INTERNATIONAL JOURNAL OF
ADVANCED SCIENTIFIC RESEARCH AND INNOVATION
VOLUME 5, ISSUE 2, 2022, 49 – 71.

Molecular characterization of extended spectrum β-lactamase resistance genes for isolated Escherichia coli from calves diarrhea

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Abstract

Neonatal calf diarrhea (NCD) is a major disease in calves. *Escherichia coli* is considered the most common and economically important cause of diarrhea in young calves.This work is conducted to determine the extended spectrum ß-lactamase (ESBL) producing Escherichia coli (E. coli) isolated from calf diarrhea and select the most effective antibiotic used to treat this problem. A total of two hundred fecal samples of diarrheic calves were collected for the presence of ß-lactamase encoding genes (blaTEM, blaSHV, and blaOXA) 84(42%) out of those samples were *E.coli* positive.The commonly identified *E.coli* serogroups were O26,O111,O128,O91,O114,O103,O17, and O127. *Escherichia coli* isolates showed an obstacle of antimicrobial resistance for different antimicrobials while most of these isolates cleared susceptibility for ciprofloxacin and Ceftriaxone. Depending on the results of PCR, the *Stx1*and *Stx2* were negative in all the isolates. Only one isolate O26 carried *Stx1* gene and O17 carried *Stx2* gene. It was found that all representative serotypes were negative for *eae* gene except O111, O26, and O127.*blaTEM*, *blaSHV* were the most predominant genes (100%) but *blaOXA* had the lowest prevalence (12.5%). The results proved that multiple antibiotic resistances are widely spread among isolated strains of *E. coli*.

Regular screening of antibiotics sensitivity before actual application to animals is essential to reduce the possibility of dissemination of resistance genes.

Keywords: *Escherichia coli*, virulence genes, □-lactamase, quinolones, calves.

Introduction:

Neonatal calf diarrhea (NCD) is a major disease worldwide when calves are reared intensively, and constitutes substantial costs in terms of calf mortality, opportunity costs for labor and capital, veterinary costs, and loss in calf value. The term NCD refers to a disease complex characterized by acute, undifferentiated diarrhea in young calves (Kerstin *et al.*, 2012). *Escherichia coli* is considered the most common and economically important cause of diarrhea in young calves (Muktar *et al.*, 2015). *E. coli* is a gram-negative, non-sporulating, rod-shaped, flagellated, and facultative anaerobic bacterium of the family Enterobacteriaceae. There are several diarrheagenic *E. coli* pathotypes including Shiga-toxin producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enterohaemorrhagic *E. coli* (EHEC)**.** (Zinnah MA *et al.*,2007; Donnenberg.,2013).

The emergence of extended-spectrum b-lactamase (ESBL) producing Escherichia coli represent a contemporary public health threat (lee et al.,2020). Antimicrobials are used in pre-weaned calves to treat diseases such as diarrhea and pneumonia (McEwen and Fedorka-Cray., 2002). Evidence suggests that the incorrect use of antimicrobials in animal production is the most important factor for the emergence, selection, and dissemination of antimicrobial resistance (Sayah et al.,2005; Tempini et al.,2018)**.** Due to the increased use of b-lactams and subsequent relocation of ESBL, ESBL producing E. coli are widely disseminated into the environment and into the food-producing animals (Blaak et al.,2014;Ma et al.,2018,2019). An important mechanism of resistance that can be found in *E. coli* is the production of extended-spectrum β-lactamases (ESBL) (Rubin and Pitout .,2014)or both, resulting in the enzymatic inactivation of β-lactams.

The global prevalence of β-lactamase genes *(blaTEM, blaSHV and blaOXA)* has increased considerably over the last 30 years throughout the world in animals. ESBLs are often encoded by genes located on large plasmids, and these also carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines, and chloramphenicol (Paterson.,2000).

The purpose of this study is to determine the antibiotic resistance and highlights the relationship between phenotypic and genotypic resistance of pathogenic E. coli isolates. So must be applying a nationwide surveillance program to monitor antimicrobial resistance where most of administration of these antimicrobials is unnecessary.

Materials and Methods

1-Sample Collection:

A total of two hundred fecal samples of diarrheic calves were collected from different commercial cattle farms in Assiut governorate,Egypt. All the sampled calves should be between one day and three months. Calves should not have been treated with antimicrobials within two weeks prior to sampling. Faecal samples were collected as rectal swabs and submitted to laboratory. The Samples were processed within 24 -48 hours after reception.

