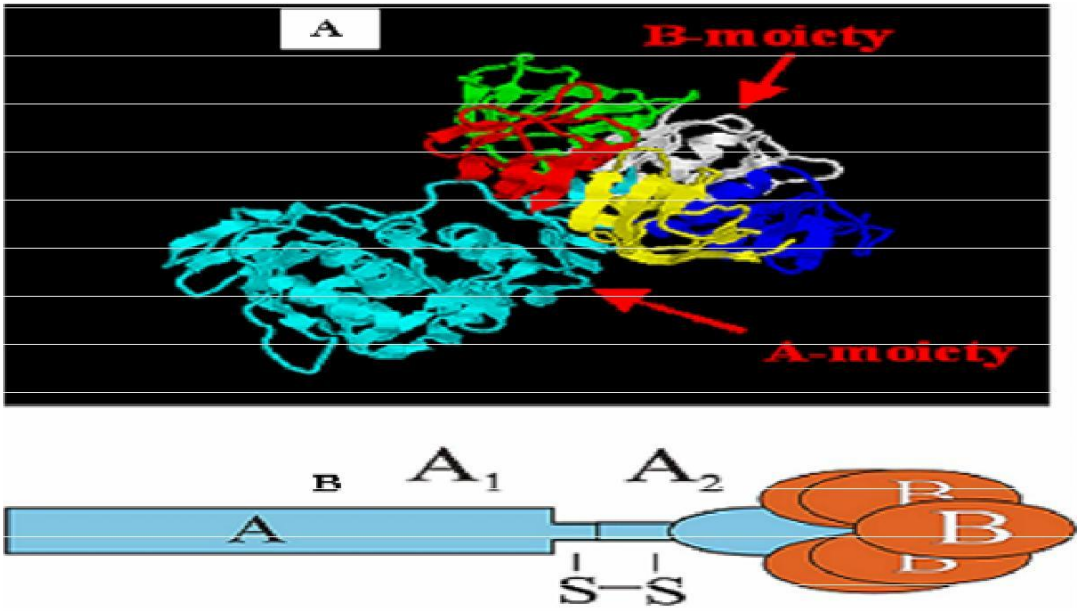

Figure 2. Schematic (A) and crystallographic (B) structure of Shiga toxin. As indicated the A fragment of the toxin is cleaved into the A1 and A2 fragments (held together by disulphide bonds S-S), and the A1 fragment can then inactivate ribosomes. The five small B fragments are responsible for binding to Gb3. (Source; Schmitt and Schaffrath, 2005).

Caprioli et al., 2005). One of the moiety of the toxin molecule (the B-moiety) is responsible for binding to cell surface receptors and the other moiety (the A-moiety) for inhibition of protein synthesis (Figure 2a; Chapman, 1995; Caprioli et al., 2005; Schmitt and Schaffrath, 2005). Cleavage of the A-moiety is achieved via the disulfide bond resulting into 2 fragments (A_1 and A_2) that are linked by a disulfide bond that are responsible for the cytotoxic effect of the toxin (Figure 2b).

Epidemiology and pathogenesis of Shiga toxins (verocytotoxins)

Shiga toxin-producing bacteria are ubiquitous microorganisms known to cause infection of varying severity in hu-

