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Virulence Genes, Antibiotic Resistance and Phylotyping of *Escherichia coli* O157 Recovered from Diarrheic Calves

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ABSTRACT

E. coli O157 is one of the main food-borne pathogens that attributed to the attaching and effacing Shigatoxigenic *E. coli* pathotype (AE-STEC). The occurrence of *E. coli* O157 in diarrheic calves investigated through molecular detection of *rfbE*O157 encoding gene was 8.02% within *E. coli* strains. Detection of AE-STEC virulence genes in *E. coli* O157 strains using multiplex PCR showed the presence of *eae*, stx_1, stx_2 and *ehylA* in percentages of 93.3, 73.3, 20 and 13.3%, respectively. The virulence genes profile of *E. coli* O157 revealed the predominance of *eae*+stx_1 combination in 66.7% of these strains. All *E. coli* O157 strains exhibited antibiotic multi-resistances with higher resistance (100%) to amoxicillin/clavulanic acid, cefalexin, cefuroxime and tetracycline, while the lowest resistance was detected for gentamicin (40%). Phenotypic resistance to extended spectrum cephalosporins (ESCs) indicated that 60% of these strains were resistant to ceftriaxone and cefotaxime, while 53.3% were resistant to cefquinome. Molecular detection of extended spectrum β-lactamases (ESBLs) encoding genes recorded the superiority of bla_{TEM} gene (100%), whereas the bla_{SHV} and bla_{CTXM} genes were detected in percentages of 40 and 20%, respectively. The genetic profiling of resistance genes revealed the role of bla_{CTXM} gene in ESCs resistance as bla_{TEM}+bla_{SHV}+bla_{CTXM} combination was detected only in ESC resistant strains. Finally, B2 phylogroup was the most prevalent one (80%) within *E. coli* O157 strains. This implicates diarrheic calves as a source of highly pathogenic multi-resistant *E. coli* O157 strains.

Key words: *E. coli* O157, Virulence & Resistance genes, Diarrheic calves, Antimicrobial resistance, ESBLs.

INTRODUCTION

Escherichia coli O157 related to the attaching and effacing Shigatoxigenic *E. coli* (AE-STEC) pathotype, is considered one of the most significant food-borne pathogens associated with economic losses worldwide (Kamel et al. 2015; Thiry et al. 2017).

Infection of neonatal calves with AE-STEC O157 is characterized by diarrhea and hemorrhagic enteritis, while adult animals infected asymptomatically and act as a reservoir, the consumption of dairy and meat products contaminated with ruminant feces is linked to severe human infection characterized by bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (Mainil and Daube 2005; Stenkamp-Strahm et al. 2017).

AE-STEC virulence factors are attributed to the release of Shiga toxins (Stx1 and Stx2) (encoded by stx_1 and stx_2 genes), Intimin (Adhesin) protein (encoded by *eae* gene) that adheres bacteria to the intestinal villi forming attaching and effacing lesion and Enterohemolysin (encoded by *ehylA* gene) that enhances the effect of Shiga toxins and induces epithelial and endothelial cell damage (Naylor et al. 2005; Bielaszewska et al. 2014).

For antibiotic therapy of neonatal calf diarrhea, β-lactams, aminoglycosides, fluoroquinolones and tetracyclines, are frequently used (Constable 2004). This therapy is not preferred for AE-STEC O157 associated calf diarrhea, as the exposure to certain antibiotics may increase the severity of the disease due to the lysis of bacteria with the release of Shiga toxins (Mohsin et al. 2010; Saeedi et al. 2017).

Antibiotic multiple resistant AE-STEC O157 strains are of major concern, as they harbor several resistance genes that play a role in the spread of antibiotic resistance within various pathogenic and commensal *E. coli* (Srinivasan et al. 2007; Mehmood et al. 2020). Extended spectrum cephalosporin (ESC) resistant *E. coli* is the most important one and is mediated by the production of extended spectrum β- lactamases (ESBLs) enzymes that confer resistance to penicillins, aminopenicillins, 3rd and 4th generation cephalosporins (Poirel et al. 2018; CDC

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2020). The treatment of ESC resistant *E. coli* is limited, as resistance genes encoding for other antibiotic classes, including fluoroquinolones, aminoglycosides, sulfonamides and trimethoprim, are carried on the same plasmid of ESBLs encoding genes (Hawkey and Jones 2009).

