Research Paper

Characterization of verotoxigenic *Escherichia coli* (VTEC) isolates from faeces of small ruminants and environmental samples in Southern Jordan

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This study was conducted to determine the prevalence rate of VTEC in slaughtered sheep and goats and to evaluate the contamination rate of VTEC in slaughterhouses and butchers' shops in southern Jordan. 201 *E. coli* isolates from animals' faecal samples and 33 *E. coli* isolates from slaughterhouse/butcher shop samples were characterized by multiplex PCR (mPCR) reaction for detection of *stx1, stx2*, *eae A* and *E-hly A* virulent genes. Twenty-six virulent *E. coli* isolates were characterized by mPCR to seven different virulent patterns: *stx1*, *stx1+stx2*, *stx1+eae A*, *stx1+E-hly A*, *stx1+eae A+E-hly A*, *eae A* and *E-hly A*. It was found that VTEC comprised 6.4% and 21% of the total *E. coli* isolates from slaughtered small ruminants and slaughterhouses/butchers' shops, respectively. The VTEC comprised 76.2% of the virulent isolates. The proportion of *stx1*:*stx1+stx2* patterns was 19:1. It was found that the characterized complex VTEC (containing *eae A* and/or *E-hly A*) possessed three virulence patterns, including (VTEC) *stx1+eae A*, (VTEC/EHEC) *stx1+E-hly A* and (VTEC/EHEC) *stx1+eae A+E-hly A* in percentages of 30%, 25% and 10%, respectively, in relation to the total VTEC isolates. Only two VTEC isolates were characterized as *E. coli* O157 and O26 serotypes, as highly pathogenic strains. Each of the O157 and O26 VTEC isolates was in a percentage of 0.4% in relation to the total *E. coli* isolates with virulent patterns *stx1, eae A* and *E-hly A*. The rest of the VTEC isolates were non-O157 VTEC. The antibiotic sensitivity test showed that the isolated VTEC was highly sensitive to gentamicin and co-trimoxazole and highly resistant to tetracycline and ampicillin.

Keywords: *Escherichia coli* / O157 / VTEC / PCR

Received: February 15, 2008; accepted: August 08, 2008

DOI 10.1002/jobm.200800060

Introduction

Verotoxigenic (Shigatoxeginic) *Escherichia coli* (VTEC) strains have wide notoriety [1]. More than 200 different O, H serotypes of STEC/VTEC serotypes have been isolated and the number associated with human illness exceeds 100 [2, 3]. A majority of the reported outbreaks and sporadic cases of such illnesses have been associated with serotype O157:H7 [4]. In a survey of cattle and sheep presented for slaughter at abattoirs in Great Britain, 4.7% of cattle and 1.7% of sheep contained *E. coli* O157 in the faeces [5].

A few studies have demonstrated that animals including cows, sheep, goats and pigs are reservoirs for different VTEC strains [6–8]. Evidence has suggested that VTEC is better adapted to persist in the alimentary tract of sheep than other pathotypes of *E. coli* [9]. Most VTEC infections in humans are connected to the consumption of contaminated food and water, but transmission by contact with infected farm animals and person-to-person contact has also been documented [10]. VTEC is capable of producing toxins active in Vero-cells *in vitro* named verotoxins (also called shiga-like

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toxins "*stx*") [11]. Two kinds of *stx* can be produced by VTEC: *stx*1 and *stx*2 [12]. The biological effects of *stx* take place generally once it is bound to a target eukaryotic cell membrane. Toxin molecules are thought to be internalized by a process of receptor-mediated endocytosis [13, 14].

The production of *stx* alone may not be sufficient for VTEC to cause disease [15]. Other virulence factors may play a role in VETC pathogenicity, including 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 [16]. The formation of A/E lesions is mediated by multiple genes called the Locus of Enterocyte Effacement (LEE) [17]. Another virulence factor that contributes to VTEC pathogenicity is the 60-MDa plasmid-borne enterohaemolysin *A gene* (encoded by the E-*hly* A gene).

The VETC of serotype O157 or those that have specific combinations of virulence factors appear to be more virulent in mankind [12]. VTEC can be associated with different diseases in humans, including haemorrhagic colitis (HC), which is characterized by bloody diarrhoea, haemolytic-uraemic syndrome (HUS), which may result in life-threatening sequelae (renal failure) and thrombotic thrombocytopenic purpora (TTP), through which the central nervous system is mainly affected [18].