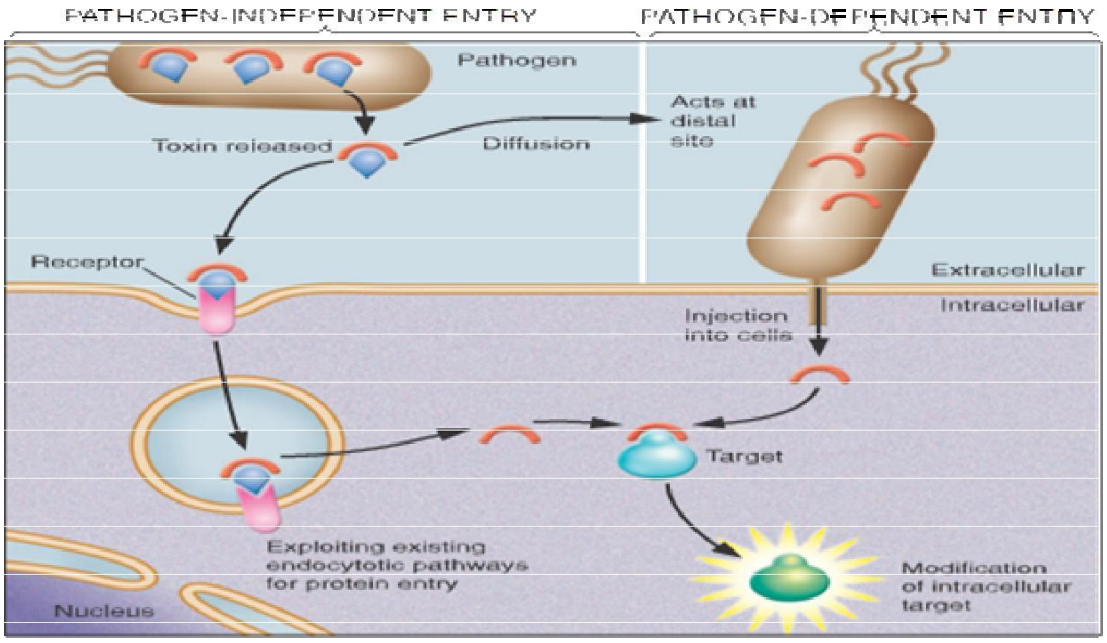

Figure 3. Model of toxin entry into the host cell (Source; Blanke, 2006).

severity in humans and animals (WHO, 1999; Collaway, 2004). It is now recognized that there is a very broad spectrum of human disease associated with Stx-producing organisms. Stxs are not only causal agents of diarrhea in humans in some geographical areas, but are also significant agents in at least two other life threatening infections, hameolytic colitis (HC) and haemolytic uremic syndrome (HUS) (Parry and Salmon, 1998; Agbodaze, 1999; Pulz and Matussek, 2003). The infections are, therefore, a public health problem of serious concern. HC, also referred to as “ischemic colitis", is a distinct clinical syndrome that appears typically with abdominal cramps and watery diarrhoea, followed by a grossly haemorrhagic discharge resembling lower gastrointestinal bleeding. This is accompanied by little or only low grade fever with no inflammatory exudates in the stool. The lack of fever and the absence of inflammatory exudates in the stool differentiate this illness from the dysentery described in shigellosis, campylobacter enteritis, or invasive *E. coli* gastroenteritis. HUS, which was first described as a distinct clinical entity by Gasser et al. (1955), is defined by a triad of features: acute renal failure in childhood, thrombotic thrombocytopenia purpura (TTP), and microangiopathic haemolytic anemia (MAHA) or Moschowitz's disease. The last condition is a disease characterized by thrombocytopenia, haemolytic anaemia, bizarre neurological manifestations, azotemia (uremia), fever and thrombosis of the terminal arterioles and the other capillaries (Agbodaze, 1999; Collaway, 2004; Grotiuz et al., 2006).

Pathogenesis of Shiga toxins is a multistep process, involving a complex interaction between a range of bacterial and host factors. After oral ingestion of the bacteria through contaminated food or water, the none-invasive bacteria adhere to the intestinal epithelial cells of the distal small bowel and colon (Collaway, 2004). As in most Gram-negative bacteria and the AB toxin system, the pathogens inject their toxins into the cytosol of host cells through bacterial transport machines that function as macro molecular syringes (Figure 3). This leads to a rearrangement or modification of the morphology of the cells and initiation of inflammation (Paton and Paton, 1998; Collaway, 2004; Colpoys et al., 2005).

The bacteria (often in very low initial doses), has an average incubation period of 3 - 4 days and must initially survive the harsh (acidic) environment of the stomach and then compete with other gut microorganisms to establish intestinal colonization, as well as release toxins. With the help of bacterial flagellin, the toxins are first absorbed by the intestinal epithelium and then translocated to the bloodstream (Miyamoto et al., 2006).

Shiga toxin can probably reach the circulation because of active transport in these cells and also passively after damage to the intestinal cells (Figure 4a; Acheson et al., 1996) . Subsequently, it is transported in the circulation to reach its primary target, the renal endothelium of the kidney. At the renal endothelium, the toxins attaches to the specific toxin receptors, the globotriaosylceramide (Gb_3, Pk Antigen, CD77; Figure 4b) present on target cell surfaces (the receptors are also found on red blood cells, platelets and B lymphocytes). Consequent of this attachment, the toxin induces both local and systemic effects (Figure 4c; Geelen et al., 2007).

