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Distribution of antimicrobial resistance and some widespread extended-spectrum beta-lactamase genes in different phylogroups of Shiga toxin-producing *Escherichia coli* (STEC) isolates of ruminant origin

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ABSTRACT

Limited data is available on the prevalence of ESBL genes in the STEC isolates of ruminant origin. This study investigated the molecular prevalence of ESBL-encoding genes (bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA}) and AMR of 58 STEC isolates recovered from cattle (n = 32), sheep and goats (n = 26). In the current study, ESBL genes were identified by the molecular technique. Moreover phenotypic AMR was tested by disc diffusion method against six antibiotics , namely amoxicillin-clavulanic acid, tetracycline, neomycin, florfenicol, enrofloxacin, and sulfamethoxazole-trimethoprim. Phylogenetic groups were also determined by a PCR scheme. Isolates were categorized into five phylogroups of (A, B1, C, D, and E), with B1 being the most prevalent phylogenetic group (43; 74.1%). Statistical analysis revealed a significant association between phylogroup D and small ruminants (sheep and goats, $p = 0.014$). Moreover, the highest rates of antimicrobial resistance were related to tetracycline (25.9%) and neomycin (22.4%). Isolates resistant to tetracycline ($p = 0.001$), trimethoprim-sulfamethoxazole ($p = 0.013$) and neomycin ($p = 0.00$) were significantly prevalent among strains recovered from cattle. In addition, the majority of multidrug-resistant strains also had a significant distribution among cattle isolates ($p = 0.001$). In the current study, the prevalence of ESBL positive STEC was 12.06% (7/58). Genes bla_{CTX-M} and bla_{TEM} were detected separately and in combination in bovine isolates. However, only one STEC strain of small ruminants harbored bla_{TEM}. In conclusion, it seems that cattle isolates are notable sources of different AMR traits which could be a threat to veterinary sections, public health and food hygiene, in particular.

Keywords

E. coli, STEC, antimicrobial resistance (AMR), ESBL, bla_{CTX-M}, bla_{TEM}, Phylogroup.

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Abbreviations

ESBL: extended-spectrum beta-lactamase
STEC: Shiga toxin-producing Escherichia coli
AMR: antimicrobial-resistance
HC: hemorrhagic colitis
HUS: hemolytic uremic syndrome

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Introduction

Human disease caused by STEC range from mild diarrhea to HC and potentially life-threatening HUS [1]. The STEC was the third most prevalent foodborne pathogens in the European Union which increased during the last decade [2]. Ruminants, particularly cattle, have been identified as the most major STEC reservoir. Furthermore, sheep and goats also play a key role in the spread of STECs into the food chain [3]. The STEC has also been isolated from wild animals, and has been reported as a safety risk in the production of fresh fruits and vegetables [4].

Antimicrobial misuse in animal production systems has sent a warning signal to the world's public health. This event resulted in the evolution of antibiotic-resistant strains, and it was with estimated that AMR caused disease mortality to rise from 700,000 in 2014 to 10 million by 2050 [5]. The AMR is regarded as a severe problem in healthcare settings because its mobile genetic elements can alter antibiotic resistance patterns in pathogenic and commensal *E. coli*, as well as the intestinal microbiota of animals and humans [6]. In contrast, little is known about AMR in STEC, particularly when it comes to broad-spectrum beta-lactamases, such as ESBLs from the bla_{CTX-M}, bla_{TEM}, bla_{OXA}, and bla_{SHV} families of ESBL variations [7]. Another issue with resistant-STEC is the spread of these ESBL-coding genes across other *Enterobacterales,* endowing them with antibiotic resistance [8]. Although the use of antibiotics to treat STEC infections is still controversial, antibiotics given early in the course of the infection may help to avoid HUS according to some studies [9]. In this scenario, the frequency of resistant-STEC strains is concern since disease progression continues unabated. The STEC has a high level of genomic plasticity, with mobile genetic components including plasmids, bacteriophages, and genomic islands playing a key role in the transmission of genes, particularly those involved in virulence [10].