2-Bacteriological examination

-Samples were cultured on nutrient broth and incubated for 24 hours at 37ºC, according to (Quinn, et al.,2004). After incubation, a loopful from inculated broth was seeded onto MACCONKEY'SAGAR (Oxoid) plates for 24 h at 37 °C. Rose pink colonies were picked up and streaked onto EOSIN METHYLENE BLUE

AGAR (Oxoid) and incubated overnight at 37 °C. Green metallic sheen colonies of *E. coli* were picked up for biochemical tests, Indole, Triple Sugar Iron Agar, Citrate utilization, Methyl Red-Voges-Proskauer and Urease production tests.

3-Serological identification of *E. coli*:

- The positive isolates were serologically identified according to (Kok et al. 1996).Serotyping of *E. coli* isolates were done at the Reference Laboratory of Veterinary Quality Control on Poultry Production, Dokki, Egypt using commercially available kits (Escherichia coli Antisera set 1 for O antigen, DENKA SEIKEN, Tokyo, Japan) which consists of 8 polyvalent sera and 44 monovalent sera.

4- Congo red binding test for detection of pathogenicity:

All serotypes of *E.coli* isolates were examined for detection of pathogenicity.The medium used for CR dye binding was tryptose agar with 0.2 % galactose and 0.03% CR due to *E.coli* isolates were streaked onto CR agar plates and incubated at room temperature for additional 48 hours. The colonies were examined at 18, 24, 48, and 72 hours of incubation. The *E.coli* that produced red colonies between 18 and 72 hours of incubation were recorded as Congo red positive and the ones that produced grayish-white colonies and remained so throughout the incubation period were recorded as Congo red negative (Panigrahy and Yushen ., 1990).

5-Antimicrobial sensitivity test:

The antimicrobial sensitivity test was performed according to Finegold and Martin (1982) by using the disc diffusion method. Different antimicrobials were used such as Ceftriaxone, Ceftiofur, Amoxacillin+Clavulanic acid, Ciprofloxacin, Trimethoprim+Sulphamethoxazole, Streptomycin, Oxytetracycline, Ampicillin, Amikacin and Gentamycin. The interpretation of the measured zone was done according to (CLSI.,2019).

6-Molecular detection of virulence genes and ESBL encoding genes:

Multiplex PCR was applied for detection of virulence genes involving Shiga toxin 1 (Stx1), Shiga toxin 2 (Stx2) and intimin (eaeA) genes as well as (ESBL) genes blaTEM, blaSHV and blaOXA.

-DNA Extraction:

Escherichia coli DNA was extracted according to QIAamp DNA mini kit instructions that provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total handson preparation time is only 20 minutes. They have specific sequence of primers and amplify specific products as shown in Table(1).

Table 1. showing different genes detected by PCR.

Gene	Sequence	Amplified product	Reference
eaeA	ATG CTT AGT GCT GGT TTA GG	248 bp	Bisi-Johnson *et al.*, 2011
	GCC TTC ATC ATT TCG CTT TC		
BlaTEM	ATCAGCAATAAACCAGC	516 bp	Colom *et al.*, 2003
	CCCCGAAGAACGTTTTC		
BlaSHV	AGGATTGACTGCCTTTTTG	392 bp	
	ATTTGCTGATTTCGCTCG		
BlaOXA	ATATCTCTACTGTTGCATCTCC	619 bp	
	AAACCCTTCAAACCATCC		
Stx1	ACACTGGATGATCTCAGTGG	614 bp	Dipineto *et al.*, 2006
	CTGAATCCCCCTCCATTATG		
Stx2	CCATGACAACGGACAGCAGTT	779 bp	
	CCTGTCAACTGAGCAGCACTTTG		

-Cycling conditions of the primers during cPCR:

The thermal profile and primer annealing temperature for multiplex PCR are shown in Table 2.

Table 2: Cycling conditions of the primers during cPCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
eaeA	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
blaTEM	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
blaSHV	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.

blaOXA	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
Stx1, stx2	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

-Agarose gel electrophoreses

PCR products were separated on 1% agarose gel (AB gene). A 100 bp DNA Ladder (Qiagen, USA) defined the fragment sizes. The gel was visualized through a documentation system. As in table(1).