Phylotyping using PCR is considered a simple and rapid method for grading *E. coli* strains to one of the main phylogroups (A, B1, B2 and D) based on the presence or absence of three genetic markers (ChuA, YjaA and TspE4.C2) (Clermont et al. 2000).

This study aimed to investigate virulence genes profile, antibiotic resistance with the characterization of ESBLs encoding genes and phylotyping of AE-STEC O157 strains recovered from diarrheic calves in Egypt.

MATERIALS AND METHODS

Animals and samples

A total of 137 rectal swabs were collected from diarrheic calves up to 4 weeks old from different farms during the period from January 2019 to September 2019. Sterile cotton swabs were used for sampling, then inserted into the Amies transport medium (Oxoid, UK) and sent to the laboratory on the ice box.

Bacteriological isolation and biochemical identification

Fecal swabs were cultured on Tryptone Soya Broth (TSB) (Oxoid, UK) and incubated at 37°C for 18 hours. Subcultures were made on Eosin Methylene Blue (EMB) agar medium (Oxoid, UK) and incubated at 37°C for 24-48 hours. Different colonies with a characteristic green metallic sheen were characterized as typical *E. coli* either O157 or non O157 strains and confirmed by biochemical identification according to Quinn et al (2002).

Molecular identification of *E. coli* O157

The genomic DNA of all *E. coli* strains was extracted from overnight inoculated TSB using the boiling method as mentioned by Kang et al. (2004). The supernatant was used as a DNA template, stored at -20°C, for detection of gene encoding for the O157 somatic antigen ($rfbE_{O157}$) using uniplex PCR (Table, 1).

For identification of *E. coli* O157, a uniplex PCR assay was applied in a 25 µl mixture of DNA (3 µl), 2X *Taq* PCR Master Mix (Qiagen, Germany) (12.5 µl), $rfbE_{O157}$ forward and reverse primers (1 µl each) and PCR grade water (Jena Bioscience, Germany) (7.5µl). The amplification condition was applied according to Possé et al. (2007). *E. coli* serotype O157/H7 reference strain (ATCC®700927™) was used as a positive control for $rfbE_{O157}$ and other AE-STEC virulence genes, whereas double distilled water was used as a negative control. PCR products were analyzed by agarose gel electrophoresis using 1.5% agarose gel-stained with ethidium bromide, viewed under UV light and documented with a GelDoc 1000 fluorescent imaging system (Bio-Rad).

Molecular detection of AE-STEC virulence genes

All genomic DNA of the identified *E. coli* O157 strains were subsequently PCR tested for AE-STEC virulence genes (stx_1, stx_2, *eae* and *ehylA)* using two multiplex PCR assays. The first PCR assay was used for the detection of *eae* and *ehylA* genes according to Chandra et al. (2013), whereas the second assay was applied for the detection of stx_1 and stx_2 genes according to Fagan et al. (1999). Target genes, oligonucleotide primer sequences and the expected product size in different PCR assays were mentioned in Table 1.

Antimicrobial resistance test

Antimicrobial resistance test was conducted on *E. coli* O157 strains using the Kirby-Bauer disk diffusion method (Bauer et al. 1966). The panel of these antibacterial agents consisted of β-lactams including, ampicillin (AMP, 10), amoxicillin/clavulanic acid (AMC, 30), cefalexin (CL, 30), cefuroxime (CXM, 30), ceftriaxone (CRO, 30), cefotaxime (CTX, 30), cefquinome (CFQ, 30) and cefoxitin (FOX, 30), and non β-lactams including, ciprofloxacin (CIP, 5), gentamicin (CN, 10), nalidixic acid (NA, 30), sulfamethoxazole/trimethoprim (SXT, 25) and tetracycline (TE, 30). Antibiotic disks were purchased from HiMedia, India, except for Cefquinome disks (Oxoid, UK). Antibiotic resistance results were interpreted according to CLSI (2013) and CLSI (2020).

