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Antimicrobial determinants of multidrug resistant Escherichia coli serotypes isolated from diarrheic calves

Eman E. Abdeen[1], Mohammed A. Nayel[2], Hannan H[3], Ahmed Elsify[2], Akram A. Salama[2], Ahmed A. Zaghawa[2], Abdulaziz M. Almuzaini[4], Ayman Elbehiry[1,5] and Walid S. Mousa[2]

[1]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Menoufia, **Egypt.**
[2]Department of Animal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, University of Sadat City, Menoufia, **Egypt.**
[3]Department of Medical Laboratories, Faculty of Medical Applied Science, Al Majmaah University, **Saudi Arabia.**
[4]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Buraydah, **Saudi Arabia**.
[5]Department of Public Health, College of Public Health and Health Informatics, Qassim University**,** Buraydah, **Saudi Arabia**

***Correspondence:** mohamed.aboalez@vet.usc.edu.eg; **Received: 14 Nov. 2019, Revised: 19 Dec. 2019 Accepted: 20 Dec. 2019 e-Published: 26 Dec. 2019, Reviewed by: Prof. Dr. Adel Khadr, Prof. Dr. Yamen Hegazy**

Calf diarrhea is one of the most important devastating enteric problems that threats the bovine industry worldwide. Pathogenic Escherichia coli (*E. coli*) is considered the most problematic etiology. Therefore this study was conducted to investigate the antibiogram pattern and antibiotics resistance genes of *E. coli* strains isolated from calf diarrhea. Sixteen (21.33%) *E. coli* strains were isolated from 75 diarrheic calves and serotyping revealed that O26 and O157 were the most common serotypes (25%) and (18.75%), respectively. The results antibiogram profile indicated that all isolates found to be resistant to multiclass of antimicrobial groups. All isolates exhibit resistance to streptomycin and cefaclor 100%, amoxycilline 81.25%, sulpha/trimethoprim 62.5%, gentamycin 56.25%, cefotaxime 50%. Meanwhile high susceptible to chloramphenicol, enrofloxacin and cefotaxime was recorded. In the same context, *bla*TEM, *tet*A, *aad*A2, *sul*1 and *drf*A were the most frequently detected antibiotic resistance genes. In conclusion, our findings revealed that *E. coli* is considered one of the important bacterial etiology causing diarrhea in newborn calves. In addition to, most of *E.coli* serotypes express high resistance to multi-groups of antimicrobials in phenotypic and genotypic profile. Our results suggested a relationship between the phenotypic and antimicrobial resistance genes which could contribute the emerging and evolution of antibiotics resistance pattern by *E.coli* strains that complicated the control of such organism.

Keywords: *Escherichia coli*, antibiotic, resistance genes, diarrhea.

INTRODUCTION

Calf diarrhea represents a prevalent financial dilemma in the animal industry worldwide (Shahrani et al., 2014). Diarrhea is a common reason for high morbidity and mortality rates, particularly in newborn calves. Various microorganisms, such as protozoa (*Cryptosporidium parvum*), viruses (coronavirus and rotavirus) and bacteria (*Salmonella*, *E.Coli*,

Clostridium perfringes), can cause diarrhea (Izzo et al., 2011). These pathogen can cause infection alone or in combination with other associated pathogens (Cho and Yoon, 2014).

Although several types of bacteria have been recovered from calves with diarrhea*, E. coli* remains one of the most noteworthy pathogenic bacteria in diarrheic calves, and its prevalence varies according to geographic area (García et al., 2000). Accordingly, *E. coli* is considered a leading cause of some clinical syndrome such as septicemia, diarrhea, pneumonia, meningitis and panophthamitis especially in newborn calves and death in complicated dehydrated cases (Shahrani et al., 2014).

