African Journal of Biotechnology Vol. 10(64), pp. 14160-14168, 19 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1534
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Virulence factors and antibiotic susceptibility among verotoxic non O157: H7 *Escherichia coli* isolates obtained from water and wastewater samples in Cape Town, South Africa.

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Accepted 7 September, 2011

Forty eight samples (30 wastewater and 18 river water) were collected between July and November, 2010, from different sources in Cape Town, South Africa in order to characterize verotoxic non O157: H7 *Escherichia coli* strains. Samples (1 ml) were inoculated into MacConkey broth (MB, 9 ml) and incubated at 37°C for 24 h, after which a loopful of the MB was then spread onto Eosin Methylene Blue (EMB) and further incubated for 24 h at 37°C in order to isolate *E. coli.* The identification of isolates was done using standard biochemical procedures; and confirmed serologically using *E. coli* polyvalent antisera (Bioweb, SA). Isolates were also characterized for virulence factors such as verotoxin, haemolysin, gelatinase, extended spectrum beta lactamases (ESBLs), cell surface hydrophobicity and bacterial serum resistance, as well as susceptibility (using disc diffusion method) to stem bark extracts of *Curtisia dentata.* Results showed the presence of different serotypes of *E. coli* (69 isolates in all) including 026: H11, 055, O111: NM, O126, O44, O124, O96:H9, O103:H2, O145: NM and O145:H2. Over 60% of the isolates exhibited serum resistance, haemolysin and gelatinase production, 81% exhibited cell surface hydrophobicity and over 52% produced ESBLs. Results also showed that while 60% of the isolates showed various levels of resistance to different antibiotics [ampicillin (10 µg), cefuroxime, cephalexin, ceftazidime and tetracycline (30 µg in each case) (multidrug resistance index (MDRI) values 4.20 to 5.60%)], only 28% were resistant to ethanol stem bark extracts of *C. dentata* (MIC, 70 to 150 mg/ml). The presence of pathogenic verotoxic antibiotic resistant *E. coli* in these water sources is a threat to water quality and food security and *C. dentata* has a potential for sourcing novel antibiotic substances for chemotherapy against these resistant pathogenic strains of *E. coli.*

Key words: *Curtisia dentata, Escherichia coli,* haemolysins, cell surface hydrophobicity, gelatinase, plant extracts, verotoxins.

INTRODUCTION

Escherichia coli (Enterobacteriaceae) are short Gram-negative bacilli, non-spore forming fimbriate with peritrichous flagellum, with capsule or microcapsule often present. The bacteria grow readily on simple culture or synthetic media with glycerol or glucose as the sole carbon source and energy. On solid media, colonies are circular and smooth with a complete edge; some strains produce mucoid colonies (Villaseca et al., 2005). *E. coli* are widespread intestinal parasites of mammals, birds and humans and are present wherever there is faecal contamination (Doughari et al., 2009). They are usually considered to be opportunistic pathogens, which constitute a large portion of the normal intestinal flora of humans. The bacteria can, contaminate, colonize, and subsequently cause infection of extra intestinal sites, and are a major cause of septicemia, peritonitis, abscesses, meningitis, and urinary tract infections (UTI) in humans.

E. coli is an incredibly diverse bacterial species with the

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ability to colonize and persist in numerous niches both in the environment and within animal hosts (Wiles et al., 2008). The organisms are known to cause enteric infections and diarrhea (gastroenteritis) in humans and animals, and many strains have been identified to produce verotoxins or shiga toxins. These toxins are responsible for lethal acute bloody diarrhea (haemolytic colitis and haemolytic uremic syndrome) in humans (Karmali et al., 1983; Karch et al., 1999). Five classes or virotypes of *E. coli* are recognized as causative agents of these diarrheal diseases amongst which include enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC) and enterohemorrhagic *E. coli* (EHEC) (Doughari et al., 2009). Each class falls within a serological subgroup and manifests distinct features in pathogenesis.

Diarrheal diseases remain the second leading cause of death worldwide, which is responsible for an estimated 2,219,000 deaths in 1998, representing approximately 4.1% of all deaths mostly among children under the age of five, and also accounts for 5.3% of the disease burden in developing countries (Doughari et al., 2009). Concerns for acute diarrhea have been further heightened since the emergence of verotoxin (shiga toxin) producing bacteria that contaminates water and food sources. *E. coli* O157:H7 or 'hamburger bug' strain was the strain most associated with shiga toxin production (Doughari et al., 2009) and therefore widely studied. However, currently several other distinct serotypes of *E. coli* other than the O157 are recognized as shiga toxin *E. coli* (STEC) associated with human diseases. These serotypes including *E. coli* O111:H8, O26:H11, O103:H2 have been reported in some parts of developed countries (Guth et al., 2000, 2003; Doughari et al., 2009). The association of various *E. coli* serotypes with disease of varying severity in humans and with sporadic disease or outbreaks has led to the proposal that verotoxin producing *E. coli* be classified into five seropathotypes, with seropathotype A comprising of O157:H7 and O157:NM, the serotypes considered to be most virulent. Seropathotype B comprises serotypes O26:H11, O103:H2, O111:NM, O121:H19, and O145:NM, that are similar to the O157 in causing severe disease and outbreaks but occur at lower frequency. Seropathotype C comprises of serotypes that are infrequently implicated in sporadic haemorrhagic uremic syndrome (HUS) but are not associated with outbreaks and include O91:H21 and O113:H21. Seropathotype D is composed of numerous serotypes that have been implicated in sporadic cases of diarrhoea, and seropathotype E comprises of the many verotoxin producing serotypes that have not been implicated in human diseases (Karmali et al., 2003). Though reports abound on *E. coli* O157:H7 outbreaks in developed countries, and despite their increasing medical significance, only a handful of reports are available in developing countries especially in Africa and little or none at all is available on the other verotoxic non O157:H7 *E. coli* strains.