Ruminants are an important reservoir of pathogenic VTEC and human infections are frequently associated with direct or indirect contact with ruminant faeces. Strategies to reduce the prevalence of VTEC in ruminants should lower the incidence of human infection [19]. The need for hygienic meat production has gained importance, due to awareness among consumers about health risks associated with contaminated meat [20].

The main objective of this study was to characterize VTEC strains isolated from the faeces of small ruminants and environmental samples from the southern part of Jordan.

Materials and methods

Study design

During a period between June and October 2006, a single faecal sample was obtained from 233 slaughtered sheep and 169 goats respectively (Fig. 1). Samples were collected randomly from six slaughterhouses and 16 butchers' shops, located in the southern part of Jordan. Faecal samples were collected by obtaining colon contents from each slaughtered animal; by ligation of the colon in two sites 5–10 cm long, then transections from these ends. Thus, we avoided the contamination of faecal samples from slaughterhouse or butcher shop sites. The intestinal sample (in between the two ends) was transferred immediately, on ice, within two to four hours to the laboratory. Samples were collected over a period of five months, during which two collection sites per week were selected randomly within one governorate. Information on the sources of slaughtered animals was recorded.

In addition to the faecal contents, 57 drag swabs (Solar Biologicals Inc., New York, USA) from six different slaughterhouses and 16 butchers' shops were collected and tested for VTEC presence. Drag swabs were obtained from the walls and floors of the premises. The visits to the premises were sudden, and no cleaning was intentionally practised before sampling. Drag swabs were carefully opened and floors and walls were swabbed in a circular motion. The swabs were placed directly in bags that contained special transfer media. After incubation for 24 hours, swabs were sub-cultured in the enrichment media.

Sample processing

In the case of the intestinal samples, the colon was opened aseptically and the faecal matter was harvested. Approximately 1 g from each sample was inoculated into 9 ml MacConkey broth (DIFCO, MD, USA) supplemented with cefixime (0.05 mg/l). After incubation at 37 °C for 24 h, a loopful was streaked on sorbitol MacConkey (DIFCO, MD, USA) agar medium supplemented with cefexime (0.05 mg/l) (CSMAC) and SMAC agar [21]. The plates were incubated at 37 °C for 24 h. Both sorbitol-fermenting colonies (small red/pink) and non-fermenting colonies (colourless) were selected for conformation. The number of colonies selected varied for each sample, but as a general rule one to three colonies were taken. *E. coli* suspected colonies were inoculated in tryptic soy slants and incubated for 24 h at 37 °C and then stored at 4 °C. *E. coli* isolates were identified by phenotypic characterization and conventional biochemical activities using an IMViC test (a positive identification of *E. coli* required results to be positive for the indole and methyl red test, negative for the Voges-Proskauer and citrate utilization tests). All materials for the IMViC tests were obtained from DIFCO, MD, USA.

Drag swabs were processed similarly. The morphological characteristics of bacterial isolates were studied and their biochemical identity was determined using the REMEL kit (RapID^{TM} ONE and RapID^{TM} NF plus systems) procedure. All the VTEC isolates were screened for lack of β-glucuronidase production, which is con-

sidered one of the major biochemical markers of the serotype O157.

DNA isolation and purification

DNA was isolated and purified from all the obtained *E. coli* isolates (234 isolates) using a commercially available kit (Bio-Basic LtD, Ontario, Canada). Primarily, bacterial isolates and reference strains were cultivated in 10 ml MacConkey broth and incubated at 37 °C for 12 h. A suspension of 8×10^8 cells/ml was used in the DNA isolation processes. The quantity of the isolated DNA was measured by DNA spectrophotometry (Biochrom, London, U. K.) at a 260 nm wavelength.

Multiplex PCR for detection of *stx*1, *stx*2, *eae A* and *E-hly A*

Polymerase chain reaction (using MyCycler, Bio-Rad, USA) was carried out for each sample in a total volume of 25 μl, containing 1 μl of extracted nucleic acid templates (either from the original DNA samples (70–100 ng/μl) or diluted samples 1:10, 1:20, 1:30 and 1:40) from both cultures and reference strains. In addition to DNA, the PCR mixture was composed of 2.5 μl of Taq reaction buffer (final concentration 1 mM), 4 μl of $MgSO_4$ (Boehringer Mannheim, Germany) (final concentration 3.2 mM), 0.5 μl of deoxynucleoside triphosphates (dNTPs) mixture (Bio-basic Ltd, Ontario, Canada) (final concentration 50 μM) and 0.2 μl of Taq DNA polymerase (Bio-basic Ltd, Ontario, Canada) (1U). The primers used in this study and PCR conditions were as reported by Fagan et al. 1999 [22] (see Table 1). Ten pmol from each of the primer templates were added to the PCR mixture mentioned above.