The role of genetic background in antibiotic resistance has also been widely investigated in *E. coli*, but to the best of our knowledge has not been studied in ESBL genes among STEC. Based on some previous research, certain members of phylogenetic groups A and D are prone to acquire resistance against third-generation cephalosporins, while B2 strains are more vulnerable [11]. A multiplex PCR, which can classify *E. coli* isolates into eight phylogenetic groups, is the most practical approach for identifying phylogroups A, B1, C, E, D, F, B2, and E. Clades. *E. coli* strains are not randomly dispersed among bacterial populations. Therefore, phylotyping is a useful tool in different genotyping studies. Pathogenicity, niche, and resistance features of the members of the same group tend to be similar [12]. In the present study, we evaluated the STEC isolates of ruminant origin (sheep, goats, and cattle) to build a clear picture of status of AMR, and some important resistance genes in 58 STEC isolates recovered in recent years. Results would help combat AMR in both veterinary and public health sections.

Results

Phylogenetic groups

We classified 58 STEC isolates into five phylogroups (A, B1, C, D, and E) according to Clermont's phylogrouping method. Members of groups A, B1, and C were identified among all the sources, while group D was only related to sheep and goats (5/26) and E was only detected in cattle (1/32). Moreover, group B1 was the most prevalent phylogenetic group in all sources (sheep and goats: 17/27, 29.3%; cattle: 26/32, 44.8%) and overall (43/58; 74.1%). Statistical analysis revealed a significant association between phylogroup D and small ruminants ($p = 0.014$). No other notable relations were observed. The results are represented in details in table 1.

Antibiotic resistance

Phenotypic resistance:

A total of 58 isolates were investigated for phenotypic resistance to six different antibiotics, including: tetracycline (TET), trimethoprim-sulfamethoxazole (SXT), amoxicillin-clavulanic acid (AMC), neomycin (NEO), florfenicol (FLO) and enrofloxacin (ENFX), by disk diffusion method. The highest rates of antimi-

Table 1.
Distribution of STEC isolates in five phylogenetic groups.

Source (n)	Phylogenetic groups				
	A	B1	C	D	E
Cattle (32)	4 (6.9%)	26 (44.8%)	1 (1.7%)	0	1 (1.7%)
Sheep / Goats (26)	2 (3.4%)	17 (29.3%)	2 (3.4%)	5 (8.6%)	0
Total (58)	6 (10.3%)	43 (74.1%)	3 (5.2%)	5 (8.6%)	1 (1.7%)
***p*-value**	0.681	0.231	0.582	**0.014***	1.000

a. *significant difference ($p < 0.05$).

Table 2.
Frequency of phenotypic antimicrobial resistance and ESBL genes of STEC isolates (n, %).

Source (n)	Antibiotics						MDR	ESBL genes	
	TET	SXT	AMC	NEO	FLO	ENFX		bla_{CTX-M}	bla_{TEM}
Cattle (32)	14 (24.1%)	7 (12.1%)	3 (5.2%)	13 (22.4%)	3 (5.2%)	0	13 (22.4%)	5 (8.6%)	2 (3.4%)
Sheep/Goats (26)	1 (1.7%)	0	3 (5.2%)	0	0	1 (1.7%)	1 (1.7%)	0	1 (1.7%)
Total (58)	15 (25.9%)	7 (12.1%)	6 (10.3%)	13 (22.4%)	3 (5.2%)	1 (1.7%)	14 (24.1%)	5 (8.6%)	3 (5.2%)
***p*-value**	**0.001***	**0.013***	1.000	**0.000***	0.245	0.448	**0.001***	0.058	1.000

a.*significant difference ($p < 0.05$)

crobial resistance were related to tetracycline (25.9%) and neomycin (22.4%). Moreover, resistant isolates to tetracycline ($p = 0.001$), trimethoprim-sulfamethoxazole ($p = 0.013$), and neomycin ($p = 0$) were significantly prevalent among strains recovered from cattle. In addition, the majority of MDR strains also had a significant distribution among cattle isolates ($p = 0.001$). Table 2, represents the results in details.

ESBL/β-Lactamase genes:

Isolates were screened for four widespread ESBL/β-Lactamase genes, namely bla_{CTX-M}, bla_{TEM}, bla_{SHV}, and bla_{OXA} gene families. The genes bla_{SHV} and bla_{OXA} were absent among the STEC isolates. Moreover, only seven isolates (12.06%) possessed ESBL genes. Among them, one isolate harbored bla_{CTX-M} and bla_{TEM} simultaneously, while the remaining six strains carried only one gene. Furthermore, the gene bla_{CTX-M} was only present in cattle isolates. Figure 1 and table 2, represent the results in details.

Correlations:

Correlations between AMR, ESBL genes, and MDR were measured and presented in details in table 3. Notable strong correlations were observed between tetracycline and neomycin with each other, and MDR.