Svarm , 2006 illustrated that most of the *E. coli* strains of dairy cow origin were vulnerable to all investigated antimicrobial drugs, while Swedres-Svarm, 2013 revealed that calves aged 6 to 11 months were susceptible to various antimicrobial drugs. However, the occurrence of *E. coli* resistance to various antimicrobial agents is age-dependent. The antibiotic resistance rates in *E. coli* recovered from neonatal calves and adult cows were studied by Yamamoto et al., (2013), who found that calves are considerably more resistant than older cattle. The high incidence of multidrug-resistant *E. coli* in pre-weaning calves may be due to repeated therapy with different types of antibiotics (Yamamoto et al. 2013; Pereira et al., 2014).

Consequently, repeated antimicrobial therapy may promote a large community of antibiotic-resistant bacteria in farm animals (Berge et al., 2005). Similarly, the data on the incidence of antimicrobial resistance genes in *E. coli* recovered from young calves are sparse. Thus, the current aimed to investigate the distribution of the *E. coli* serotypes recovered from diarrheic calves. Also to study their antibiogram against different groups of antibiotics and resistance to *bla*TEM, *bla*CTX, *dfr*A, *tet*A, *sul*1 and *aad*A2 as antibiotic resistance genes.

MATERIALS AND METHODS

Sampling and clinical examination

The samples included two-hundred calves under three month old that were monitored clinically for incidence of diarrhea cases from Behera Province, Egypt. The clinical examination of the observed diarrheic calves included the evaluation of clinical signs, degree of dehydration, pulse and respiratory rates, and systemic reactions. Seventy-five fecal samplings were aseptically gathered from diseased calves using sterilized rectal swabs and directly transferred to a lab for bacteriological examination.

Isolation and identification of *E. coli* strains

The swab contain fecal samples were inoculated in tryptic soy broth (mTSB- Difco La Jolla, CA, USA). Incubation of all samples were done at 37°C for 12 hours and then sub-cultured on a selective culture medium (MacConkey agar, MAC; Difco) at 37°C for one day. Lactose fermenting colonies that appeared in the plate were picked up and cultured in eosin methylene blue (EMB; Difco) medium. Metallic green colonies were considered *E. coli*. All isolates were recognized as *E. coli* according to their morphological features and gram staining. Moreover, several standard biochemical tests, including indole, citrate utilization, Voges-Proskauer methyl red, triple sugar iron agar (TSI), and urease tests, were used for *E. coli* confirmation as described by Cowan (1985). Serotyping of *E. coli* isolates were performed by slide agglutination test according to (Edwards and Ewing, 1972)

Antibiotic susceptibility tests

The vulnerability of *E. coli* strains to various antibiotics was determined using a Kirby–Bauer disk diffusion assay as described by the Clinical and Laboratory Standards Institute (CLSI, 2002). A suspension of the organism is prepared to equal the turbidity of a 0.5 McFarland standard (1.5 × 108 colony forming units (CFU) ml−1) .The isolates were screened, and the results were interpreted according to the CLSI. The subsequent commercial antibiotic discs (Oxoid) were utilized: tetracycline (TET), 30 mg; chloramphenicol (CHL), 30 mg; ampicillin (AMP), 10 mg; streptomycin (S), 10 mg; enrofloxacin (ENR), 5 mg; sulfamethoxazole/trimethoprim (SXT), 25 mg; gentamicin (GEN), 10 mg; cefaclor 30 mg (CEC); ciprofloxacin (CIP), 5 mg; and cefotaxime (CTX) 30 mg. The findings were recorded as susceptible, intermediate, or resistant in relation to the inhibitory zone diameter as interpreted by the CLSI.