Gb_3 consists of a ceramide long chain fatty acid embed-

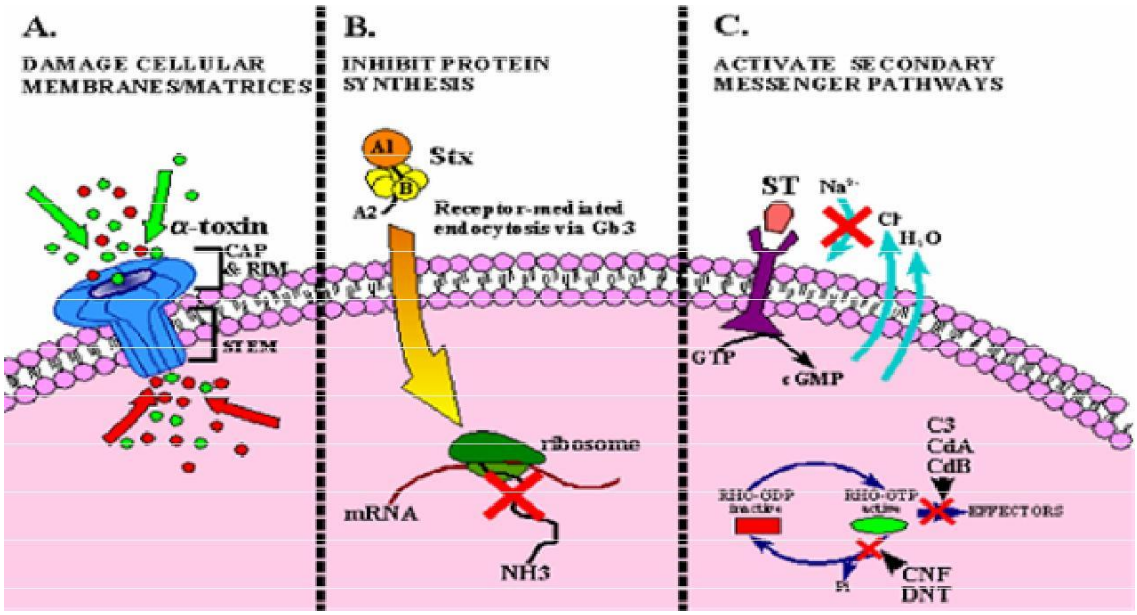

Figure 4. Diagrammatic representation of the mode of action of Shiga toxins (Stx) (b) and other bacterial toxins. Stx binds to Gb_3 resulting in inhibition of protein synthesis. Other mechanisms of action commonly used among Stx and other bacterial toxins include: damage to cellular membranes (a) and activation of secondary messenger pathways (c) (Source; Schmitt et al. 1999).

ded in the plasma membrane and a short extracellular trisaccharide chain terminated by a digalactose residue. The B subunit of Stx (Kd = 0.1 nM) facilitate high affinity binding of the holotoxin to the two terminal binding sites (Site I and Site II) digalactose residue of Gb_3. Stx1 and Stx2c exhibits optimum binding to Gb_3 with a fatty acyl chain lengths of 20 to 22 carbons and 18, respectively (Rivera-Betancourt et al., 2004). Once bound to a target cell membrane, toxin molecules are thought to be internalized by a process of receptor-mediated endocytosis. Internalization involves the formation of a clathrin-coated pit within the cell membrane, which subsequently pinches off to form a sealed-coated vesicle with toxin bound to the internal surface. Shiga toxins, which all have one A- fragment and five B-fragments regardless of the source organisms, enter the cytosol of cells and act enzymatically on a cytosolic target (Schmitt and Schaffrath, 2005).

Once inside the cell, the low endosomal pH triggers conformational changes in the toxin molecules. The internalized toxins in some instances are successfully degraded by host lysosomes, while toxins not successfully degraded proceed to cause biological effects (Doyle and Schoeni, 1987; Rivera-Betancourt et al., 2004; Ge et al., 2002). Successfully internalized toxins undergo membrane insertion forming endosomal vesicles that translocates them to the cytosolic side via the endoplasmic reticulum (ER) (Lencer and Tsai, 2003). During this process, the A_1 subunit is nicked at the trypsin-sensitive site near the amino terminus by a membrane bound protease furin, generating two fragments - 28 kDa N-terminal A_1 fragment and a 4 kDa C terminal A_2 fragment (Tesh and O'Brien, 1991). The A_1 terminal fragment (28 kDa N-terminal) is catalytically active and is released from the A_1 skeleton into the cytosol, while the A_1 C terminal remain attached to the B moiety by the disulphide bonds (Figure 2). The released catalytically active fragment has RNA N-glycosidase activity and therefore cleaves a specific N-glycosidic bond in the 28S rRNA which mediates peptide bond elongation in cellular protein synthesis. This cleavage prevents elongation factor 1-dependent binding of the aminoacyl-tRNA to the 60S ribosomal subunit, thereby inhibiting the peptide chain elongation step of protein synthesis (Khan et al., 2003).