Determination of ESC resistant *E. coli*

Strains of *E. coli* that confer resistance to one or more of third and fourth generation cephalosporins were considered ESC resistant *E. coli* according to CDC (2020).

Molecular detection of ESBLs encoding resistance genes

A multiplex PCR assay was conducted to detect ESBLs encoding genes (bla_{TEM}, bla_{SHV} and bla_{CTXM}) using universal primers (Table, 1) according to Fang et al. (2008).

Determination of *E. coli* phylogroups

Phylogenetic groups of *E. coli* were determined using the Clermont phylotyping method based on the presence or absence of *chuA, yjaA* and TspE4.C2 genes combinations. For detection of these genes (Table, 1), a triplex PCR assay was conducted on *E. coli* O157 strains, which were allocated to one of the phylogroups A (*chuA*-/TspE4.C2-), B1 (*chuA*-/TspE4.C2+), B2 (*chuA*+/*yjaA*+) and D (*chuA*+/*yjaA*-) according to (Clermont et al. 2000).

RESULTS AND DISCUSSION

AE-STEC O157 is regarded to be one of the causes of neonatal calf diarrhea and is perceived to be a serious human pathogen. A 187 *E. coli* strains were recovered from 137 diarrheic calves up to 4 weeks old relying on the selection of more than one strains from the same sample. Based on the detection of *rfbE*O157 encoding gene using uniplex PCR (Fig. 1), 15 *E. coli* strains (8.02%) were confirmed as *E. coli* O157. This is nearly agreed with Kang et al. (2004), Manna et al. (2006), and Kuyucuoğlu et al. (2011) who recovered *E. coli* O157 from diarrheic calves in percentages of 9.8%, 7.59% and 10.6%, respectively. On the other hand, Stenkamp-Strahm et al. (2017) recovered *E. coli* O157 in a percentage of 0.25%.

Genetic profiling of virulence markers was performed on 15 *E. coli* O157 strains using two multiplex PCR assays (Fig. 2 and 3). The virulence genes *eae*, stx_1, stx_2 and *ehlyA* were detected in percentages of 93.3%, 73.3%, 20%, and 13.3%, respectively (Table, 2). The predominance of stx_1 than stx_2 was also detected by Mainil and Daube (2005).

Table 1: Primer names, target genes, oligonucleotide primer sequences and the expected product size used in different PCR assays

Primer name (Target gene)	Oligonucleotide primer sequences (5-3`)	Product; size (bp)	References
E. coli O157 gene			
O157 (*rfbE*$_{O157}$)	CGG ACA TCC ATG TGA TAT GG TTG CCT ATG TAC AGC TAA TCC	259	Paton and Paton (1998)
AE-STEC virulence genes			
EAE (*eae*)	TCA ATG CAG TTC CGT TAT CAG TT GTA AAG TCC GTT ACC CCA ACC TG	482	Vidal et al. (2005)
HlyA (*ehlyA*)	AGC TGC AAG TGC GGG TCT G TAC GGG TTA TGC CTG CAA GTT CAC	569	Wang et al. (2002)
Stx1 (*stx*$_1$)	CGA TGT TAC GGT TTG TTA CTG TGA CAG C AAT GCC ACG CTT CCC AGA ATT G	244	Müller et al. (2007)
Stx2 (*stx*$_2$)	CCA TGA CAA CGG ACA GCA GTT CCT GTC AAC TGA GCA GCA CTT TG	779	Gannon et al. (1992)
ESBLs encoding genes			
SHV (*bla*$_{SHV}$)	CTT TAT CGG CCC TCACTCAA AGG TGC TCA TCA TGG GAA AG	237	Fang et al. (2004)
TEM (*bla*$_{TEM}$)	CGC CGC ATA CAC TAT TCT CAG AAT GA ACG CTC ACC GGC TCC AGA TTT AT	445	Monstein et al. (2007)
CTX-M (*bla*$_{CTXM}$)	ATG TGC AGY ACC AGTAAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	Boyd et al. (2004)
Phylogroup encoding genes			
ChuA (*chuA*)	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	279	Clermont et al. (2000)
YjaA (*yjaA*)	TGA AGT GTC AGG AGA CGC TG ATG GAG AAT GCG TTC CTC AAC	211	
TspE4.C2 (TspE4.C2)	GAG TAA TGT CGG GGC ATT CA CGC GCC AAC AAA GTA TTA CG	152	