Results:

1-Incidence of *E. coli* in fecal samples:

From total of 200 diarrhetic samples there were 84 (42%) *E. coli* positive.

2-Serotyping of E. coli isolates recovered from diarrhetic samples:

The isolates of 84 *E.coli* isolates were serologically identified by using rapid diagnostic *E.coli* antisera sets. The serotyping of *E. coli* strains isolated from diarrheatic samples (42%) belonged to 8 different 'O' groups. The most commonly detected E. coli serogroups were O26 (17.8%), O111(15.4%), O128(13.09%), O91(14.8%), O114(10.7%), O103(11.9%), O17(7.1%), O127(9.5%).

3-Congo red binding test:

In this test the most serotypes gave positive reaction were O26 (13%), O128 (11.9%), O111(10.7%) followed by O91 and O127 were (9.5%) and O103 (8.3%) but O114 (7.1%).The lowest serotypes for Congo red reaction was O17 (4.7%).

4-Susceptibility of *Escherichia coli* for different antimicrobials

Multidrug resistance was detected in all isolated serotypes (100%) of E. coli was observed with Ampicillin, but 87.5% of E.coli was resistant to tetracycline. Trimethoprim+Sulphamethoxazole and Streptomycin were 75%, 62.5% followed by Gentamycin, and Ceftiofurwere resistance (50%), On the other hand, the highest sensitivity was observed against Ceftriaxone and Amikacin that have very low resistance (25%) followed by Ciprofloxacin (0%) so they were considered the most influential antibiotics as in table (3).

Table 3: The results of antibiotic sensitivity test of E. coli serogroups isolated from calves diarrhea **S**: Sensitive; **R**: Resistant; **I**: Intermediate; **AK**:amikacin; **CIP**:ciprofloxacin; **CRO**: ceftriaxone; **CF**: cetiofurwere; **AMC**: amoxicillin-clavulanic acid; **SXT:** Trimethoprim+Sulphamethoxazole; **S:** Streptomycin; **TE**: tetracycline; **AM:** Ampicilli

O groupes	**CRO**	**CF**	**AMC**	**CIP**	**SXT**	**S**	**TE**	**AM**	**AK**	**GM**	**Total resistant**
O128	S	S	R	S	R	R	R	R	S	S	5(50%)
O111	S	I	R	S	R	I	R	R	S	S	4(40%)
O26	S	S	I	S	R	R	R	R	S	I	4(40%)
O114	I	R	I	S	R	I	R	R	R	R	6(60%)
O103	R	R	R	S	I	R	R	R	S	S	6(60%)
O91	R	I	R	S	R	R	I	R	S	S	5(50%)
O17	S	S	I	S	R	S	R	R	S	R	4(40%)
O127	S	R	R	S	I	R	R	R	R	R	7(70%)
Number of isolates %	2 (25%)	3 (37.5)	5 (62.5%)	0 (0%)	6 (75%)	5 (62.5%)	7 (87.5%)	8 (100%)	2 (25%)	3 (37.5%)	

5-Molecular detection of some virulence genes and ESBL encoding genes

Depending on the results of PCR, the *Stx1*and *Stx2* were negative in all the isolates(figure 1). Only one isolate O26 carried *Stx1* gene and O17 carried *Stx2* gene(figure2). It was found that all representative serotypes were negative for *eae* gene except O111, O26, and O127.*blaTEM*, *blaSHV* were the most predominant genes (100%) but *blaOXA* had the lowest prevalence (12.5%) (figure3,4,5).

Table 4. Results of Molecular Detection of some virulence and drug resistance genes of *Escherichia coli* isolates.