Identification of antibiotic resistance genotypes

Extraction of DNA

DNA extraction was conducted by using QIAamp DNA Mini test kits (Qiagen, GmbH, Germany) according to the manufacturer's procedures.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions of antibiotics resistance genes of *E. coli*

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	(Colom et al., 2003)
blaTX	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	(Archambault et al., 2006)
Aada2	TGTTGGTTACTGTGGCCGTA GATCTCGCCTTTCACAAAGC	622	94˚C 5 min.	94˚C 30 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	(Walker et al., 2001)
tetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Randall et al., 2004)
Sul1	CGG CGT GGG CTA CCT GAA CG GCC GAT CGC GTG AAG TTC CG	433	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	(Ibekwe et al., 2011)
dfrA	TGGTAGCTATATCGAAGAATGGAGT TATGTTAGAGGCGAAGT CTTGGGTA	425	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	(Grape et al., 2007)

All *E. coli* strains were analyzed for six antibiotic resistance genes *bla*TEM (beta-lactams), *bla*CTX (Cephalosporin), *dfr*A (trimethoprim), tetA (tetracycline), *sul*1 (sulfonamides) and *aad*A2 (streptomycin) by PCR. Several PCR protocols were applied to investigate the target genes of the *E. coli* isolates. Lists of primers used, the PCR conditions, and the amplified products are presented in Tables 1.

All reactions were carried out on a PCR thermocycler (Applied Biosystems 2720). Amplification of DNA was conducted in a total volume of 25 µl including 12.5 µl of PCR Master Mix, 1 µl of each primer of 20-pmol concentration, 4.5 µl of purified water, and 6 µl of DNA template. The amplified products were subjected to gel electrophoresis (1.5% agarose), then stained with ethidium bromide and finally captured with a UV transilluminator.

RESULTS

Prevalence and clinical examination of diarrheic calves

In the present investigation, the frequency of diarrhea in the examined 200 calves was 37.5 % (75/200) from sporadic cases. Clinical examination of diseased calves revealed variable degree of profuse watery and mucoid diarrhea with a variable degree of dehydration that was detected by a decrease in skin elasticity, sunken eyes, cold extremities and inability to stand. All affected calves were depressed, had rapid pulse and respiratory rates and rough coats. Systemic reactions were observed in some of the affected animals although some cases were normal and subnormal body temperature. Bacteriological examination revealed that 16 out of 75 (21.33%) diarrheic calves were positive for *E.coli* isolation.

Serotyping identification of *E. coli* isolates and antibiotic sensitivity test

Sixteen *E. coli* strains were isolated from the feces of 75 diarrheic calves according to the morphological features and biochemical analysis. The serogroups of *E.coli* isolates reveled that O26 (25%) followed by O157 (18.75%), (12.5%) for (O78, O125, O146, O44) and O18 (6.25%). The identified *E. coli* strains demonstrated a high degree of resistance to ampicillin (81.25%), gentamycin (62.5%), sulfamethoxazole/trimethoprim (62.5%) and tetracycline (31.25%). Hence, all *E. coli* strains in our study had strong resistance to both streptomycin and cefaclor. Susceptibility was observed to ciprofloxacin (81.25%), followed by chloramphenicol (81.25%), enrofloxacin (75%), and cefotaxime (62.5%). In contrast, our results proved that the *E. coli* strains were highly vulnerable to chloramphenicol, enrofloxacin and cefotaxime, with incidence rates 81.25%, 75% and 62.5%, respectively.

Table (2): Distribution of phenotypic resistance and Antibiotic resistance genes genes among *E.Coli* serogrouping recovered from neonatal calves.