Table 2: Virulence genes profile of *E. coli* O157 strains

Virulence genes; Strain. no	1	2	3	4	5	6	7	8[a]	9[b]	10	11	12	13	14	15	Total (%)
eae	+	+	+	+	+	+	+	+		+	+	+	+	+	+	14 (93.3)
stx$_1$	+	+	+	+	+		+			+	+	+	+		+	11 (73.3)
stx$_2$						+							+	+		3 (20)
ehylA													+	+		2 (13.3)
eae+*stx*$_1$	●	●	●	●	●		●			●	●	●			●	10 (66.7)
eae+*stx*$_2$						●										1 (6.7)
eae+*stx*$_2$+*ehylA*														●		1 (6.7)
eae+*stx*$_1$+*stx*$_2$+*ehylA*													●			1 (6.7)

[a] Strain no. 8 carry *eae* gene only; [b] Strain no. 9 not carry any of the AE-STEC virulence genes.

Table 3: Antibacterial resistance testing, ESC resistance, ESBLs encoding genes profile and phylogroups of *E. coli* O157 strains

Strain no.	Antibacterial resistance testing													ESC resistance Strain no. (%)	ESBLs encoding genes						Phylogroups				
	AMC	CL	CXM	TE	SXT	AMP	FOX	NA	CIP	CRO	CTX	CFQ	CN		*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{CTXM}$	Profile 1	Profile 2	Profile 3	A	B1	B2	D	untypeable
1	+	+	+	+	+	+	+	+	+	+	+	+	+	Resistant	+			●					●		
2	+	+	+	+	+	+	+	+	+	+	+	+	+	9 (60)	+	+	+			●			●		
3	+	+	+	+	+	+	+	+	+	+	+	+	+		+			●					●		
4	+	+	+	+	+	+	+	+	+	+	+		+		+	+			●				●		
5	+	+	+	+	+	+		+	+	+	+	+	+		+			●					●		
6	+	+	+	+	+	+	+	+	+	+	+	+			+			●							●
7	+	+	+	+	+	+	+			+	+	+			+	+	+			●			●		
8	+	+	+	+	+	+	+			+	+	+			+	+			●					●	
9	+	+	+	+	+	+	+			+	+	+			+	+	+			●			●		
10	+	+	+	+	+	+	+	+	+				+	Sensitive	+			●					●		
11	+	+	+	+	+	+	+	+	+					6 (40)	+			●					●		
12	+	+	+	+	+		+	+	+						+			●					●		
13	+	+	+	+	+	+		+	+						+	+			●				●		
14	+	+	+	+			+								+			●					●		
15	+	+	+	+	+										+			●				●			
Total	15	15	15	15	14	12	12	10	10	9	9	8	6		15	6	3	9	3	3	0	1	12	1	1
%	100	100	100	100	93.3	80	80	66.7	66.7	60	60	53.3	40		100	40	20	60	20	20	0	6.7	80	6.7	6.7

ESBLs profile: 1= *bla*$_{TEM}$, 2= *bla*$_{TEM}$+*bla*$_{SHV}$ and 3= *bla*$_{TEM}$+*bla*$_{SHV}$+*bla*$_{CTXM}$.