Because of its clinical significance, *E. coli* has been the subject of numerous investigations in an attempt to define those virulence factors which allow it to initiate and sustain infections. It is now believed that virulence in *E. coli* is multifactorial (Cavalieri et al., 1984) and attributes such as verotoxin production, production of haemolytic enzymes, and gelatinases, cell surface polysaccharides that facilitate adhesion or ability to resist the phagocytic properties of human and animal serum are associated primarily with virulent strains. These factors often interact in a complicated manner so that the precise mechanisms still remain to be established (Hedge et al., 2008). For the successful development of new therapies and for the effective prevention and control of diarrhea, identification of pathobiologic mechanisms is important since the presence of a microorganism in any sample does not prove a causal relationship to disease (diarrhea) (Cavalieri et al., 1984).

The source of *E. coli* pathogens in most cases is believed to be the host's own intestinal flora, thus transmission is largely via the oral-faecal route through the consumption of food or water contaminated with the organisms. Water or food contamination is often encountered when faeces containing the bacteria gain access to these food and water sources. This phenomenon is an existing threat to food and water safety in the developing countries (WHO, 2002). It is therefore important to investigate food and water sources in order to determine whether pathogenic *E. coli* are present, with the view to developing proactive preventive or control measures. This work therefore investigated some water samples in South Africa for the presence of verotoxic *E. coli* other than *E. coli* O157, their virulence potentials, as well as the effect of stem back extracts of *Curtisia dentata* on the isolates.

MATERIALS AND METHODS

Sample collection

Forty eight water samples (duplicates) were collected from four different sources: wastewater treatment plant, River Berg, River Plankenberg and Winelands Pork Abattoir, all in Cape Town, South Africa, for a period of five months (July to November, 2010). To collect the water samples, the shoreline sampling method as described by Obire et al. (2005) was adopted. In this procedure, 250 ml volume sized sterilized sample bottles were held at the base and dipped downwards below the water surface (20 to 30 cm deep), opened and allowed to fill up, then corked while still under water (Health Protection Agency, 2007). The collected water samples were maintained in a cooler box with ice packs (4 to 10°C) and then immediately transported to the University laboratory where they were analyzed within 3 to 6 h.

Isolation and identification of *E. coli*

For isolation of bacteria, water samples were first filtered using membrane filter, then the filter paper was inoculated into MacConkey broth (MB; DIFCO, MD, USA) and incubated at 37°C for 24 h, after which a loopful of the MB was spread onto plates of Eosin

Methylene Blue (EMB, Oxoid, SA) and further incubated at 37°C for 24 h. Isolates were further purified by picking discrete colonies (green metallic sheen) and subculturing onto fresh plates of EMB and further incubating for 18 to 24 h at 37°C. After incubation, 1 to 2 discrete colonies were inoculated into the presumptive diagnostic medium sulfide-indole-motility medium (SIM) and incubated at 35°C for 24 h. Further characterization of isolates was carried out using the IMViC (DIFCO, MD, USA) test kit. Isolates that were indole positive, hydrogen sulfide negative, non-motile, as well as negative for methyl red, Voges- Proskauer and citrate utilization tests were identified as *E. coli.* Slide agglutination tests were performed on selected 5 to 10 presumptive single colonies using polyvalent *E. coli* antisera 2, 3 and 4 (Bioweb PTY, SA). *E. coli* ATCC 25922 was used as the control. Serotyped (confirmed) *E. coli* isolates were inoculated onto tryptic soy (TS) slants, incubated for 24 h at 37°C, and then stored at 4°C (Roy et al., 2004; Taraweh et al., 2009) until use.

Detection of virulence factors on the bacterial isolates

Screening of isolates for verotoxin production

All the bacterial isolates were screened for verotoxin production using antibody-based rapid slide agglutination assays with the Duoperth kit (Merck, SA) according to the manufacturer's instructions. The bacterial isolates were first precultured in 1 ml casaminacid yeast extract (CAYE) broth (20 g of casamino acid, 6 g of yeast extract, 2.5 g of NaCl, 8.71 g of KH_2PO_4, and 1 ml of trace salt solution - 0.5% $MgSO_4$, 0.5% $MnCl_2$ and 0.5% $FeCl_3$ dissolved in 0.0005 M H_2SO_4) and incubated at 37°C with rotation at 100 rpm for 24 h. After incubation, 10 µl of the precultured broth (approximately 1 $\times 10^7$ cells/ml) was inoculated into fresh CAYE broth and further incubated for 16 h with rotation at 100 rpm at 37°C. The culture was centrifuged at 5000 x *g* for 5 min to separate the supernatant and cell pellets. The cell pellets were then washed three times with phosphate buffered saline (PBS, 5 ml) and then suspended in 0. 25 ml 0.01 M Tris-HCl (pH 7.5). To the pellets, 0.5 ml distilled water containing 50 µg/ml polymyxin B was added and the suspension incubated at 37°C for 30 min. 200 µl of the culture suspension was then transferred onto the test device using a sterile Pasteur pipette and the result read after 10 min. The appearance of red bands on the vtx1 or vtx2 bands denoted the presence of either one of or both verotoxins.