The PCR conditions consisted of an initial 95 °C denaturation step for 3 min followed by 35 cycles of 95 °C for 20 sec (denaturation step), 58 °C for 40 sec (annealing step), and 72 °C for 90 sec (elongation step). The final extension cycle was followed by 72 °C incubation for 5 min. Amplified DNA fragments were resolved by gel electrophoresis (Labenet, Taiwan) using 2% agarose gels in Trisborate EDTA (TBE) buffer. Gels were stained with 0.5 μg of ethidium bromide per ml. The running time was 90–120 min at voltage 120 mV. Finally, gels were visualized and photographed by UV illuminator system (Alphainnotech, USA) [22]. For each gel, the first lane was specified for a 100 bp DNA ladder (Bio-Basic, Canada), the second lane and the third lane were specified for positive and negative control. All other PCR reagents were purchased from Bio-Basic, Canada.

Table 1. Primers used for RT-PCR.

Primer	Direction	Primer sequence (5′ → 3′)	Fragment size (bp)
E-*hly A*	Forward	ACGATGTGGTTTATTCTGGA	165
	Reverse	CTTCACGTGACCATACATAT	
*stx*1	Forward	ACACTGGATGATCTCAGTGG	614
	Reverse	CTGAATCCCCCTCCATTATG	
*stx*2	Forward	CCATGACAACGGACAGCAGTT	779
	Reverse	CCTGTCAACTGAGCAGCACTTTG	
eae A	Forward	GTGGCGAATACTGGCGAGACT	890
	Reverse	CCCCATTCTTTTTCACCGTCG	

Serotyping of the isolated VTEC

Serotyping for the O157, O26, and O111 serotypes of VTEC that were characterized by mPCR *E. coli* was performed using antibody-based rapid slide agglutination assays (Denka-Seiken, Tokyo, Japan).

Antimicrobial susceptibility testing

The isolated bacteria were tested for their susceptibility to eight antimicrobials, as set by the National Committee for Clinical Laboratory standards, using a disc diffusion method [23]. The susceptibility of characterized isolates to the most commonly used antibiotics in the treatment of VTEC/EHEC infection in Jordan was tested. A total of eight antimicrobial agents were included in this study. The concentration (μg/disc) of the used antibiotics are: Ampicillin (AMP, 10), co-trimoxazole (trimethoprim/sulfamethoxazole) (COT, 1.25/23.75), ciprofloxacin (CIP, 5), gentamicin (GEN, 10), nalidixic acid (NAL, 30), nitrofurantoin (NIT, 30), tetracycline (TET, 30) and chloromphenicol (CHL, 30). All antibiotic discs were purchased from SPAN Diagnostic Ltd, Udhna, India.

Results and discussion

A total of 464 bacterial isolates were recovered from the sheep and goat faecal samples. From the 464 recovered bacteria, 287 (62%) were identified as *E. coli* isolates (Fig. 1). By the conventional method, 88 (30%) of *E. coli* isolates were suspected as O157 through their phenotypical characteristics on CSMAC agar (non-sorbitol fermenters forming; colourless colonies on CSMAC agar). A total of 79 bacterial isolates were recovered from slaughterhouses and butchers' shops. Fifty (64%) of the 79 isolates were identified conventionally as *E. coli*.