No	Sample	blaTEM	blaSHV	blaOXA	eaeA	Stx1	Stx2
1	O128	+	+	-	-	-	-
2	O111	+	+	-	+	-	-
3	O26	+	+	-	+	+	-
4	O114	+	+	-	-	-	-
5	O103	+	+	-	-	-	-
6	O91	+	+	+	-	-	-
7	O17	+	+	-	-	-	+
8	O127	+	+	-	+	-	-

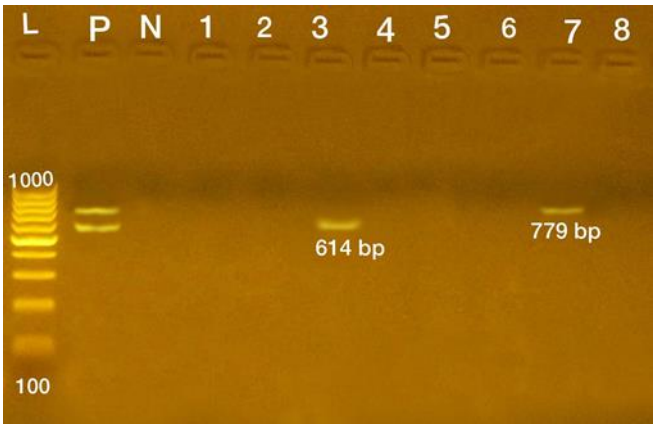

Figure 1: Agarose gel electrophoresis of multiplex PCR using shiga toxin1gene *(Stx1*)(614 bp) and shiga toxin2*(Stx2)* (779 bp).

Lane L: 100 bp ladder as molecular size DNA marker. Lane p: Control positive *E. coli* for *Stx1* and *Stx2*. Lane N: Control negative .All representative serotypes are negative for shiga toxin1 except lane3 (O26) has this gene but all representative serotypes are negative for shiga toxin2 except lane 7 (O17).

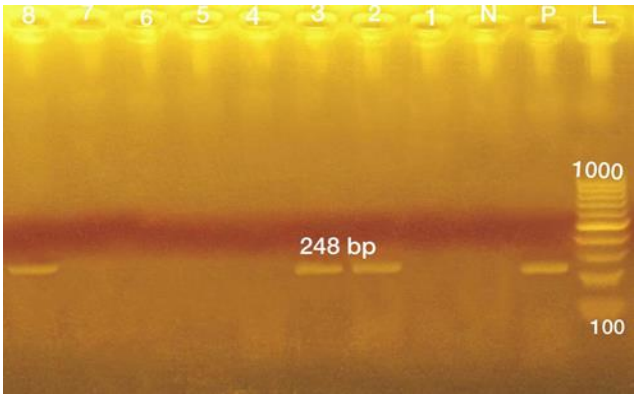

Figure 2: Agarose gel electrophoresis of uniplex PCR using intimine gene *eaeA*(248 bp).

Lane L: 100 bp ladder as molecular size DNA marker. Lane p: Control positive *E. coli* for *eaeA*, . Lane N: Control negative. Lanes 2 (O111), 3 (O26) & 8 (O127): Positive *E. coli* strains for *eaeA.* Lanes 1(O128), 4 (O114),5 (O103), 6 (O91) &7 (O17).

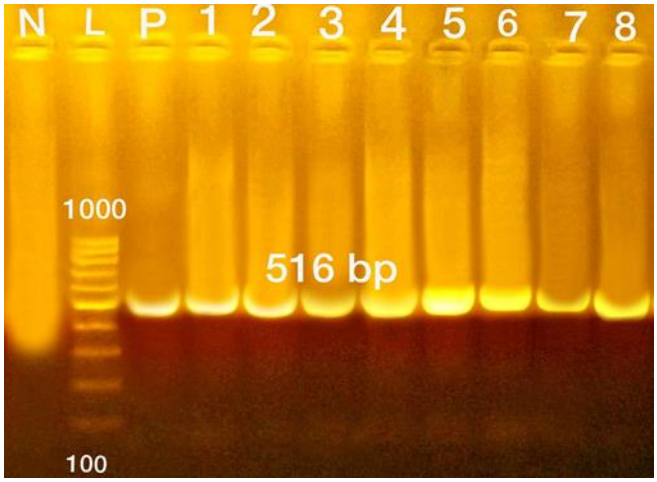

Figure 3: Agarose gel electrophoresis of uniplex PCR using *blaTEM* gene (516 bp).

Lane L: 100 bp ladder as molecular size DNA marker. Lane p: Control positive *E. coli* for *blaTEM*. Lane N: Control negative. All representative serotypes are positive for this gene.