The resulting disruption of protein synthesis leads to the death of renal endothelial cells, intestinal epithelial cells, Vero cells or Hela cells or any cells which possess the Gb_3 (or Gb_4 for Stx2e) receptor. Subversion of the protein synthesis machinery results in cytotoxicity to the human renal endothelial cells, consequently damage to the glomerular (Lencer and Tsai, 2003; Calderwood et al., 1987; Caprioli et al., 2005; Schmitt and Schaffrath, 2005). There is also occlusion of microvascular function. These pathological effects are characterized by a lowered glomerular filteration, bloody urine and acute renal failure that characterize HUS. In the intestinal mucosa, ulceration of the intestinal walls results in bloody diarrhea, a symptom characterizing HC (Te Loo et al. 2001; Collaway, 2004; Schmitt and Schaffrath, 2005). Although the major extra intestinal target organ is the kidney, virtually any organ can be involved (Amirlak and Amirlak, 2006). Consequently, the binding of toxins to

glycolipid receptors on vascular endothelial cells of the central nervous system and the intestinal colon results in neurological complications and hemorrhagic colitis (or bacillary dysentery) respectively. The role of Shiga toxin in the invasion process of S. *dysenteriae* type 1 and SLT-I and SLT-11 in attachment of EHEC to colonic epithelial cells, remains unclear. However, the capacity of the bacteria to invade or adhere to colonic epithelial cells is thought to reduce dilution of the toxins in the gut and allow the toxins to be delivered to the cells in a focal, concentrated manner. Both *in vitro* and *in vivo* experiments with Shiga toxin and the SLTs have demonstrated multiple potent effects. The toxins have been shown to be: (i) Directly cytotoxic for certain cell lines; (ii) Enterotoxic, mediating fluid accumulation in ligated ileal loops; and (iii) Paralytic-lethal when injected intravenously into mice and rabbits (Tesh and O'Brien, 1991).

Other virulence factors may play a role in Shiga toxin pathogenicity, these include; intimin (encoded by the *eae* A gene), which is required for intimate adherence of these pathogens to tissue culture cells and formation of the attaching and effacing (A/E) lesion (Khan et al., 2003; Collaway, 2004) . The formation of A/E lesions is mediated by multiple genes called the Locus of Enterocyte Effacement (LEE). Another virulence factor that contributes to verocytotoxin pathogenicity is the 60-MDa plasmid borne enterohaemolysin A gene (encoded by the E- *hly* A gene) . The toxins from *E. coli* of serotype O157 or those that have specific combinations of virulence factors appear to be more virulent in mankind (Khan et al., 2003; Tarawneh et al., 2009).

Structure and organization of Shiga toxin (*Stx*) or verocytotoxin (*Vtx*) genes

The Stx or Vtx genes are invariably chromosomally located. The genes that encode Stx1 and Stx2 are carried chromosomally or by lysogenic bacteriophages. The genes that code for the A and B subunits of Stxs, stxA and stxB, are organized within an operon. The operator region of Stx/Stx1 (but not Stx2) contains a consensus fur box that is responsible for the iron regulation of Stx and Stx1 production (Karmali et al., 1986; Schmitt et al., 1999). The operons of the nucleotide sequences of the genes encoding Stx from *S. dysenteriae*, as well as Stx1 and Stx2 from *E. coli*, have a common structure consisting of a single transcriptional unit, encoding first the A subunit followed by the B subunit. The *stx* B-subunit gene has a stronger ribosome binding site than that of the A-subunit gene, resulting in increased translation of B subunits, thereby satisfying the 1:5 A/B-subunit stoichiometry of the holotoxin. The predicted amino acid sequences were 315, 315 and 318 amino acids long for the A subunits of Stx, Stx1 and Stx2, respectively and 89 amino acids for the B subunits of all three toxins (Calderwood et al., 1987; Parry and Salmon, 1998; Bettelheim, 2001; Cherla et al., 2003). Both A and B subunits had hydrophobic N-terminal signal sequences characteristic of secreted proteins and the predicted *M*r values for the processed A and B subunits were in accordance with previous estimates based on analysis of purified toxins (Parry and Salmon, 1998). Interestingly, a 21-bp region of dyad symmetry spanning the 210 region was found upstream of *stx* and *stx*1 and this motif is thought to be associated with iron regulation of toxin expression (Paton and Paton, 1998). Stx and Stx1 are virtually identical (differing only in a single amino acid in the A subunit) but Stx2 had only 56% identity to the other toxins for both the A and B subunits.

There is also a significant degree of amino acid homology between the A subunits of Stx and the plant toxin ricin (Paton and Paton, 1998). An enzymatically active A subunit is none covalently associated with a binding, or B, component. The B subunit pentamer directs the binding of the holotoxin to sensitive eukaryotic cells via specific glycolipid receptors. Once internalized, the A polypeptide is cleaved into an enzymatically active A1 portion and an A2 portion; these fragments remain asso-ciated through a disulfide bond. The A2 portion serves as a link between the A1 fragment and the B pentamer. Other toxins that share this AB structure are the *E. coli* heat-labile toxin, cholera toxin and pertussis toxin (Schmitt et al., 1999).