Screening of isolates for haemolysin production

The plate haemolysis technique as described by Sharma et al. (2007) was used to screen for the presence of cytolytic protein toxins known as the alpha haemolysin secreted by most haemolytic bacteria. In this procedure, discrete bacteria colonies (2 to 3) from nutrient agar (NA) plates were subcultured onto 5% sheep blood agar plates supplemented with 10 mM $CaCl_2$ and incubated at 37°C for 24 h. After incubation, enterohemolysin production was detected by the appearance of a complete zone of erythrocytes lysis around each bacterial colony on the plates.

Cell surface hydrophobicity test

The cell surface hydrophobicity of the bacterial isolates was determined using the salt aggregation test (SAT) as described by Raksha et al. (2003) and Sharma et al. (2007). Briefly, a loopful (10 µl) of bacterial suspension in 1ml of phosphate buffer (pH 6.8) (equivalent to 5×10^9 colonies/ml) was mixed with equal volumes of ammonium sulphate solution of different molarities (1.4, 2.0 and 4.0 M) on a glass slide. The suspensions were rotated carefully for 1 min and then microscopically observed for agglutination. The highest dilution of ammonium sulphate solution giving a visible agglutination of bacteria was scored as the SAT value. Bacterial suspension clumping at the lowest dilution (1.4M) was considered autoaggregative, while those with SAT values of ≤ 2 M were considered hydrophobic.

Screening of isolates for gelatinase production

To screen the bacterial isolates for gelatinase production, gelatin agar was inoculated with the test bacteria and then incubated at 37°C for 24 h, after which the plate was flooded with mercuric chloride ($HgCl_2$) solution. The development of opacity in the medium and a zone of clearing around the bacterial colonies was considered positive for the presence of gelatinase (Sharma et al., 2007).

Bactericidal serum resistance assay

Bacteria were first grown on blood agar for 18 to 24 h at 37°C. The cells were then harvested and suspended in Hank's balanced salt solution (HBSS). Equal amounts (0.05 ml) of the bacterial suspension and serum was mixed in a test tube and then incubated at 37°C for 180 min and absorbance read at 600 nm. Viable count (%) was determined by calculating the differences in absorbance value before and after incubation. Resistance of the bacteria to serum bactericidal activity was expressed as the percentage of bacteria survival after 180 min of incubation with serum in relation to the original count. Bacteria were termed serum sensitive if viable count dropped to 1% of initial value, and resistant if >90% of organisms survived after 180 min of incubation.

Screening of isolates for extended spectrum beta-lactamase (ESBL) production

The screening of isolates for ESBL was carried out using the disc diffusion method according to the criteria recommended by NCCLS (2005). Briefly, two discs (30 µg in each case), ceftazidime and cefotaxime were placed on Muller Hinton agar (MHA) plates previously seeded with the test bacteria and the plates incubated at 37°C for 18 h. After incubation, ESBLs production was determined by the appearance of zone diameters of inhibition (≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime) against the test bacteria.

Phenotypic confirmation of ESBLs production

The double disc synergy test (DDST) as described by Iroha et al. (2008) and Sharma et al. (2007) for confirming ESBLs production was used for this purpose. 0.1 ml of each bacterial isolate suspension equivalent to 0.5 McFarland turbidity standard was spread on Mueller-Hinton agar plates using a sterile swab stick. A combination disc containing amoxicillin (20 µg) and clavulanic acid (10 µg) was placed at the centre of the Petri-dish and ceftazidime (30 µg) and cefotaxime (30 µg) was placed 15 mm apart center to center on the plates and then incubated at 37°C for I8 to 24 h. An enhanced zone of inhibition (synergy, regardless of size) between any one of the beta-lactam discs compared to the combined amoxicillin-clavulanic acid disc was considered to be positive for ESBL enzyme production (Iroha et al., 2008).

Determination of multi-drug resistance index (MDRI) for bacterial isolates

This test was carried out using the disc diffusion method as

described by Perilla et al. (2003). MHA plates were inoculated with the test organisms (0.5 McFarland turbidity standard) using a sterile swab stick and the plates were incubated at 37℃ for I8 to 24 h. After incubation, the zone diameters of inhibition (mm) were measured. The bacteria were tested for susceptibility against ampicillin (10 µg), cefuroxime (30 µg), cephalexin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), aztreonam (30 µg), nalidixic acid (30 µg), amikacin (30 µg), tetracycline (30 µg), gentamicin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg) (Oxoid UK) (WHO, 2002) and stem bark extracts of *C. dentata* (250 µg). Resistance to more than four antibiotics was taken as multidrug resistance (MDR). MDR index (MDRI) of individual isolates was calculated by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics to which the isolate was exposed (Chandran et al., 2008). Isolates with MDRI values of more than 0.2 or 20% were considered highly resistant.