Isolate number	Serotype	Phenotypic resistance	Antibiotic resistance gene
1	O26	CN, S,AMP,TE, SXT, CEC	*bla* $_{TEM}$, *tet*A , *aad*A2,
2	O78	CN,S,ENR,AMP, TE, CEC	$bla_{TEM,}$ *tet*A,
3	O125	S,AMP, SXT, CEC	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, *drf*A
4	O26	S, AMP, TE, SXT,CEC	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1
5	O157	CN, CIP, S,AMP, SXT, CEC, CTX	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, *drf*A
6	O44	CN, CIP, S, AMP, SXT, CEC, C	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, *drf*A
7	O146	S, ENR, AMP, CEC, CTX,	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, *drf*A, bla_{CTX}
8	O146	CN, S, ENR, SXT, CEC	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, *drf*A, bla_{CTX}
9	O157	CN, S, ENR, AMP, CEC	$bla_{TEM,}$ *tet*A
10	O18	CIP, S, CTX, CEC	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, bla_{CTX}
11	O44	CIP, S, SXT, CEC	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, bla_{CTX}
12	157O	CN, S, AMP, TE, CEC, CTX, C	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, *drf*A
13	O125	CN, S, AMP, CEC, CTX	bla_{TEM} , *sul*1, *aad*A2 ,
14	O78	S, AMP,SXT, CEC, CTX	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, *drf*A, bla_{CTX}
15	O26	CN, S, AMP, SXT, CEC, CTX	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1, *drf*A
16	O26	S, AMP, SXT, CEC, CTX	$bla_{TEM,}$ *tet*A, *aad*A2, *sul*1

PCR results of antibiotic resistance genes in *E. coli* strains

All *E. coli* strains were examined for antibiotic resistance genes by using PCR assays. Based on the molecular screening of the antibiotic resistance genes, all tested *E. coli* isolates exhibited resistance to the *bla*TEM gene (100%) (Fig. 1A). Moreover, the genotypes that encode resistance to tetracycline (*tet*A), streptomycin (*aad*A2), sulfonamides (*sul*1) and trimethoprim (*drf*A) were the main resistance genes detected in the *E. coli* strains recovered from diarrheic calves, with an incidence rate of 93.75%, 87.5%, 81.25% and 43.75%, respectively (Fig. 1B-E), while the *bla*CTX gene was detected in five strains (31.25%) (Fig. 1F) of *E. coli* serotypes. The obtained results in table (2), showed that a strong relationship was observed between phenotypic resistance and antibiotic resistance genes among the screened *E.coli* serotypes.