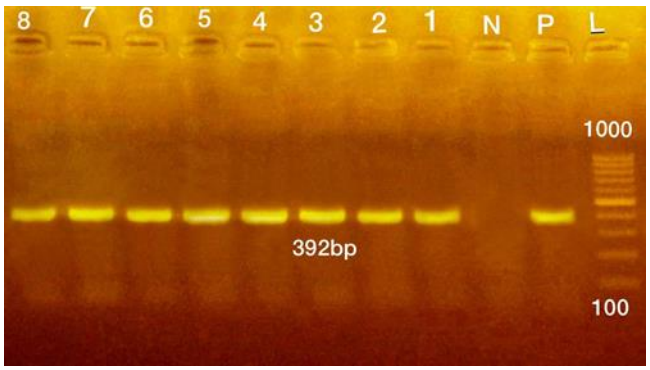

Figure 4: Agarose gel electrophoresis of uniplex PCR using *blaSHV* gene (392 bp).

Lane L: 100 bp ladder as molecular size DNA marker. Lane p: Control positive *E. coli* for *blaSHV*. Lane N: Control negative. All representative serotypes are positive for this gene.

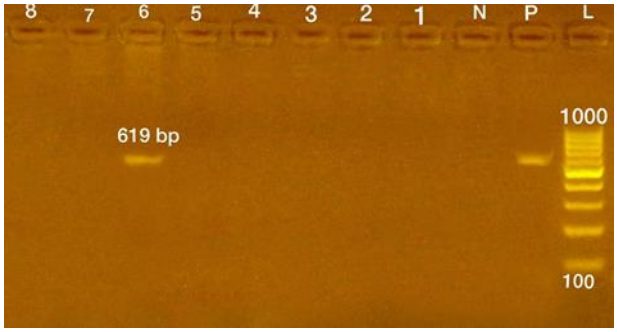

Figure 5: Agarose gel electrophoresis of uniplex PCR using *blaOXA* gene (619 bp).

Lane L: 100 bp ladder as molecular size DNA marker. Lane p: Control positive *E. coli* for *blaOXA*. Lane N: Control negative. All representative serotypes are negative for this gene except lane 6 (O91) serotype.

Discussion:

E. coli is considered the most common and economically important cause of diarrhea in young calves (Muktar *et al.*, 2015). Among the bacterial enteropathogens causing calf diarrhea is enterotoxigenic *E. coli* (ETEC) which is considered the most economically important pathogen.

In the present study, a moderate frequency was obvious of *E.coli* isolates that were obtained from fecal samples of diarrheic calves with an incidence of 42%. These results might be attributed to the low level of hygiene prevailing in the farm where the study was applied and no routine cleaning or disinfection of milk utensils or equipment used for feeding and drinking the calves which favor the growth of such bacteria causing diarrhea. These obtained results are in harmony with that detected by (El Bably *et al.* 2016) who demonstrated that the most predominant diarrhea-causing bacteria in calves was E. coli (68.3%) in the examined farm. Furthermore,

(Raihan *et al.,* 2014) found that the frequency distribution of *E.coli* isolates from diarrheic calves was (28%). (El-Hamamy *et al.,* 1999) demonstrated that the predominant isolate from diarrheic calves was E. coli (52.5%).

Serogrouping of *E.coli* was carried out to give an idea about the most important and predominant serogroups found in diarrhetic calves. In this study, the results showed that *E.coli* isolates belong to 10 different serotypes (O26, O111, O128, O91,O114 , O103, O17,and O127). These results agree with (Blanco *et al.*,2004) who found different serotypes of Shiga toxin *E.coli* including (O26, O91); (Shahrani *et al.*,2014) recorded different serotypes including (O128, O26) and (El Bably *et al.* 2016) who found the most serotype of pathogenic *E.coli* isolates was O26. (Mohammed *et al.,* 2016) declared that *E. coli* serotypes O91 (41.17 %),

and O145 (29.41 %) were the most predominant serogroups obtained from flies in cattle farms.