Diagnostic methods

There are a number of difficulties associated with the diagnosis of verocytotoxin infections. Diagnostic procedures are based on detection of the presence of verocytotoxin producing genes in fecal extracts or fecal cultures and/or isolation (culture) of the organisms. Other procedures include immunological methods, immunomagnetic separation (IMS), polymerase chain reaction (PCR) and serological methods. These procedures differ in complexity, speed, sensitivity, specificity and cost (Te Loo et al., 2001; Roy et al., 2004).

Culture and isolation of bacterial agent can be carried out on a wide range of specimens including both clinical and environmental samples. Samples such as urine, stool, rectal swabs (Vu-Khac and Cornick, 2008), drag swabs (Tarawneh et al., 2009), food (Ge et al., 2002), blood, meat (Vu-Khac and Cornick, 2008) hides and carcasses (Gilbert et al., 2008), cloacal swabs from pigeons (Pedreson et al., 2006), water (Heijnen and Medema, 2009), wastewater from treatment plants, animal water troughs and sewage (Luo et al., 2002; Heijnen and Medema, 2006) have been employed. Samples are often cultured on sorbitol MacConkey agar (SMAC) and incubated at 37ºC and examined after 18 to 24 h of incubation for the presence of colorless, sorbitol-negative colonies (Leotta et al., 2006; Pizza and Rappuoli, 2006; Heijnen and Medema, 2009). The test is based on the property of most faecal bacteria not being able to ferment sorbitol, which distinguishes them from the majority of

other fecal bacteria especially *E. coli* belonging to other serotypes. The sensitivity of SMAC is limited by the capacity to recognize non-fermenting colonies against the background of other organisms on the plate; this is particularly difficult when the O157 strain forms less than 1% of the flora. Improvements on the isolation rate have been made by supplementing SMAC with cefixime, to inhibit *Proteus* spp. and with cefixime and potassium tellurite (CT-SMAC) (Roy et al., 2004; Tarawneh et al., 2009). Recently, further improvements have been made especially for the isolation of *E. coli* 0157, by the development of a commercial agar medium, Rainbow Agar O157 containing selective agents for *E. coli.* In addition, chromogenic substrates for -D-glucuronidase and -galactosidase have become available for the isolation of Shiga toxin producing *E. coli* (STEC). Glucuronidase-negative, galactosidase-positive O157 strains appear as black colonies on this medium, whereas commensal *E. coli* strains are pink (Cherla et al., 2003; Heijnen and Medema, 2006).

Immunomagnetic separation (IMS) techniques have been developed to assist in the isolation of Shiga toxin *E. coli* (principally O157) and other Stx producing bacteria from low-abundance specimens (Chapman et al., 1994). The procedure involves coating magnetic beads (by use of commercial magnetic bead reagent e.g. Dynabeads, Dynal, Oslo, Norway) with anti-lipopolysaccharide (LPS) antibody and mixing them with broth cultures or suspensions of feaces or suspect food homogenates (Karmali et al., 1983; Calderwood et al., 1987; Parry and Salmon, 1998; Bettelheim, 2001; Cherla et al., 2003; Heijnen and Medema, 2006). 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 were plated and the resultant colonies were tested for reactivity with the appropriate O antiserum and, more importantly, for Shiga toxin production. IMS was reported to be 100 times more sensitive than direct culture on either cefixime-rhamnose SMAC or CT-SMA (Leotta et al., 2006; Pizza and Rappuoli, 2006; Heijnen and Medema, 2009; Sepehriseresht et al., 2009).

For polymerase chain reaction (PCR), specific primers for detection of stx1 and stx2 genes were employed (Te Loo et al., 2001). The mixture for the amplification of stx1 and stx2 genes usually consisted of PCR buffer, salt solution medium, deoxynucleoside triphosphates (dNTPs), primers and Taq DNA polymerase. The amplification conditions consisted of an initial denaturation step (e.g. at 94°C for 4 min), repeated cycles of denaturation (e.g 30 cycles of 94°C for 1 min), followed by the annealing step (e.g 55°C for 1 min) and the extension cycle (e.g 72°C for 1 min and a final extension at 72°C for 10 min). PCR products were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining with two tubes serving as negative and positive controls (e.g a tube containing all PCR reaction mixture except template DNA used as negative control and three bacterial standard strains producing Shiga toxins; strain 1 producing Shiga toxin 1, strain 2 producing Shiga toxin 2 and strain 3 producing both of toxins used as positive controls) . This then is followed by electrophoresis on agarose gel in TAE buffer (Chapman et al., 1994; Blanco et al., 2003; Zahraei et al., 2007). Various modifications including multiplex-PCR or multiplex-real-time PCR have been employed in detection and identification of the toxin genes in various samples (Te Loo et al., 2001; Zahraei et al., 2007; Tarawneh et al., 2009; Sepehriseresht et al., 2009).