In a total of 201 *E. coli* that were isolated from slaughtered sheep (150) and goats (51), 18 (9%) were characterized by mPCR as having one or more of the virulence genes: *stx*1, *stx*2, E-*hly* A and *eae A* (Figs. 2 and 3). VTEC comprised 13 (72%) of the total number of the charac-

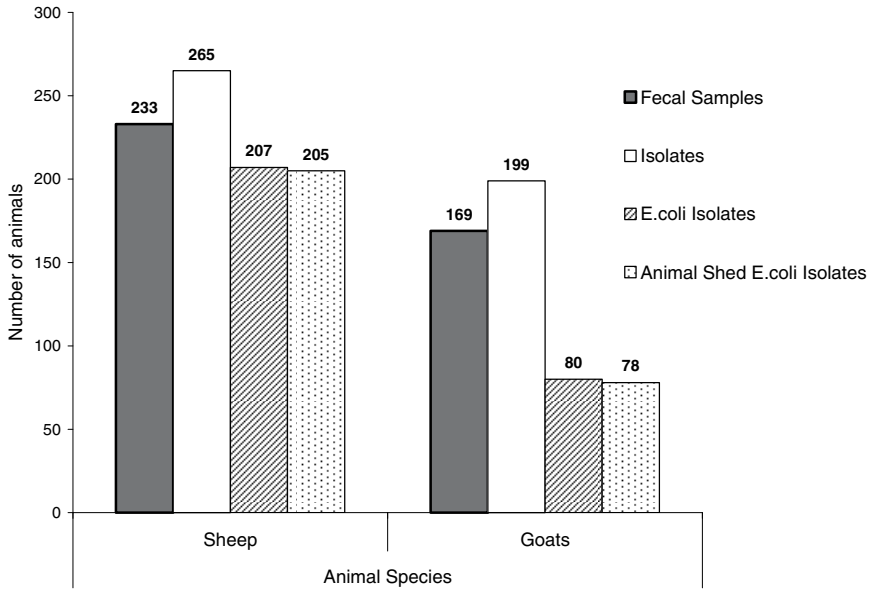

Figure 1. Relative numbers of the collected slaughtered sheep and goats fecal samples, suspected isolates, identified *E. coli* isolates and the number of animals that shed *E. coli* from the southern part of Jordan.

terized *E. coli* isolates (Table 2). VTEC was isolated from slaughtered sheep and goats with the same percentage (6%). VTEC was also isolated from slaughtered animals regardless of the sources of those animals, that is, either local or imported (Table 3). Other pathotypes of *E. coli* were characterized by mPCR-like, EPEC (+*eae A*) and EHEC (+*hly* A) in a total of two (11%) and three (16%), respectively, from the total number of virulent isolated *E. coli* (Table 2).

According to the slide agglutination serotyping, of the 13 suspected VTEC that were isolated, one belonged to the O157 serotype and another belonged to the serotype O26. Both O157 and O26 have the genotype *stx*1, E-*hly* A and *eae*. The rest of the isolates were serotypes other than O157 or O26. All the VTEC isolates were screened for β-glucuronidase production. Only the O157 isolate lacked the β-glucuronidase production.

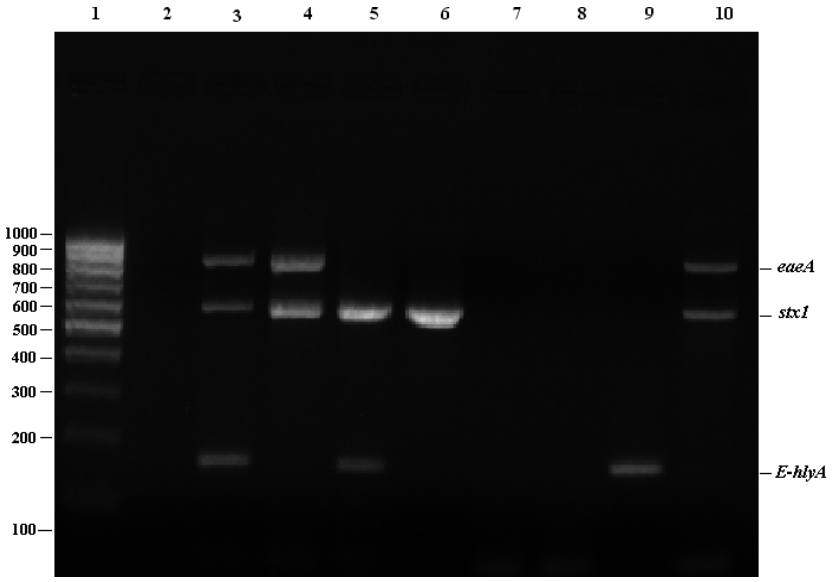

Figure 2. Amplification products of *stx1*, *eae A* and *E-hly A*. lane 1; 100 bp ladder, lane 2: negative control (ATCC 25922), lane 3; positive control (EHEC C4193-1), lane 4; +*stx1* and +*eae A*, lane 5; +*stx1* and +*E-hly A*, lane 6; +*stx*1, lane 7 and 8; negative, lane 9; +*E-hly A* and lane 10; +*eae A* and *stx1*.