Distribution of antibiotic- resistant isolates among phylogenetic groups:

The majority of the isolates resistant to five antibiotics (all the antibiotics except enrofloxacin) and MDR were observed in group B1 as it was the most frequent phylogroup. Interestingly, all the bla_{CTX-M} + and 66.6% of bla_{TEM} + (2/3) strains also belonged to the isolates in phylogroup B1. The rest of the resistant isolates were scattered among groups A, D and E. Group C did not show any phenotypic resistance, while one of the bla_{TEM} + strains was a member of group C. Statistical analysis revealed no significant difference in the distribution of antibiotic resistant isolates between phylogenetic groups; except for enrofloxacin, an important quinolone, which was significantly related to phylogenetic group D. However, based on the scarcity of the group D in our study such a difference cannot be conclusive. The results are represented in details in table 4.

Table 3.
Correlations between AMR, ESBL genes, and MDR

rho / p-value	TET	SXT	AMC	NEO	FLO	ENFX	bla_{CTX-M}	bla_{TEM}	MDR
TET	-	0.627*	0.187	0.910*	0.395*	-0.078	0.239	0.218	0.955*
SXT	0.000	-	-0.126	0.689*	0.630*	-0.049	0.075	-0.087	0.657*
AMC	0.159	0.347	-	0.089	-0.079	-0.045	0.097	0.432*	0.205
NEO	0.000	0.000	0.507	-	0.435*	-0.071	0.277*	0.248	0.953*
FLO	0.002	0.000	0.554	0.001	-	-0.031	0.206	-0.055	0.414*
ENFX	0.559	0.715	0.737	0.595	0.818	-	-0.041	-0.031	-0.075
bla_{CTX-M}	0.070	0.577	0.467	0.035	0.121	0.762	-	0.206	0.257
bla_{TEM}	0.101	0.518	0.001	0.061	0.684	0.818	0.121	-	0.232
MDR	0.000	0.000	0.122	0.000	0.001	0.577	0.051	0.080	-

a.The table simultaneously represents *p*-values (numbers on the left side of the table diameter) and Spearman's correlation coefficients (numbers on the right side of the table diameter); Colored cells: strong correlations ($rho > 0.8$); *: Correlation is significant ($p < 0.05$)

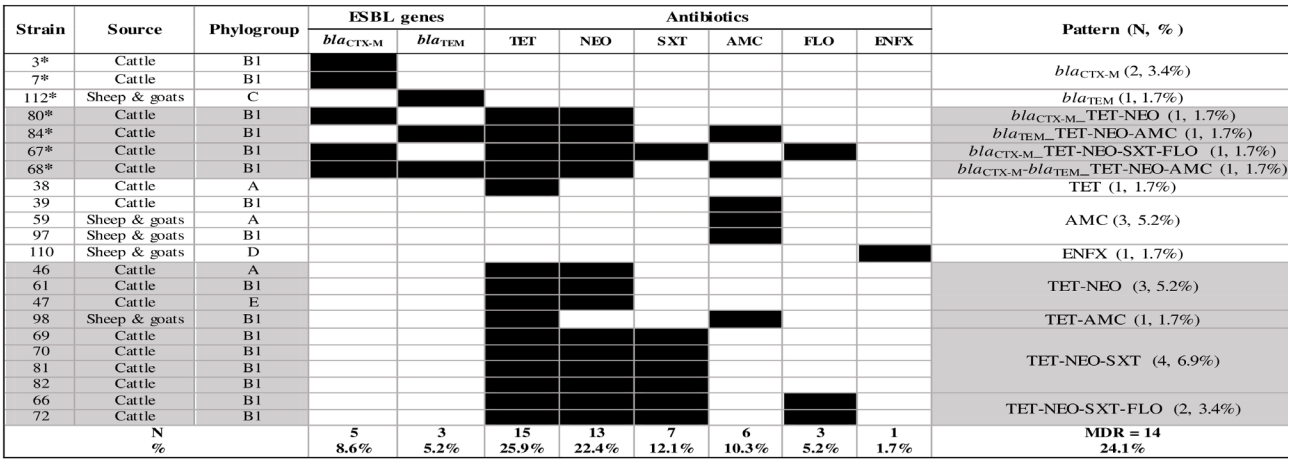

Figure 1.
AMR patterns for 22 resistant STEC isolates. Black = having resistance genes/not susceptible; White = no gene/susceptible; Gray = MDR; * = ESBL +