$$\text{MDRI (\%)} = \frac{\text{Number of antibiotics resisted}}{\text{Total number of antibiotics used}} \times 100$$

Extraction and determination of phytoconstituents from stem bark extracts of *C. dentata*

To extract phytoconstituents from the plant material, 5 g ground plant stem barks was soaked in 200 ml of solvent (water and ethanol) for 2 h followed by filtration; the procedure was repeated three times. The filtered extracts obtained from extraction with any one solvent was combined, and dried under vacuum at 25℃. The percentage yield of the extract was calculated and then used to screen for the presence of phytoconstituents as described by Doughari and Ioryue (2009).

Determination of antibacterial effects and minimum inhibitory concentration (MIC) of the stem bark extracts of *C. dentata* against *E. coli.*

Antibacterial activity determination was carried out using the filter paper disc diffusion method as described by Doughari and Obidah (2008). Filter papers (4 mm in diameter) were cut using a paper punch and then sterilized by autoclaving. The sterilized filter papers were then soaked in different concentrations of extracts (100 to 3000 µg/ml and 2.5 to 200 mg/ml/disc) and then allowed to dry. To test for susceptibility, dried extract-soaked filter paper discs were placed on different MHA plates earlier seeded with different test organisms (0.5 ml McFarland turbidity standard) and left on the table for 5 min to dry. The plates were then incubated at 37℃ for 24 h, after which the antibacterial activity was determined as relative inhibition zone diameters (mm) against each test bacteria. Dried filter paper discs soaked with ethanol or 30 µg/ml ampicillin were used as negative and positive controls respectively.

To determine the MIC of the plant extracts against the test bacteria, the organisms were inoculated into test tubes containing varying concentrations (50, 100, 150, 250, 500, 1000 µg/ml and 2.5, 10, 50 and 100 mg/ml) of plant extract and 1 ml of nutrient broth (NB) was added. A loopful of the test bacteria previously diluted to 0.5 McFarland turbidity standard, was introduced into each broth sample. The procedure was repeated on the test organisms in test tubes containing NB and the standard antibiotic ampicillin (as negative control), or NB only (as positive control). All the culture tubes were then incubated at 37℃ for 24 h. After incubation, they were examined for bacterial growth by observing/measuring of turbidity.

RESULTS

Results of physical parameters of the water samples [mean pH and temperature values 6.4, 17.8℃ (waste water), and 7.4, 17.3℃ (river water), respectively], biochemical and cultural characteristics, serotypes, number of resistant isolates and multidrug resistance index values of the *E. coli* isolates obtained from the wastewater and river samples are shown in Table 1. Cultural and biochemical characterization of the isolates showed that *E. coli* exhibited green metallic sheen on EMB agar with variable haemolysis on sheep's blood agar. Results also showed that out of the 69 non 0157 *E. coli* isolates obtained, different serotypes including O26:H11, O55, O111:NM, O126, O44, O124, O96:H9, O103:H2, O145:NM and O145:H2 were present (Table 1). Results of multidrug resistance index (MDRI) showed that the MDRI values ranged between 7.00 to 33.00%, with isolates from wastewater samples exhibiting the highest MDRI values.

Table 2 shows the results of percentage extraction, phytochemical analysis and MIC values of the aqueous and ethanol stem bark extracts of *C. dentata.* The results showed that the highest amount of extracts (58.82%) was obtained from water compared to that of ethanol (38.72%). For phytochemicals, although saponins, tannins, anthraxquinones, steroids and phenols were common to both ethanol and aqueous extracts, only ethanol extracts contained alkaloids, while aqueous extracts contained glycosides and flavonoids. Result also revealed that the MIC values of the water extracts ranged between 100 to 2500 mg/ml and 70 to 150 mg/ml for aqueous stem bark and ethanol stem bark extracts, respectively.

Figure 1 shows the results of the presence of virulence factors in the various *E. coli* serotypes isolated. Results shows that 47% of the isolates produced verotoxins (both Vtx1 and Vtx2, 38%, Vtx1, 6% and Vtx2, 3%), 81% exhibited cell surface hydrophobicity, over 60% exhibited serum resistance, haemolysin and gelatinase production, while over 52% produced extended spectrum beta-lactamases (ESBLs). Results also shows that while 60% of the isolates showed various levels of resistance to different antibiotics [ampicillin (10 µg), cefuroxime, cephalexin, ceftazidime and tetracycline (30 µg in each case)] (Figure 2), only 28% showed various resistance to ethanol stem bark extracts of *C. dentata.*

DISCUSSION

E. coli is a consistent inhabitant of the human intestinal tract, and it is the predominant facultative organism in the human gastrointestinal tract. The frequency of *E. coli* in the human intestine and faeces has led to its usage amongst other coliform bacteria as an indicator of faecal pollution and water contamination. Thus, the presence of *E. coli* in any given food or water source is an indication of faecal contamination by intestinal parasites

Table 1. Cultural and biochemical characteristics, percentage resistant to four or more antibiotics and multidrug resistance index (MDRI) values of *Escherichia* serotypes obtained from wastewater and river water samples.