For serological methods, the gold standard assay for the presence of toxin in faecal specimens and isolates remains Vero cell culture. However, several commercial toxin kits are now available including two Enzyme Immunoassay (EIA) kits; Prospect Shiga Toxin *E. coli* (STEC) Microplate assay and Premier EHEC immunoassay, both of which have been registered by the United States Food and Drug Administration for use on stool specimens directly or on overnight broth culture. Two other toxin assays, VTEC–RPLA and Duopath Verotoxin (DV) are recommended only for use on colony sweeps or isolates (Park et al., 2003). Neutralisation tests in Vero cell cultures have shown that there is little, if any, cross reactivity between antibodies raised against each of the toxin types (Parry and Salmon, 1998; Cherla et al., 2003; Heijnen and Medema, 2006). The VTEC–RPLA is a reverse-passive latex agglutination assay which differentiates between Stx1 and Stx2 and also quantifies the amount of toxin present. Anti Stx1 and anti Stx2 rabbit antibodies were located on latex particles (Yokoyama et al., 2006). If Shiga toxin 1 or Shiga toxin 2 were present in bacterial supernatant, the toxins gets attached to their specific antibodies and produce a lattice at the end of ELISA microplate wells. But if there were not any of these toxins, lattice would not be formed and the latex is therefore precipitated at the end of the wells (Rivera-Betancourt et al., 2004; Sepehriseresht et al., 2009). Duopath Verotoxin (DV) immunochromatographic test was originally intended to confirm STEC isolates from foods (Park et al., 2003). The DV test uses colloidal gold-labelled monoclonal antibodies to "trap" any Stx1 and Stx2 present in samples as they migrate over a mem-brane. A positive result appears as a red line within 10 min. Like the VTEC–RPLA, the DV test is recommended for testing colony sweeps or isolates rather than primary faecal broth cultures (Bettelheim, 2001; Pulz and Matussek, 2003). Commercial serological diagnostic reagents ELISAs specific for antibodies to Stx1, Stx2 and O157 LPS have been developed to detect the minutest concentrations of toxins undetected by PCR present in a sample (Sepehriseresht et al., 2009).

Symptoms and transmission

Many domestic animals particularly ruminants and wildlife

carrying verocytotoxin producing bacteria are asymptomatic (McClure, 2000; Collaway, 2004). Certain STEC strains however, are capable of causing diarrhea in cattle particularly calves, cats and dogs (Anon, 2006a; 2006b). Piglet edema disease is another serious, frequently fatal STEC-related illness characterized by neurological symptoms including ataxia, convulsions and paralysis; edema is typically present in the eyelids, brain, stomach, intestine and mesentery of the colon (Paton et al., 2001). This disease is associated with particular STEC serotypes (most commonly O138:K81, O139:K82 and O141:K85) that are not known to infect humans (Rivera-Betancourt et al., 2004; Tarawneh et al., 2009).

In humans, many infected patients initially suffer a watery diarrhea, but in some, this progresses within 1 or 2 days to bloody diarrhea and hemolytic colitis (HC) (Table 1). Also, severe abdominal pain is also frequently reported (Anon, 2006a). In a proportion of patients, infection progresses to hemolytic uremic syndrome (HUS), a life-threatening sequela characterized by a triad of acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia. Some individuals with HUS experience neurological symptoms including lethargy, severe headache, convulsions and encephalopathy (Tarawneh et al., 2009). Although HUS occurs in all age groups, its incidence is higher in infants, young children and the elderly. Indeed, it is a major cause of acute renal failure in the pediatric population. The age distribution of HUS may be a consequence of the immunological naivety of young children and declining immune system function in the elderly, although age related differences in receptor expression may contribute. Healthy individuals can become infected, but 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 faeces (Wells et al., 2001; Rivera-Betancourt et al., 2004; Ge et al., 2002; Schmitt and Schaffrath, 2005).