Table 4.
Distribution of phenotypic antimicrobial resistance and ESBL genes in STEC belonging to different phylogroups (n, %)

Phylogroups (n)	Antibiotics						MDR	ESBL genes	
	TET	SXT	AMC	NEO	FLO	ENFX		bla_{CTX-M}	bla_{TEM}
A (6)	2 (33.3%)	0	1 (16.7%)	1 (16.7%)	0	0	1 (16.7%)	0	0
B1 (43)	12 (27.9%)	7 (16.3%)	5 (11.6%)	11 (25.6%)	3 (7%)	0	12 (27.9%)	5 (11.6%)	2 (4.7%)
C (3)	0	0	0	0	0	0	0	0	1 (33.3%)
D (5)	0	0	0	0	0	1 (20%)	0	0	0
E (1)	1 (100%)	0	0	1 (100%)	0	0	1 (100%)	0	0
Total	15	7	6	13	3	1	14	5	3
***p*-value**	0.205	0.596	0.849	0.189	0.894	0.029*	0.184	0.753	0.237

a.*significant difference ($p < 0.05$)

Discussion

Today, it is well established that livestock is an important reservoir of pathogenic *E. coli* with public health significance [13]. The presence of STEC strains which are responsible for a wide range of clinical manifestations from mild diarrhea to HC and HUS in humans, has been shown in food-producing animals, especially cattle [14,15]. Emerging AMR in the STEC strains of animal and food sources is a public health threat, as the possibility of resistant genes acquisition by other bacteria is increased [16]. Among different AMR, ESBL has gained a lot of attention during the last decade and ESBL-producing *E. coli* strains have been isolated from livestock as well [17,18]. However, there is a lack of knowledge on the occurrence of ESBL among STEC strains in cattle, sheep and goats as they are one of the main suppliers of milk and meat in most parts of the world. From this perspective, the current study has been conducted to evaluate the prevalence of ESBL-encoding genes among the STEC isolates of ruminant origin.

In the present study, the prevalence of ESBL positive STEC was 12.06% (7/58) which is higher than the reports of Ewers et al., (2/149; 1.34%) and Elmonir *et al.*, (7/100; 7%) [19, 20]. In fact, there is a lack of literature relevant to ESBL in STEC for comparison, as most studies on ESBL-producing E. coli have been performed in the non-STEC isolates of ruminants. Furthermore, bovine is the main subject in such studies, whereas ovine and caprine are mostly neglected. In other words, although livestock is known as STEC reservoirs, only a few studies have addressed the ESBL-producing STEC in cattle, sheep, and goats [21–25]. Moreover, some of the mentioned studies have focused on food hygiene aspects [21,24,26], while attention to gut-isolated pathogens is also valuable, because the intestinal tract is a 'melting pot' and one of the suitable milieu for gene exchange among bacterial

species [27].

To date, several types and subtypes of ESBL-encoding genes have been detected in meat, milk and stool samples of ruminants. For example, ESBL genes such as bla_{CMY} [24,28], bla_{TEM} [18,23,24,26,29], bla_{SHV} [18,23,24,26], and ampC [22,24] have been reported in cattle as well as sheep and goats samples, while bla_{OXA} has been only reported from bovine *E. coli* strains [18]. Moreover, combination of bla_{CTX-M} + bla_{TEM} seems to be more common in *E. coli* with animal origin [24,30]. It seems that bla_{CTX-M} is the most prevalent ESBL-encoding gene in both bovine and small ruminant *E. coli* strains [18,21–24,26,28,29]. in the present study, bla_{CTX-M} and bla_{TEM} were detected separately and in combination in bovine isolates, whereas only bla_{TEM} was identified in one strain of STEC of small ruminants. Our finding are in line with the mentioned reports.

In the present study, most of the ESBL-producing strains which were recovered from cattle belonged to group B1 (6/7; 85.71%). It has been shown that the ESBL positive *E. coli* strains of livestock are mostly related to phylogenetic groups A and B1 and a lesser extent are related to B2 and D which is similar to our results [23, 26, 28, 30, 31]. However, one of our isolates (1/7; 14.28%) which was recovered from small ruminants belonged to phylogenetic group C. As shown by Atlaw *et al*., (2021), the ESBL positive strains of sheep and goats could be rarely scattered among non-commensal groups such as C and E [31].