*Water source/temperature(℃)/ pH/Number of sample	*E. coli* serotypes from each water sample	Cultural and biochemical characteristics of *E. coli* strains isolated from the water sample								Number of isolate	Number (%) of isolates resistant to four or more antibiotics	MDRI (%) values range for isolate
		EMB	ShB	G	S	I	M	E	O			
#Wastewater treatment plant/17.8/6.4)/18 samples	O103:H2, O86, O145:H2, O96:H9,O126, O4,O55,O111:NM,O124,O44,O124,O44	+	+/-	-	-	+	-	+	+	25	17(68.00)	7.00-33.00
!Abattoir wastewater/ 17.8/6.4/12 samples	O4,O145:H2,O111:NM,O103:H2,O113, O86,O26:H11,O96:H9,O124	+	+/-	-	-	+	-	+	+	24	12(50.00)	7.00-33.00
River Plankenberg/ 17.3/7.2/18 samples	O86,O113,O145:H2,O4, O103:H2,O96:H9	+	+/-	-	-	+	-	+	+	7	0(0.00)	7.00-20.00
River Berg/17.3/7.2/18 samples	O4,O26:H11,O86,O103:H2	+	+/-	-	-	+	-	+	+	13	4(38.78)	7.00-13.33

EMB, Eosin Methylene Blue; ShB, haemolysis on sheep blood agar; G, Gram reaction; S, sulphide production; I, indole production; M, motility; E, Erchlich's reagent; O, oxidase reaction; - = negative; + = positive; +/- = variable haemolysis *48 samples in total; #from Athlone Wastewater Treatment Plant; !from Winelands Pork Abattoir.

Table 2. Phytochemical constituents and mean minimum inhibitory concentration (MIC) values of aqueous and ethanol stem bark extracts of *Curtisia dentata*.

Extract	Extraction (%)	Phytoconstituent								Mean MIC range for *C. dentata*
		Saponin	Tannin	Alkaloid	Glycoside	Anthraquinone	Flavonoid	Steroid	Phenol	
WE	58.82	+	+	-	+	+	+	+	+	100-2500 mg/ml
EE	38.72	+	+	+	+	+	-	+	+	70-150mg/ml

WE, Aqueous extract; EE, ethanol extract; + = present; - = absent

of humans. Results of this study revealed the presence of various verotoxin producing non O157 serotypes of *E. coli* (O26:H11, O55, O111:NM, O126, O44, O124, O96:H9, O103:H2, O113 and O145:H2) from the river and wastewater samples investigated. The wastewater samples contained more serotypes of *E. coli* compared to the river samples. The wastewater samples contained mixtures of wastes emanating either from animal or human excreta, industries or the hospitals, and these wastes contained a high load of enteric bacteria including *E. coli* that therefore accounts for a higher number of *E. coli* serotypes. Majority of these serotypes exhibited the presence of virulence factors. Virulence factors enable *E. coli* to colonise selectively the mucosal uro-epithelium and to evoke an inflammatory reaction from the host and eventually making the host tissues susceptible to invasion. Therefore the capacity of *E. coli* to produce many virulence factors contributes to its pathogenicity consequently causing a variety of infections such as gastrointestinal and urinary tract infections, soft tissue infections, bacteraemia and neonatal meningitis. These virulence factors enable the bacteria elicit an infection by overcoming the host defence mechanisms. In this study, many of the *E. coli* isolates investigated exhibited verotoxins, cell surface hydrophobicity, serum resistance, haemolysin, gelatinase production and ESBLs production. Verotoxin or shiga toxin-producing bacterial strains are associated with a broad spectrum of human