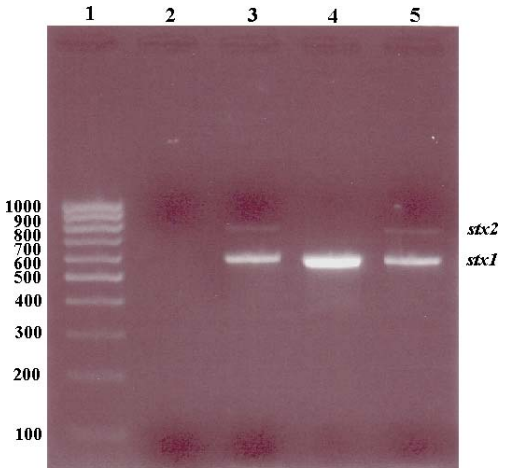

Figure 3. Amplification products of *stx*1 and *stx*2 genes. Lane 1; 100bp ladder, lane 2; negative control (ATCC 25922), lane 3; positive control (EHEC C 4193–1), lane 4; *stx1*, lane 5; *stx1* & *stx2*.

Table 2. Virulence genes profile(s), detection rates and sources of VTEC isolates.

Virulence genes	sheep	goat	(slaughterhouses/ butcher shops)	total
*stx*1	3	0	3	6
*stx*1+*stx*2	1	0	0	1
*stx*1+*eae A*	3	1	2	6
*stx*1+*E-hly A*	1	2	2	5
*stx*1+E-*hly* A+*eae A*	2	0	0	2
E-hly A	2	1	0	3
eae A	0	2	1	3
TOTAL	**12**	**6**	**8**	**26**

In this study, VTEC was found in 13 (6.5%) of the investigated *E. coli* isolates from slaughtered sheep and goats (Tables 2 and 3). This supports the hypothesis that sheep and goats are important reservoirs of VTEC. A number of studies have demonstrated that animals, including cows, sheep and goats, are reservoirs for different VTEC strains that have been associated with human illness [24, 25]. In fact, the prevalence rates of VTEC in sheep and goats varied among different studies – 32.3% [26], 16% [27] and 6.1% [28] – which is in accordance with our result. In the case of slaughtered animals, VTEC was isolated from sheep and goats with the same percentage (about 6%) (Table 2). This result demonstrates that both sheep and goats are important reservoirs of VTEC, as has been suggested [1]. Furthermore, VTEC isolates comprised 21% (7/33) of the total *E. coli* isolates from slaughterhouses and butchers' shops (Table 2). None of the seven VTEC isolates from slaughterhouses and butchers' shops were stereotyped as O157, O26 or O111. VTEC contamination in slaughter sites is related to poor hygiene and non-activated sanitary measurements.

VTEC isolated either from slaughtered animals, slaughterhouses or butchers' shops possessed genes for *E-hlyA* and/or *eaeA* in 65% of the total VTEC isolates (Table 2). This type of VTEC is called complex VTEC. Complex VTEC is more commonly recovered from the faeces of humans with HUS and HC than VTEC strains that do not possess these accessory virulence genes [29]. Complex VTEC possessing the *E-hly A* gene has been described as EHEC; the role of *stx* in EHEC pathogenesis in ruminants is unclear [30]. Contamination of slaughterhouses with complex VTEC, regardless of the source of VTEC, and the high prevalence rate of complex VTEC in slaughtered sheep and goats will increase the rate of meat contamination. Consequently, the probability of human infection with complex VTEC will increase. Epidemiological and comprehensive studies have suggested that the level of contamination with complex VTEC is linked primarily to the level of intestinal infection, the faecal excretion of these pathogens and the general hygiene of slaughterhouses and butchers' shops [31]. Thus, routine microbiologic testing performed in slaughterhouses and butchers' shops can be beneficial in terms of diminishing food contamination before it reaches consumers.

Among the isolated VTEC, only one isolate had the *stx2* gene, in a percentage of 5%, while the other isolates genetically had the *stx1* gene in a percentage of

Table 3. Characterization of suspected VTEC isolates on CSMAC agar, clinical findings and sources of isolates.