Although antibiotic therapy in infections caused by STEC is now contraindicated due to the elevated risk of HUS in some cases, research on using antibiotics that inhibits transcription or translation, such as rifamycins (alone or in combination with fluoroquinolones), showed promising results. This, may lead to changes in the treatment regimen using antibiotics in future [32,33]. Currently, the importance of the emergence and spread of AMR in STEC is getting clear. The more resistant traits STEC has, the poorer the response to therapeutic strategies will be. One of the well-known factors in the emergence of AMR in STEC is the extensive use of antibiotics in clinical and agricultural environments. Today, the occurrence and increase of AMR in the STEC of various populations (human, livestock, companion animals,and the environment) have been documented [34,35]. In our research, resistance to tetracycline (25.9%), neomycin (22.4%), trimethoprim-sulfamethoxazole (12.1%) and amoxicillin-clavulanic acid (10.3%) was observed which is in line with other studies that have noted resistance to tetracycline, aminoglycosides, sulfonamides and β-lactams as the most horizontally acquired AMR in STEC [34,35].

Occurrence a positive strong correlation of resistance to tetracycline with MDR is one of the notable observation in the current study. This, can be partially confirmed by the results recorded by Bourely *et al.* (2019), which proposed the resistance to tetracycline and amoxicillin as an indicator for MDR in *E. coli* recovered from animals [36]. We indicated a strong correlation between neomycin resistance and MDR as well. However, there is a lack in literature relevant to this finding to be compared with.

In conclusion, ESBL-encoding STEC strains were detected in cattle, sheep, and goats in the present study. Moreover, bovine strains showed higher AMR in both ESBL positive and ESBL negative STEC isolates which could be due to the extensive application of antibiotics in the cattle industry for therapeutic and non-therapeutic purposes, such as growth promotion [36,37]. As antibiotic use can lead to a pressure for the emergence and spread of AMR, it seems that more caution should be taken in the veterinary field for antibiotic application especially in sections related to cattle.

Materials & Methods

E. coli Isolates

A panel of 58 non-duplicate archived STEC *E.coli* isolates was chosen from the bacterial collection (Ferdowsi University of Mashhad, Iran), including 32 isolates from cattle, and 26 isolates from sheep and goats. These bacteria were isolated during 2010 - 2018 in the context of different previous studies and surveyed for the presence of *stx* genes. In brief, the original sampling procedure included collecting fecal samples using sterile cotton swabs from the rectum of animals. In cases with a sample transfer time of more than 24 h, Amies (Oxoid, UK) transfer medium was used. The samples were cultured on MacConkey agar (Merck, Germany) and a pure isolate from each sample was confirmed as *E. coli* using standard biochemical tests [38]. All mentioned isolates were cryopreserved as stocks at -70 ˚C and recovered on Brain Heart Infusion broth (Merck, Germany) with the subsequent additional streak on MacConkey agar to confirm the purity.

To confirm the identity of the STEC isolates, a PCR protocol proposed by Lin *et al*., (1993) was applied based on the recognition of a common sequence of different stx types or subtypes. Each PCR reaction was performed in a volume of 20 µl containing: 10 µl Taq DNA Polymerase Master Mix RED 2x (Amplicon, Denmark) containing 1.5 mM $MgCl_2$, 1 µl of each primers (Macrogen, Seoul, South Korea), ultrapure water and 300 ng of template DNA. Primer characteristics and thermal conditions are shown in Table 5. Finally, PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel and Green Viewer safe stain (0.01 v/v) [39].

DNA Extraction

The crude DNA was extracted using a boiling method as described before [40]. In brief, a suspension of three colonies from an overnight culture (18-20 h) was selected and prepared in sterile tubes containing 300 µl of sterile distilled water. The tubes were boiled in a boiling water bath for 10 min and after cooling on ice buckets centrifuged at 800×g for 5 min. The supernatant was used as a DNA template in the PCR.

Determination of phylogenetic groups

Phylogenetic groups of the isolates were investigated using

the updated method developed by Clermont *et al*., (2013). The method enables an *E. coli* isolate to be assigned to one of the eight phylogroups (A, B1, B2, C, D, E, F, Clade I) and also allows isolates that are the members of other cryptic clades (II - V) of *Escherichia* to be identified. However, some isolates which cannot be categorized as mentioned groups, are known as 'unknown'. The method consists of a primary quadruplex PCR reaction and additional PCR reactions, when necessary [12].