The presence of *eae* gene in 93.3% of *E. coli* O157 strains is almost similar to that of Kang et al. (2004) who detected this gene in all *E. coli* O157 strains. This suggests the role of Intimin in the pathogenicity of *E. coli* O157 in calves and confirmed by Mainil and Daube (2005) who attributed the cause of diarrhea in calves and human infected with *E. coli* O157 to the attaching and effacing lesion caused by Intimin and not by the production of Shiga toxins.

The Enterohemolysin encoding gene (*ehylA*) was detected in 13.3% of the isolated strains, whereas Kang et al. (2004) and Kuyucuoğlu et al. (2011) detected this gene in 91.6% and 92.8% of *E. coli* O157 strains, respectively.

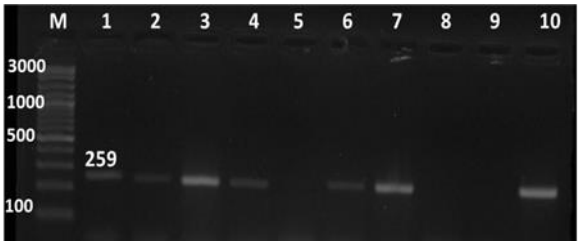

Fig. 1: PCR for detecting *rfbE*$_{O157}$ gene. Lane M: 100-3000 bp DNA marker (100 bp plus DNA ladder, Vivantis, Malaysia); Lanes 1-4, 6 and 7: Positive strains for *rfbE*$_{O157}$ gene at 259 bp. Lanes 5 and 8: negative samples. Lane 9: negative control. Lane 10: positive control.

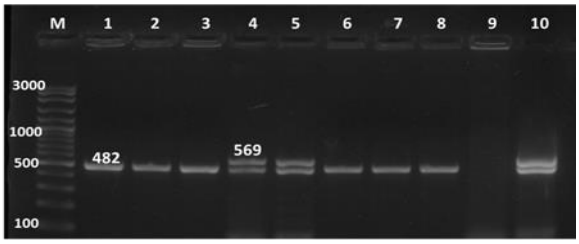

Fig. 2: Duplex PCR for detecting *eae* and *ehylA* genes in *E. coli* O157 strains. Lane M: 100-3000 bp DNA marker. Lanes 1-3 and 6-8: Positive strains for *eae* gene at 482 bp. Lanes 4 and 5: Positive strains for *eae* and *ehylA* genes at 482 and 569 bp, respectively. Lane 9: negative control. Lane 10: positive control.

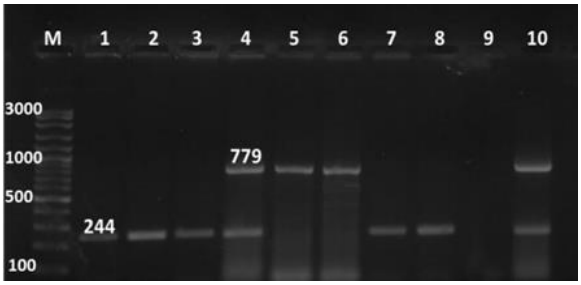

Fig. 3: Duplex PCR for detecting *stx*$_1$ and *stx*$_2$ genes in *E. coli* O157 strains. Lane M: 100-3000 bp DNA marker. Lanes 1-3, 7 and 8: Positive strains for *stx*$_1$ gene at 244 bp. Lane 4: Positive strain for *stx*$_1$ and *stx*$_2$ genes at 244 and 779 bp, respectively. Lanes 5 and 6: Positive strains for *stx*$_2$ gene at 779bp. Lane 8: negative control. Lane 9: positive control.