Antimicrobial sensitivity tests showed that *E. coli* isolated from diarrhetic calves showed high sensitivity to ciprofloxacin and ceftriaxone, intermediate sensitivity to Ceftiofur, gentamycin, streptomycin and amikacin. It was found complete resistance to ampicillin(□-lactam), oxytetracycline and Trimethoprim+Sulphamethoxazole. These obtained results agreed to some extent with (El Bably *et al.*,2016) who recorded that *E. coli* isolates from calves were mostly sensitive to enrofloxacin and neomycin, intermediate sensitivity to chloramphenicol and florophenicol; and complete resistance to β-lactams, tetracycline and sulphamethoxazole-trimethoprime. (Sadiek and Sohair.,1999), (El-Gaml *et al*.,2001) and Aba-(Alkhalil and El-Naenaeey.,2003) all indicated that enrofloxacin and ciprofloxacin were the most efficient antibiotics in treatment of

calf diarrhea. Also, (Adetunji and Isola.,2011) indicated that *E. coli* isolated were resistant to nitrofuration, tetracycline, ampicillin, gentamicin, ciprofloxacin, and chloramphenicol, and (Mohammed *et al.,* 2016) revealed that the antimicrobial resistance pattern of Gram-negative bacteria proved that *E. coli* was resistant to tetracycline (92.9 %).

According to the results produced by the multiplex PCR assay, the *Stx1*and *Stx2* were negative in all the isolates. Only one isolate O26 carried *Stx1* gene and O17 carried *Stx2* gene (figure1).. It was found that all representative serotypes were negative for *eae* gene except O111, O26, and O127 (figure 2).*blaTEM*, *blaSHV* were the most predominant genes (100%) but *blaOXA* had the lowest prevalence (12.5%). as in (Fig 3, Fig 4, Fig 5) respectively. *eaeA, Stx1, and Stx2* genes are recognized as key genes in *E. coli* virulence pathways and their occurrence can define various pathovars. Thus, based on the presence of specific genes, some isolates could be categorized (Kapper *et al.* 2004; Coura et al. 2017) as STEC (containing *eaeA* + *ehxA* + *stx1* + *stx2* genes). (Souhir *et al.*,2018) found 2 isolates, 3%)that had these virulence genes. This result is concordant with well-known features of STEC isolates (Irshad et al., 2014). For β-lactams resistance genes *(blaTEM, blaSHV,* and *blaOXA*) the current results were to some extent similar to (Jiang and Zhang.,2013) who reported that the *TEM* gene was identified in 84.6% of tested isolates while *SHV* and *OXA* were not detected 0% of the tested isolates. The result agreed with (Abd Tawab, *et al*. 2016) that found 100% carry (*blaTEM*) gene, and disagreed with (Bardoň *et al.*2018) found 26%.The (*blaSHV*) gene is disagree with (Rahman, *et al*. 2018) detected 20% of isolates carry *blaSHV* gene and (Shehata, *et al*. 2016) that found 60% carry this gene. The (*blaOXA*) gene

disagree with (Rahman, *et al*. 2018) that found 20% carry *blaOXA*, in contrast, (Shehata, *et al*. 2016) was reported 40% carry this gene.

Phenotypic multi-resistance of E. coli isolates to ß-lactams could be related to the presence of ß-lactames encoding genes. Four isolates (50%) showed a relationship between phenotype and genotype, while four isolates (50%) showed irregular relation , Table 4 and 5. This result agreed with (Reich, et al.2013) found clinical resistance to amoxicillin- clavulanic acid was more prevalent in ESBL producers and disagreed in that found rate of ceftriaxone resistance was higher in SHV-containing isolates.

Table 5: The relationship between phenotype and genotypic β-lactamase resistance E. coli

S: Sensitive; R: Resistant; **I:** Intermediate; **AMC**: amoxicillin-clavulanic acid; **AM:** pencillin; **CRO**: ceftriaxone.

serotypes	**Phenotype**				**Genotype**			
	AM	CRO	AMC	Resistance number	bla *TEM*	bla *SHV*	bla *OXA*	Number of Genes
O128	R	S	R	2	+	+	-	2
O111	R	S	R	2	+	+	-	2
O26	R	S	I	1	+	+	-	2
O114	R	I	I	1	+	+	-	2
O103	R	R	R	3	+	+	-	2
O91	R	R	R	3	+	+	+	3

O17	R	S	I	1	+	+	-	2
O127	R	S	R	2	+	+	-	2
Resistance%	100%	25%	62.5%		100%	100%	12.5%	

Conclusion:

Finally, the results proved that multiple antibiotic resistances are widely spread among isolated strains of *E. coli*. Calves can act as a source of contamination to the environment with multiple antimicrobial resistant *E.coli* which poses a high risk for human and animal populations. Regular screening of antibiotics sensitivity before actual application to animals is essential to reduce the possibility of dissemination of resistance genes.

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