Transmission of the pathogen appears to occur via three principal routes including contaminated food and contaminated drinking or swimming water sources (environmental spread), person-to-person transmission and animal contact (zoonotic) (Parry and Salmon, 1998; Tarawneh et al., 2009). Contamination of meat with STEC can occur from bovine faeces during slaughter and meat processing. Not surprisingly, consumption of raw or undercooked meat, particularly ground beef and unpasteurized milk, are the most commonly implicated foods. Cases have also been linked to the consumption of contaminated cheese, yogurt, cold cuts, lettuce, potatoes, seed sprouts, cooked maize, melon and fresh-pressed apple juice (McClure, 2000; Vernozy-Rozand, and Roze, 2003). Secondary transmission, which may involve direct hand-to- hand contact (e.g., among children in day care centres) or indirect, e.g., via contaminated water used for swimming is possible through asymptomatic carriers. Massive outbreaks is also likely to occur in fast-food restaurant chains using a common source of ground-beef patties, hamburgers/salad and sub-optimal (unhygienic) cooking and handling procedures (Anon, 2006a). Other sources of infection include foods such as unheated overnight foods, raw or inadequately pasteurized dairy products, fermented or dried meat products such as salami and jerky, fruits and vegetable products which presumably had come into contact with domestic animal manure at some stage during cultivation or handling. Stx pathogen was also detected on conveyor belts in beef-processing plants (Rivera-Betancourt et al., 2004) and was implicated in a serious outbreak associated with surface contamination and deficiencies in hygiene and meat handling practices at a supermarket (Banatvala et al., 1996).

Sources and predisposing factors to Shiga toxin infection

Cattle have long been regarded as the principal reservoir of Shiga toxin producing bacterial strains (Hyatt et al., 2001; Rivera-Betancourt et al., 2004; Anon, 2006a; 2006b). Epidemiological surveys have also revealed that gastrointestinal tracts of other domestic animals, including sheep, pigs, goats, dogs and cats and birds such as pigeons harbor these organisms (Vernozy- Rozand and Roze, 2003; Anon, 2006a; 2006b; Tarawneh et al., 2009). Soil and water contaminated with animal and human excreta also incubate these bacterial agents.

Environmental risk factors abound in the developing world. For example in Africa, majority of drinking water sources are still the traditional ones including dams, wells, rivers, streams and ponds which might harbor or are prone to contamination with water-borne and vector-borne disease agents (Oyeleke and Istifanus, 2008; Zvidzai et al., 2008). River sand and soils are often littered with animal and human excreta and since the rivers will continue to be a playing ground and source of water for nearby inhabitants, therefore, food and water -related diseases due to faecal contamination continue to be one of the major health problems globally (UNESCO, 2003; Younes and Bartram, 2001; Wright et al., 2004). Faecal matter from human or animal origin often contaminates these drinking or recreational water sources and often present further health challenges. It is estimated that 80% of all illnesses are linked to use of water of poor microbiological quality (WHO, 2002). One of the strategies for tackling this problem is the provision of protected sources such as boreholes, standpipes, protected wells and springs (Ahmed et al., 1998). Such facilities however, are located some distances requiring transportation to homes. During transportation, water gets contaminated with bacteria which grow and proliferate during storage in the homes, consequently posing a risk of infection with water-borne pathogens, Stx producing bacteria inclusive (Hoque et al., 2006; Wright et al., 2004).

Many parts of Africa have been associated with high pit latrine coverage (Taulo et al., 2008). These latrines often collapse because of poor soils (sand) on which they

are dug. Leaching of pit latrine contents and flooding of human and animal wastes into drinking or recreational water sources during rainy season could be possible sources of contamination (Mathess et al., 1988). Furthermore, recreational waters can also be contaminated considering that fingers are prone to faecal contamination during toilet use, cross contamination of water bodies is therefore very easy thus promoting occurrence of diarrhoeal disease outbreaks. The potential of water to harbour microbial pathogens and causing subsequent illness is well documented for both developed and developing countries (Taulo et al., 2008) . Dysentery caused by *Shigella* spp. (faecal bacteria) for example is a public health problem in many regions of the world and is very significant in the developing countries (Luo et al., 2002). Most African countries are associated with rearing of cattle which are often housed near settlements, coupled with poor hygiene attitude, food and water sources are easily contaminated with these bacterial agents. Other general factors include advanced age, immunosuppression, malnutrition and lack of immunity as in neonates.

Antimicrobial resistance and resistance factors among Shiga toxin producing pathogens

Antimicrobial resistance has been a public health concern globally to which for the past few decades policy makers and the academic community are preoccupied to control (Taulo et al., 2008). The evolutionary prowess of microorganisms presents serious challenges to successfully stop the development of antimicrobial resistance (Stephan and Mathew, 2005). Predisposing factors including self medication, over- the-counter sales of antibiotics and flooding the markets with fake and sub standard drugs further aggravates the situation. In recent years, increase of antimicrobial drug resistance among members of the Enterobacterioceae and Moraxellaceae has been observed in several countries (Humphrey, 2000; Cailhol et al., 2006; Grotiuz et al., 2006).