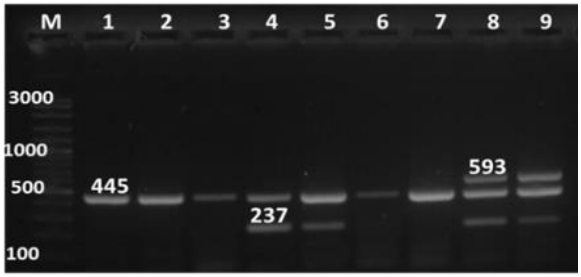

Fig. 4: Multiplex PCR for detecting *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTXM}$ genes in *E. coli* O157 strains. Lane M: 100-3000 bp DNA marker. Lanes 1-3, 6 and 7: Positive strains for *bla*$_{TEM}$, gene at 445bp. Lanes 4 and 5: Positive strains for *bla*$_{SHV}$ *and bla*$_{TEM}$ *g*enes at 237 and 445bp, respectively. Lanes 8 and 9: Positive strains for *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTXM}$ genes at 237, 445 and 593bp, respectively.

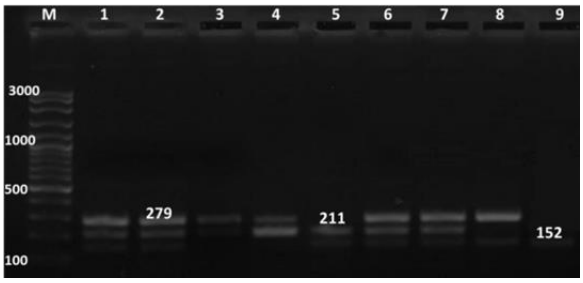

Fig. 5: Triplex PCR for detecting *chuA*, *yjaA* and TspE4.C2 genes in *E. coli* O157 strains. Lane M: 100-3000 bp DNA marker. Lanes 1, 2, 6 and 7: Positive strains for TspE4.C2, *yjaA* and *chuA* genes at 152, 211 and 279 bp, respectively. Lanes 3 and 4: Positive strains for *yjaA* and *chuA* genes at 211 and 279 bp, respectively. Lane 5: Positive strain for TspE4.C2 and *yjaA* genes at 152 and 211 bp, respectively. Lane 8: Positive strain for TspE4.C2 and *chuA* genes at 152 and 279 bp, respectively. Lane 9: Positive strain for TspE4.C2 gene at 152 bp.

Distribution of the virulence genes combination profile within *E. coli* O157 strains was demonstrated in Table (2). The predominant genes combination *eae*+*stx*$_1$ were found in 66.7% of *E. coli* O157 strains, while only one (6.7%) strain carried *eae*+*stx*$_2$ genes. Each of the genetic profiles of *eae*+*stx*$_1$+*stx*$_2$+*ehylA* and *eae*+*stx*$_2$+*ehlyA* was detected in one (6.7%) strain that regarded as a highly virulent combinations causing a severe pathological lesion (Possé et al. 2007). The presence of *eae* as a sole gene was detected in one strain (6.7%) with the absence of other virulence genes. It is not surprising to identify a strain (6.7%) that did not bear any of the AE-STEC virulence genes, as Stenkamp-Strahm et al. (2017) recorded the lack of such genes in one *E. coli* O157 strain (0.25%) recovered from calves. The existence of AE-STEC virulence genes on unstable mobile genetic elements leading to the loss of these genes during infection and strains that lost Shiga toxins were tightly linked to the other virulent Shiga toxins bearing strains based on multilocus sequence typing of these strains (Bielaszewska et al. 2007).

Regarding the antimicrobial resistance profile, all (100%) *E. coli* O157 strains showed antibiotic multiple resistances (AMR) with variation to multiple antibiotic classes (Table, 3). A higher percentage of multi-resistances within bovine *E. coli* O157 was reported by Srinivasan et al. (2007) and Iweriebor et al. (2015), while Murinda et al. (2005) recorded that 80% of such strains were susceptible to all tested antibiotics. The emergence of multi-resistant bacteria has been linked to the pervasive veterinary antibiotic treatment in recent years (Saei et al. 2012; Mehmood et al. 2020).