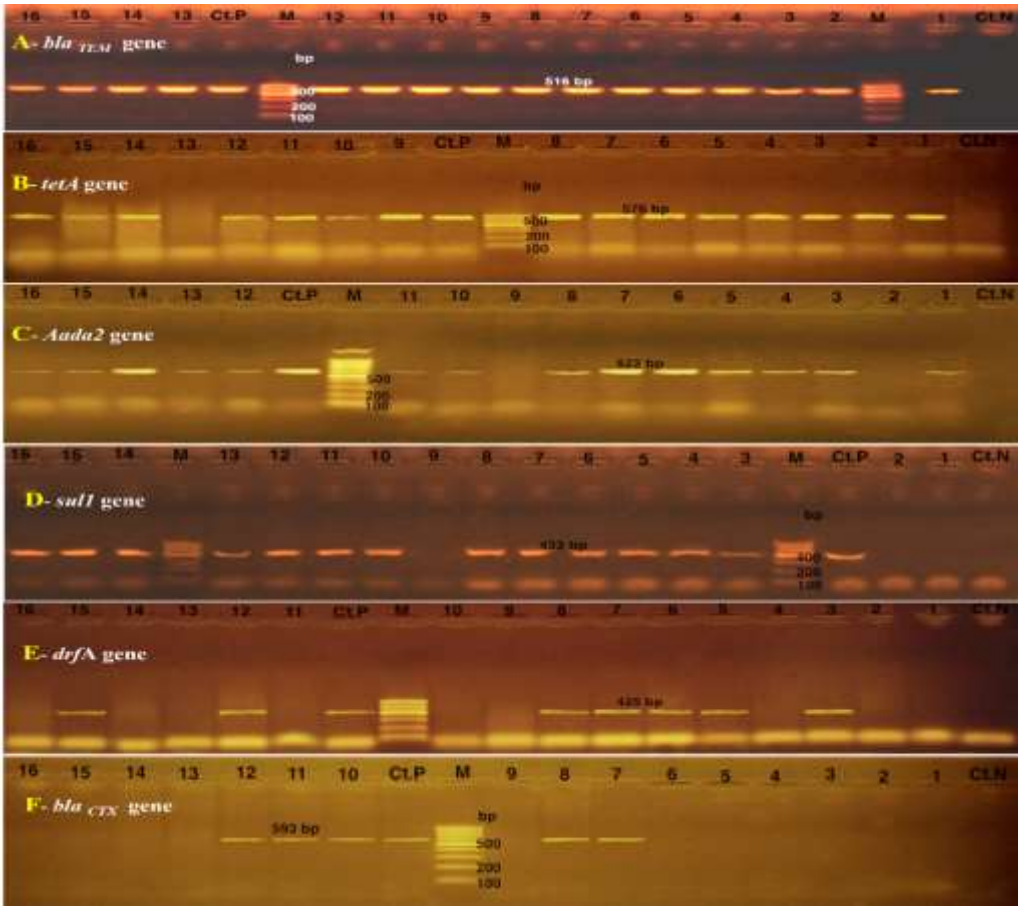

Figure 1: Uniplex PCR for the detection of 6 antibiotic resistance genes in 16 *E. coli* strains with 1.5% agarose gel electrophoresis.

(A) All *E. coli* strains (1-16) were positive for the *bla* $_{TEM}$ gene (516 bp). (B) All strains were positive for the *tet*A gene (576 bp) except lane 13. (C) Lanes 1, 3-8 and 10-16 were positive for the *aad*A*2* gene (622 bp) while lanes 2 and 9 were negative. (D) Lanes 3-8, 10-13 and 14-16 were positive for the *sul*1 gene (433 bp), while lanes 1, 2 and 9 were negative for this gene. (E) Lanes 3, 5, 6, 7, 8, 12 and 15 were positive for the *drf*A gene (425 bp), whereas lanes 1, 2, 4, 9, 10, 11, 13, 14 and 16 were negative. (F) Only five strains of *E. coli* were positive for the *bla* $_{CTX}$ gene at 593 bp (Lanes 7, 8, 10, 11 and 12), while 11 strains were negative (lanes 1, 2, 3, 4, 5, 6, 9, 13, 14, 15 and 16). Lane M: 100 bp DNA marker; lane Ct.N: Negative control; and lane Ct.P: Positive control.

DISCUSSION

Calf diarrhea is one of the principal enteric veterinary syndrome which was globally distrusted and causes high mortality and economic losses. This condition is caused by the interaction of several agents, including bacteria, viruses and other agents (Schroeder et al**.,** 2012). *E. coli* is one of the main etiological agents responsible for calf diarrhea (Nguyen et al., 2011). The present research investigated the prevalence of *E. coli* strains in neonatal diarrheic calves and studying their susceptibility to various antibiotics as well as detection of 6 antimicrobial resistance genes. In our study, 37.5% (75/200) of the calves were diarrheic, and 21.33% (16/75) of these harbored an *E. coli* strain. Similar results in Egypt were found by Galal et al., (2013), who reported an *E. coli* prevalence of 28.57%. In contrast, several studies reported higher prevalence of 50% (Osman et al., 2012) and 72.8% (Majueeb et al., 2014), while Anwarullah et al., (2014) reported a lower prevalence (14.6%). The variations in the results might be due to differences in the number of samples, regions and hygienic measurements.