The National Antimicrobial Resistance Monitoring System (NARM) for enteric bacteria began monitoring for resistance to cephalosporins and other drugs among human derived *Salmonella* and *E. coli* 0157 isolates in 1996 (NARM 2004). The increase in resistance to second and third generation cephalosporins among the Enterobacteriaceae is attributed to the acquisition and expression of extended-spectrum -lactamase (ESBL) enzymes among Enterobactericeae (El Astal and Ramadan, 2008). ESBL producing strains have variable susceptibility rates for floroquinolnes, aminoglycosides and fourth generation cephalorsporins. The carbapenems are the only class of antibiotics commonly active against ESBL, however, ESBLs are known to be multi-drug resistant (Cherla et al., 2003; Alex and Henry, 2005 Heijnen and Medema, 2006) . Since their description in the mid-80s, the incidence of ESBL-producing isolates has steadily increased showing variations between geographical areas. They are also involved in nosocomial outbreaks (as well as the Moraxellaceae) conferring multiple drug resistance and resulting in limitation in therapeutic options. ESBLs are derivatives of simple -lactamase (TEM or SHV) enzymes that are harboured mostly by Gram-negative bacilli. Selective pressure by the use of second and third generation cephalosporins favours the development of mutations that results in conformational changes in the active serine site of amino acid sequence of TEM or SHV enzymes. As a result of these mutational changes, these organisms acquire an extra gene copy that makes them to develop resistance to a wide range of antibiotics which they were previously susceptible (Wong et al., 2000; Satheesh et al., 2006; Livermore, 2005).

Though it has been reported that antibiotics which inhibit prokaryotic translation (e.g. erythromycin and doxycycline), can be used to effectively control Shiga toxin producing bacteria, the development of high rate of resistance by this group of organisms to cephalosporins, aminoglycosides and quinolones has significantly reduced treatment options of the resulting infections (Bureau of Epidemiology, 2000; Iroha et al., 2008). Furthermore, the increased secretion of toxin *in vitro* by the bacterial agents as a result of treatment with bacteriophage-inducing antibiotics, including all quinolones, trimethoprim, furazolidone, fosfomycin and Mitomycin C has made the situation more hopeless (Helms et al., 2002; Satheesh et al., 2006; Livermore, 2005; Jean et al., 2005; Satheesh et al., 2006; Abong'o and Momba, 2009).

Conclusion

The direct and indirect costs incurred by infection of humans with Shiga toxin bacteria in terms of morbidity, economic loss and loss of human lives are increasingly becoming enormous. Many cases of diarrhea in the developing countries remain undiagnosed; several of these cases might be as a result of Stx bacteria especially STEC *E. coli.* Therefore, comprehensive microbiological surveillance programs, which would provide early warning and limit the scale of outbreaks, will ultimately be cost effective, as will vaccination programs. Measures to maximize the microbiological safety of foods are also required. Meat products should be made safe by thorough cooking; dairy products and fruit juices by pasteurization and salad vegetables by adequate irradiation or blanching before consumption. Provision of portable drinking water and improved environmental sanitation by governments and individuals and on the over all, increased awareness on the benefits and strict observance of personal hygiene by the populace are the best preventive measures against such bacterial agents in the face of increasing antimicrobial resistance.

The low infectious dose of STEC infections and the consequent illness which is both serious and can lead to death has made the organisms to be a serious public

health issue. This therefore underpins the need for research into the organisms, not only for food-borne cases, but for environmentally related sources as well. Consequently, specific research approaches should encompass: i) A better understanding of the epidemiology of the transfer of STEC from animals and the environ-ment to man. For example, research to determine whether the types of STEC which are found in farm animals are the same as those found in human disease. Such research would need to ensure that all routes of human infection are addressed, including farm and wild animals (e.g. deer, rabbits etc), the environment (water sources, fields etc), food products and human-to-human transmission; ii) The agreement, internationally, of objective, standardized techniques and systems for typing the different strains of bacteria, and the use of those methods in the quantification of the toxins and producing bacteria from different environments; iii) A greater understanding of the pathogen- host interaction between the toxin producing bacteria and man; iv) Research to determine the risk of transfer of the virulence determinants of the toxins from the producing bacterium to other organisms; v) The mystery of Shiga toxins becoming more abundant in medium when exposed to antibiotics needs to be unraveled. Solution to the control of such bacteria might be embedded in novel antibiotic sources from plants with diverse novel mechanisms of action. More investigations into this area are very pertinent now with higher incidences of Shiga toxin producing bacterial infections in both the developed and the developing countries.

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