The most resistance expression was detected toward amoxicillin/clavulanic acid, cefalexin, cefuroxime and tetracyclines at a percentage of 100% followed by 93.3% for sulfamethoxazole/trimethoprim, 80% for ampicillin and cefoxitin, and 66.7% for nalidixic acid and ciprofloxacin.

Furthermore, the resistance to extended spectrum cephalosporins (ESC) was 60% for ceftriaxone and cefotaxime and 53.3% for cefquinome. The lowest resistance was detected toward gentamicin at a percentage of 40%.

The antibiotic resistance profile revealed that 60% were ESC resistant *E. coli*, while the remaining strains (40%) were ESC susceptible *E. coli* but resist to other non β-lactams. Variations in antibiotic resistance within *E. coli* O157 strains were detected by Mora et al. (2005), Srinivasan et al. (2007) and Hasan et al. (2019). The difference in resistance patterns may be attributable to the most prevalent types of veterinary antibiotics used in different geographical regions for treatment and prevention (Saei et al. 2012; El Jalil et al. 2020; Abd El-Fatah et al. 2020).

Detection of ESBLs encoding genes using multiplex PCR assay (Fig. 4) revealed the predominance of the bla_{TEM} gene in all (100%) *E. coli* O157 strains, whereas the bla_{SHV} and bla_{CTXM} genes were detected in percentages of 40% and 20%, respectively (Table, 3). On the other hand, Iweriebor *et al*. (2015) in South Africa detected the absence of bla_{SHV} gene in *E. coli* O157 strains, while bla_{TEM} and bla_{CTXM} genes were detected in percentages of 27% and 65%, respectively.

Regarding resistance genes profile as shown in Table (3), the bla_{TEM} gene was found either alone (60%) or in combination with bla_{SHV} and bla_{CTXM} genes. The bla_{SHV} gene was associated with bla_{TEM} in 20% of these strains, while the combination of bla_{TEM}+bla_{SHV}+bla_{CTXM} was detected in ESC resistant strains only (20%) and this illustrates the role of bla_{CTXM} in resistance to ESC. Furthermore, all ESC resistant strains express resistance to most of the studied antibiotics classes. This is related to the horizontal transfer of plasmid mediated ESBLs encoding genes that contributes to the emergence of antibiotic resistance within *E. coli* strains (Terentjeva et al. 2019).

Molecular phylotyping of 15 *E. coli* O157 strains revealed an unexpected high percentage of B2 phylogroup within 80% of these strains, while each of B1, D and untypeable phylogroups were comprised one strain (6.7%) as shown in Table (3). This disagreed with Tramuta et al. (2008) and Coura et al. (2017) who identified the predominant phylogroups within AE-STEC strains were B1 and A. The higher B2 level within *E. coli* strains indicates a serious concern, as the animals harboring *E. coli* strains with phylogroup B2 are a reservoir of a highly virulent extra-intestinal pathogenic strains that affecting humans (Clermont et al. 2000). In addition, identification of untypeable strain may be related to the recombination of two different phylogroups within the same strain as mentioned by Clermont et al. (2013). The relation between different phylogroups, antibiotic resistance and ESBLs genes characterization revealed that all ESBLs genes especially bla_{CTXM} bearing strains shared B2 phylogroup and multi-resistances. This association was determined by Lee et al. (2010) who stated that virulent resistant strains carrying the bla_{CTXM} gene are more prevalent in B2 phylogroup.

In conclusion, the present investigation revealed that diarrheic calves are a source of multi-resistant *E. coli* O157 that may transfer these resistance genes to other highly pathogenic strains, thereby limiting the treatment of such strains. Virulence genes profile has established that the correlation between Shiga toxins and Intimin has a crucial role in the pathogenesis of *E. coli* O157. Furthermore, the relation between ESC phenotypic resistance and ESBL genetic profiling confirmed the role of bla_{CTXM} gene in ESC resistance. Finally, *E. coli* phylotyping showed a high prevalence of B2 phylogroup that related to highly virulent extra-intestinal strains.

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