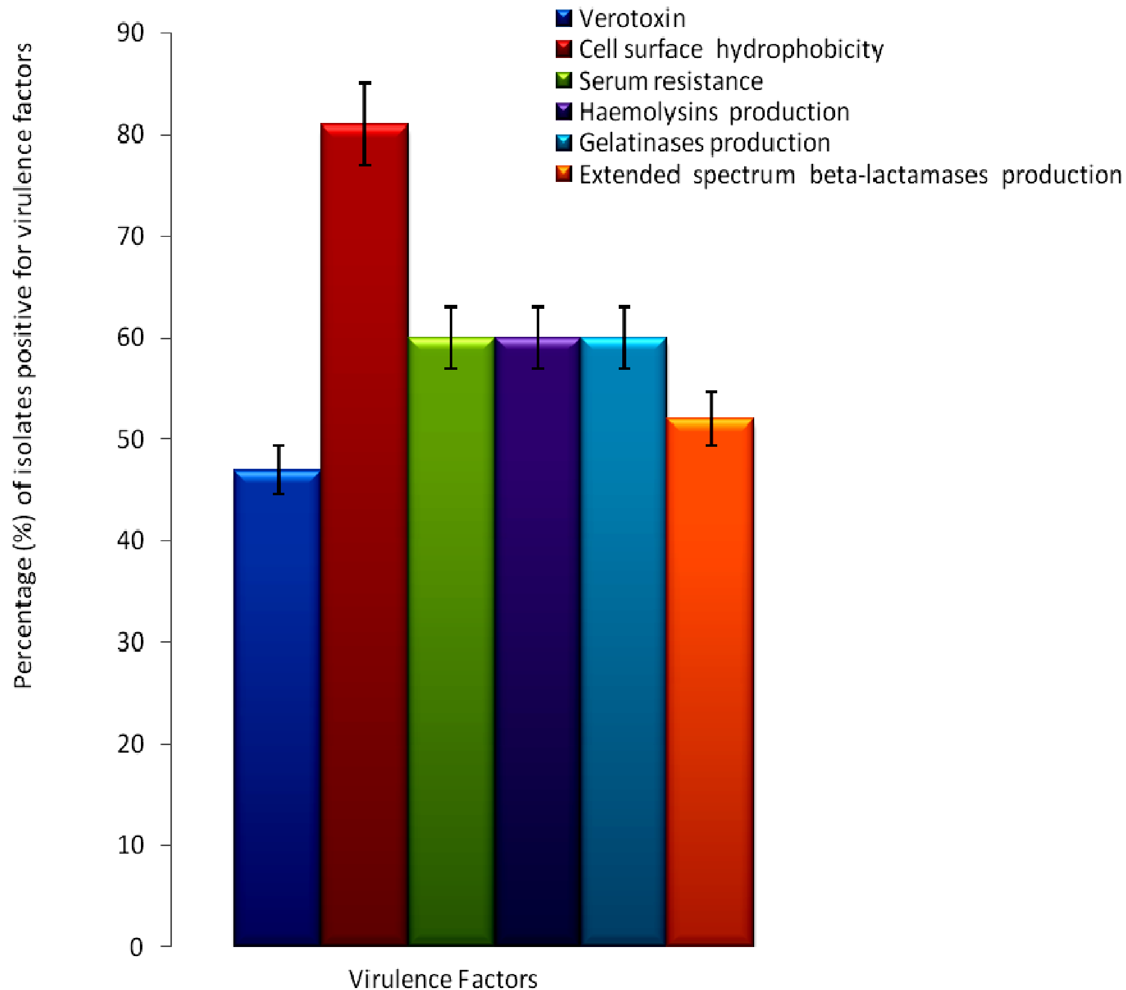

Figure 1. Virulence factors detected on *E. coli* isolates obtained from river and wastewater samples.

illnesses throughout the world, ranging from mild diarrhea to haemorrhagic colitis (HC), HUS and thrombotic thrombocytopenic purpura (TTP). Complications arising from antibiotic treatment of verotoxic related human infections have also been reported (Doughari et al., 2010). Though the wastewater samples investigated are not directly consumed, the presence of a significant proportion (47%) of verotoxin producing bacteria from the various water samples investigated is a cause for concern since the samples often gets discharged into large water bodies or are utilized for irrigation purposes. It is also a common phenomenon for rural dwellers to use river waters for both domestic and drinking purposes as well as for bathing thus increasing the possibility of contracting these bacteria.

Cell surface hydrophobicity enhances the adherence of bacterial cells to host cell surfaces including mucosal epithelial cells and confers them with resistance to phagocytosis by host cells. In this study, greater number of the *E. coli* strains demonstrated hydrophobicity. Previous studies on urinary tract infection cases reported high rate of exhibition of cell surface hydrophobicity by some pathogenic strains of *E. coli* (Sunman et al., 2001; Raksha et al., 2003). The presence of hydrophobic strains of *E. coli* in this water sources is an indication that the water could be a potential source of agents of urinary tract infections or gastroenteritis if consumed. Haemolysin production as a virulence factor by urinary isolates of *E. coli* has been reported previously (Jhonson, 1991; Raksha et al., 2003). It has been suggested that colonization of the urinary tract with haemolytic strains of *E. coli* is more likely to develop into urinary tract infections. Haemolysis may contribute to tissue injury, survival in renal parenchyma and entry into blood stream and increasing the possibility of establishment of acute pyelonephritis (Raksha et al., 2003). The mode of action of haemolysins involves pore formation on the colonized host cell (Wiles et al., 2008) and their production is associated with pathogenicity of *E. coli*, especially the more severe forms of infection (Jhonson, 1991). The higher rate of haemolysin producing strains isolated from this water samples therefore highlights the presence of invasive *E. coli* strains