VTEC/tested *E. coli* isolates	VTEC source	Serotyping			Clin. finding		Animal source	
		O157	O26	ND*	Nor.	Inf.	Loc.	Imp.
13/201	S:10	1	1	8	9	1	4	6
	G:3	0		3	3	0	3	0
7/33	SL:7	0		7	–	–	–	–
	BO:0	–	–	–	–	–	–	–

* Not determined; S: sheep; G: goat; SL: slaughterhouse; BO: butcher shop.

Table 4. Antibiotic susceptibility profile (s) of seven different isolated virulent VTEC.

Virulent patterns	Antibiotic agents							
	AMP	CIP	COT	GEN	NAL	NIF	TET	CHL
Stx1	+	+	–	–	+/–	+/–	+	–/+
Stx1+stx2	+/–	–	–	–	–	+/–	+	–
stx1+eae A	+	+/–	+/–	–	+/–	–	+/–	+/–
stx1+E-hly A	+	–	–	–	+	+/–	+	+
stx1+E-hly A+eae A	+	–	–	+/–	+	–	+	+/–
E-hly A	–	+/–	–	–	+/–	+/–	–	–
eae A	+/–	+	+/–	–	–	+	+	+/–

AMP, ampicillin; CIP, ciprofloxacin; COT, co–trimoxazole; GEN, gentamicin; NAL, nalidixic acid; NIF, nitrofurantoin; TET, tetracycline; CHL, chloromphencol; +, resistant; +/–, partially sensitive; –, sensitive.

95%. In contrast, a study that was performed in Australia suggested that 41 of 45 VTEC isolates produced *stx1* and 23 produced *stx2* [32]. In fact, a study was conducted with PCR to characterize VTEC containing *stx1* and/or *stx2*. The *stx1* family is very homogenous, but *stx2* is a more heterogeneous group than *stx1* and the *stx2* variants described so far are *stx2, stx2c, stx2d, stx2e* and *stx2f* [33]. The sources of the slaughtered animals revealed that VTEC had been shedding from slaughtered sheep and goats, regardless of their source, that is, either local or imported (Table 3). Thus, future investigation into VTEC epidemiology must be undertaken in small ruminants, regardless of the animals' sources. We found that 90% (9/10) of VTEC isolated from the sheep was shed from the young (6–10 months) rather than from old sheep. This result could be explained by a longitudinal study of VTEC in sheep which suggested that the prevalence rate of VTEC declines with an increase in age [29].

Table 4 shows the results of antibiotic sensitivity patterns for seven virulent patterns of *E. coli* isolates. The multiple antibiotic susceptibility (MAS) of different virulent patterns, *stx1*, *stx1+stx2*, *stx1+eae A*, *stx1+E-hly A*, *stx1+ E-hly A+eae A*, E-*hly A* and *eae A*, were 25%, 62.5%, 25%, 37.5%, 37.5%, 62.5% and 25%, respectively.

Resistance to ampicillin and tetracycline was present in 85% of the VTEC isolated patterns, while the resistance to chloromphenicol, nitrofurantoin and nalidixic acid occurred in 71%, to ciprofloxacin in 57%, to co-trimoxazole in 28% and to gentamicin in 14%. It is worth mentioning that ampicillin and tetracycline are used without control in livestock, which could in part explain the high resistance to these antibiotics.

We studied the effect of some antibiotics on the different virulence patterns of *E. coli* isolates, using the antibiotics commonly used in the Jordanian market for treatment of EHEC/VTEC infection (Table 4). Obviously, the controlled use of antimicrobial agents is a prerequisite to limiting the emergence of drug-resistant bacteria, as recommended [23]. Previous studies have shown a high prevalence of antibiotic resistance among some antibiotics, ampicillin and tetracycline [34, 35]. A study conducted in the USA about antibiotic resistance showed that most *E. coli* isolates were susceptible to (co-trimoxazole) trimethoprim/sulfamethoxazole, gentamicin and ciprofoloxacin [36]. This study also revealed that VTEC isolates were highly susceptible to gentamicin and co-trimoxazole, with moderate susceptibility to ciprofloxacin. However, VTEC patterns showed resistance among some antibiotics tested, such as tetracycline and ampicillin, which are commonly used in Jordan (Table 4). In fact, the resistance ability of many VTEC patterns is related to the transfer of plasmid-encoded resistance to anti-microbial agents. The possibility of transfer of resistance genes between bacteria in natural habitats has recently attracted a lot of attention [37].

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