Each PCR reactions was performed in a volume of 20 µl containing: 10 µl Taq DNA Polymerase Master Mix RED 2x (Amplicon, Denmark) containing 1.5 mM $MgCl_2$, various concentrations of each primers (Macrogen, Seoul, South Korea), ultrapure water, and 300 ng of template DNA. Thermal conditions and primer characteristics are shown in Table 5. Finally, PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel and Green Viewer safe stain (0.01 v/v).

Antimicrobial resistance

Phenotypic resistance:

Antimicrobial susceptibility was conducted according to the CLSI recommendations using the disc diffusion method [41]. The antibiotics (Padtan Teb, Tehran, Iran) were chosen from six families of widely used antibiotics in humans and/or animals including: amoxicillin-clavulanic acid (AMC, 20/10 µg), tetracycline (TET, 30 µg), and neomycin (NEO, 30 µg), florfenicol (FLO, 30 µg), enrofloxacin (ENFX, 5 µg), sulfamethoxazole-trimethoprim (SXT, 1.25/23.75 µg). The isolates that showed resistance against three or more families of antimicrobials were designated as MDR.

ESBL genes:

Molecular detection of ESBL-producing *E. coli* was carried out using using a triplex PCR reaction for bla_{TEM}, bla_{SHV},and bla_{OXA} and a uniplex PCR for bla_{CTX-M}. Each PCR reaction was performed in a volume of 20 µl containing: 10 µl Taq DNA Polymerase Master Mix RED 2x (Amplicon, Denmark) containing 1.5 mM $MgCl_2$, various concentrations of each primers (Macrogen, Seoul, South Korea), ultrapure water and 300 ng of template DNA. Primer characteristics and thermal conditions are shown in Table 5. Finally, PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel and Green Viewer safe stain (0.01 v/v).

Statistical analysis

In addition to the descriptive analysis of the results, possible relationships of genetic criteria (phylogenetic groups and ESBL genes) with phenotypic AMR were assessed by the chi-squared test and Fisher's exact test. Correlation among AMR, ESBL genes and MDR were also measured and represented using Spearman rank-order correlation coefficient (*rho*). For all the analysis, $p < 0.05$ was considered significant. Moreover, correlations with *rho* > 0.8 were categorized as "*strong correlation*". In the present study, the data were analyzed by SPSS version 16.0 (SPSS Inc., Chicago, USA).

Table 5.
Primers used in the present study (STEC, Phylogenetic groups and ESBL genes)

Panel	Primer pair	Sequence (5' to 3')	Annealing temp (°C)	Product size (bp)	Ref.
STEC	*stx*	F: GAACGAAATAATTTATATGT	43	900	[39]
		R: TTTGATTGTTACAGTCAT			
Phylogenetic grouping					
Quadruplex	*chuA*	F: ATGGTACCGGACGAACCAAC	59	288	[12]
		R: TGCCGCCAGTACCAAAGACA			
	yjaA	F: CAAACGTGAAGTGTCAGGAG		**211**	
		R: AATGCGTTCCTCAACCTGTG			
	TspE4.C2	F: CACTATTCGTAAGGTCATCC		**152**	
		R: AGTTTATCGCTGCGGGTCGC			
	arpA	F: AACGCTATTCGCCAGCTTGC		**400**	
		R: TCTCCCCATACCGTACGCTA			
Group E	*arpA*	F: GATTCCATCTTGTCAAAATATGCC	57	**219**	
		R:GAAAAGAAAAAGAATTCCCAAGAG			
Group C	*trpA*	F: AGTTTTATGCCCAGTGCGAG	59	**489**	
		R: TCTGCGCCGGTCACGCCC			
ESBL genes					
Triplex	bla_{TEM}	F: CATTTCCGTGTCGCCCTTATTC	57	**800**	[42]
		R: CGTTCATCCATAGTTGCCTGAC			
	bla_{SHV}	F: AGCCGCTTGAGCAAATTAAAC		**713**	
		R: ATCCCGCAGATAAATCACCAC			
	bla_{OXA}	F: GGCACCAGATTCAACTTTCAAG		**564**	
		R: GACCCCAAGTTTCCTGTAAGTG			
Uniplex	bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGTKATGGC	61	**593**	[43]
		R:TGGGTRAARTARGTSACCAGAAYCAGCGG			

Authors' Contributions

Conceptualization, G.H. and M.A.; Methodology, Rw.T. and H.K.R.; Software, H.K.R.; Supervision, G.H. and M.A.; Writing – original draft, R.T. and H.K.R.; Writing – review & editing, Rw.T., H.K.R., G.H., R.T. and M.A. All authors have read and agreed to the published version of the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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