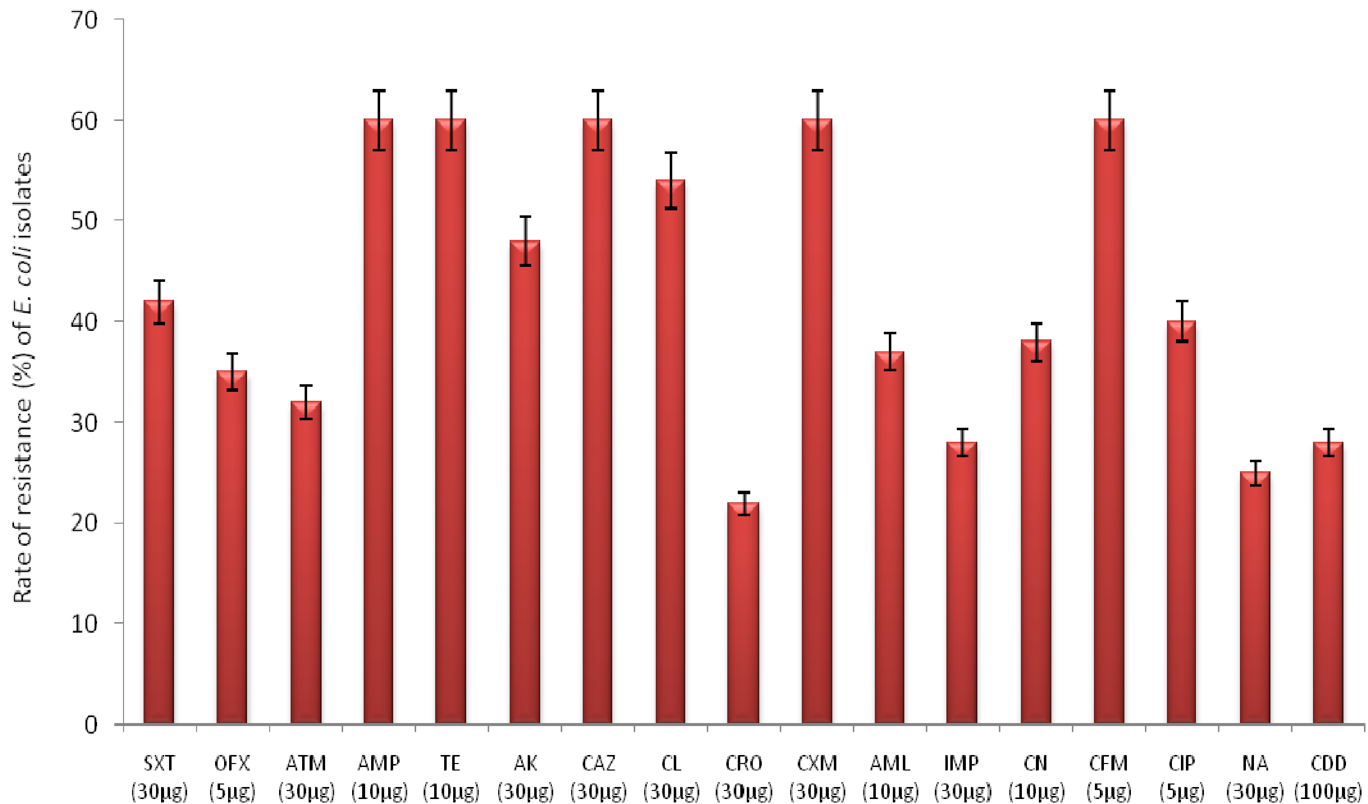

Figure 2. Antibiotic resistance rate amongst antibiotics and stem bark extracts of *C. dentata* on *Escherichia* isolates obtained from river and waste water samples. SXT- sulphomethaxazole / trimethoprim; OFX, ofloxacin; ATM, aztreonam; AMP, ampicillin; TE, tetracycline; AK, amikacin; CAZ, ceftazidim; CL, cephalexin; CRO, ceftriazone; CXM, cefuroxime; AML, amoxycillin; IMP, impenim; CN, gentamicin; CFM, cefixime; CIP, ciprofloxacin; NA, nalidixic acid; CDD = stem bark extracts of *Curtisia dentata.*

in this environment.

Serum resistance is the property by which the bacteria resist killing by normal human serum due to the lytic action of complement system. Results of this study also showed a high rate of serum resistance among the *E. coli* isolates. Siegfried et al. (1994) and Raksha et al. (2003) previously reported serum resistance among *E. coli* isolates obtained from urine samples. Among *E. coli* virulence factors, serum resistance have been shown to have a high correlation with pathogenicity. Chaffer et al. (1999) has also reported a relationship between virulence and resistance to compliment in *E. coli* serogroup O2 although additional virulence factors are required for bacteria to successfully cause an infection. The presence of serum resistant strains of *E. coli* in the water samples studied calls for more proactive measures in the control of potential infections by these bacteria. The wastewater from the waste treatment plant is not often consumed directly but released into farm areas and used for irrigation purposes, consequently if farmers and/or farm workers do not observe very high hygienic standards while using the water, may be liable to contract potentially acute bacterial infections. In 2006, an outbreak of *E. coli* O157 H:7 was reported in the United States and Canada and the source was said to be spinach contaminated with irrigation water in California (Ishii and Sadowsky, 2008). Isolation of serum resistant *E. coli* from these wastewater and river water sources has far reaching health implications. For instance, *E. coli* isolates obtained from patients with pyelonephritis, cystitis and bacteraemia were typically serum resistant whereas isolates from patients with asymptomatic bacteriuria were serum sensitive strains (Raksha et al., 2003). In addition, Gram-negative bacteria isolates that showed serum resistance demonstrated high degree of survival in the blood during bacteraemia (Raksha et al., 2003). A strong correlation between serum resistance bacterial invasion and survival in the human bloodstream amongst Gram-negative has been reported (Siegfried et al., 1994).

Gelatinase is an extracellular metalloendopeptidase capable of hydrolyzing bioactive peptides such as gelatin, pheromone, collagen, casein, fibrinogen, haemoglobin

and other bioactive peptides (Makinen and Makinen, 1994). The enzyme is an extracellular zinc endopeptidase that plays a very significant role in bacterial pathogenesis by causing direct or indirect damage to host tissue thus facilitating microbial invasion and survival in the host (Alebouyeh et al., 2005; Furumura et al., 2006). The presence of these enzymes on *E. coli* isolates is a further confirmation of their potential to cause infections.

Extended spectrum beta-lactamase (ESBL) production by *E. coli* from this study is high. This might be as a result of selective pressure imposed by extensive use of antimicrobials in animals and agriculture. Animal farming recently involves the use of antibiotics in chemotherapy. Wastewater from the wastewater treatment plant comprised mixtures of water from different sources including hospitals and animal farms where an extensive use of antibiotics in treatment of both animal and human diseases is applied. The indiscriminate use of cephalosporins is responsible for the high rate of selection of ESBL producing microorganisms. Bradford (2001) has earlier reported high prevalence rate in the production of ESBLs among *E. coli* isolates. ESBLs confer bacteria with resistance to β-lactam antibiotics. However the results of this study are in contrast with those reported by Johnson et al. (2003) where a corresponding decrease of ESBLs production with increase in virulence factors among bacteria was reported. Since ESBL production is usually plasmid mediated, it is possible, for one specimen to contain both ESBL producing and non-producing cells and, at the same time, contain other virulent factors.

In this study, most of the *E. coli* isolates have the combination of two or more of the virulent factors (cell surface hydrophobicity, serum resistance, gelatinase or haemolysin production as well as extended beta-lactamase production). The presence of multiple virulence factors increases the virulence of organisms, since such factors function synergistically in overcoming normal host defences. Thus, bacterial strains with a more extensive virulence factor complements are more effective as virulent pathogens. This therefore implies that the isolates from these water sources may be considered as potentially pathogenic.

Studies on the antibiotic susceptibility pattern for all the isolates of *E. coli* obtained showed resistance to commonly used antibiotics such as ampicillin (10 µg), cefuroxime, cephalexin, ceftazidime and tetracycline (30 µg in each case). The MDRI of some of the isolates (33.00%) is well above 20% which signifies that the bacteria are highly resistant to available antibiotics. Multi-drug resistance among *E. coli* isolates has been subjects of concern worldwide (**Chitnis** et al., 2003; Lestari et al., 2008). Dissemination of resistance genes among isolates have been held responsible for rapid and widespread multidrug resistance among not only *E. coli*, but numerous other pathogenic bacteria as well. This therefore calls for strict observance of antibiotic susceptibility test results in the prescription of antibiotics.

The demonstration of antimicrobial activity against many of the *E. coli* isolates by ethanol stem bark extracts of *C. dentata* with a low MIC values (70 to 100 mg/ml), is an indication that the plant contains bioactive components that are antagonistic to the bacteria. *C. dentata* may therefore play a very important role as source of newer chemical substances that can be used in the development of chemotherapeutic agents for the treatment of diarrhoea, urinary tract infections, bacteraemia and other infections caused by *E. coli.* The high MIC values (100 to 2500 mg/ml) recorded for the aqueous extracts compared to the ethanol extracts may not necessarily mean that the extracts do not posses antimicrobial activity, but that the phytoconstituents may be present in very low amounts at the tested concentrations. Antimicrobial activity of the acetone extracts of leaves, twigs and stem barks of *C. dentata* against *Bacillus subtilis, E. coli, S. aureus, P. aerugenosa, E. faecalis* and *C. albicans* as well as inhibition of motility in some parasitic and free living nematodes has earlier been reported (McGaw et al., 2000; Shai et al. 2008, 2009). This however, is the first report on the activity of *C. dentata* on verotoxic bacteria. Though water yielded the highest amount of extracts, the ethanol extracts showed higher activity against the test bacteria compared to the aqueous extracts. Solvents are known to have different degrees of extraction depending on their polarity (Doughari and Ioryue, 2009). The presence of phytoconstituents such as saponins, tannins, alkaloids, anthraquinones, steroids and phenols further confirms the potential application of the plant in sourcing antibiotic substances for a possible development of novel chemotherapeutic agents.

Conclusion

Though the *E. coli* strains studied in this paper were isolated from water samples, the bacteria demonstrated the capacity to adapt and survive in different tissues, by producing virulent factors and developing drug resistance. The isolates strains obtained from the water sources therefore are potential agents of human infections such as diarrhoea, urinary tract and ear infections, depending on the site of colonization. The rise in incidences of drug resistance amongst pathogenic *E. coli* strains has been demonstrated from the results of this study. This therefore calls for proper selection of antibiotics for treatment, based on an adequate detection of bacteria resistant to drug through the results of antibiotic susceptibility test as well as the judicious use of antibiotics in humans and animals. Good antibiotic policy is also required in order to limit the emergence and spread of antibiotic resistance in bacteria. Farmers should further be enlightened on the need to maintain personal hygiene, especially while handling wastewater for irrigation purposes. The public should also be educated on the need for boiling of river water before drinking, as well as maintenance of food and personal

hygiene among food handlers.

ACKNOWLEDGEMENT

The study was supported by Cape Peninsula University of Technology, Cape Town South Africa through the University Research Fund RP03.

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