The phenotypic characterization of *E. coli* isolates against 10 commonly used antimicrobials for the treatment of diarrheic calves was carried out. The results proved that the *E. coli* strains were highly vulnerable to chloramphenicol, enrofloxacin and cefotaxime, with incidence rates 81.25%, 75% and 62.5%, respectively, while high resistance was observed for ampicillin, gentamycin, sulfa/trimethoprim and tetracycline, with incidence rates 81.25%, 62.5%, 62.5% and 31.25%, respectively. Moreover, the resistance level of *E. coli* isolates to streptomycin and cefaclor was 100%. Similar results were obtained by Duse et al., (2015), who recorded high vulnerability of *E. coli* isolates to enrofloxacin and a higher resistance rate (100%) to streptomycin. Sawant et al., 2007 reported that *E. coli* strains showed strong resistance to ampicillin (48%) and tetracycline (93%), in agreement with our findings. In Egypt, similar findings confirmed that *E. coli* isolates expressed high sensitivity to enrofloxacin and tetracycline (Atwa et al., 2012). In contrast, these isolates exhibited higher degrees of resistance to ampicillin, gentamicin, erythromycin, lincomycin and penicillin-G. However, Abdulgayeid et al. (2015) studied the antimicrobial susceptibility pattern (ASP) of *E. coli* isolates from calves with diarrhea in Behera Province, Egypt, and found that the most susceptible antibiotics were cefotaxime, amoxicillin/clavulanate, and Linco-Spectin with degrees reaching 100%. Furthermore, high resistance levels were recorded against oxytetracycline and sulfamethoxazole/trimethoprim, with incidence rates of 91.6% and 50.5%, respectively. Moreover, Sato et al., (2005) demonstrated that the majority of *E. coli* strains in diarrheic calves showed high resistance to groups of antibiotics such as gentamycin, tetracycline, and ampicillin. In Bangladesh, the testing of 57 isolates of *E. coli* demonstrated a high resistance level to amoxicillin and tetracycline, with incidence rates of 59.65% and 61.40%, respectively (Islam et al., 2015), while in India, Balasubramaniam et al., (2014) illustrated that *E. coli* strains from clinical samples exhibited strong resistance (88%) to tetracycline.

Infection by E*. coli* often require antimicrobial therapy; however, a number of studies have demonstrated that the resistance of *E. coli* to antibiotics has increased over time (Solomakos et al., 2009). In the current investigation, the *bla*TEM, *tet*A, *aad*A2, *sul*1, *drf*A and *bla*CTX genes were the commonly detected antimicrobial resistance genes in the *E. coli* strains recovered from diarrheic calves, with incidence rates of 100%, 93.75%, 87.5%, 81.25%, 43.75% and 31.25%, respectively. Abdulgayeid et al. 2015 ; Balasubramaniam et al. (2014) revealed that the frequency of the *tet*A gene in *E. coli* strains of pre-weaning calves ranged from 84.2%-100%. In contrast, (Sawant et al., 2007) observed a lower frequency of this gene (7%). The *tet*A gene might be the prevalent resistance gene harbored in pathogenic *E. coli* isolated from dogs, although other tetracycline-resistant genes may express resistance via ribosomal activity and inactivation of enzymatic function (Torkan et al., 2015).

Recently, resistance to aminoglycosides and sulfonamides/trimethoprim in *E. coli* strains has been increasing. The results obtained by Shahrani et al., (2014) revealed that the *aadA1*, *sul1*, and *dfrA1-encoded genes* were the common resistance genes detected in STEC against streptomycin, sulfonamides and trimethoprim antimicrobials, respectively, and this explains the high resistance to streptomycin in our study. However, Szczepanowski et al., (2009) indicated that the aad(A1) and aac3-(IV) genes encoding resistance to streptomycin and gentamicin, respectively, were the most predominant genes in *E. coli* strains. Lower frequencies, 42.9% and 28.6%, were discovered in dogs suffering from diarrhea for the aad (A1) and aac3-(IV) genes, respectively. The high prevalence of the aad (A1) gene may be due to the frequent use of streptomycin in combination with penicillin for

wide-spectrum activity in the treatment of infectious bacterial diseases (Torkan et al., 2016). Interestingly, β-lactam antibiotics, especially ampicillin, have been widely used for the control of animal illnesses particularly caused by *E. coli* Van et al., (2008). Recently, the resistance of β-lactams among gram-negative bacteria is mediated by β-lactamase genes, which has led to the ineffectiveness of this antibiotic. The most commonly reported β-lactamase genes in *E. coli* from newborn calves with diarrhea in Egypt were CMY-, CTX-M-, OXA-, SHV- and TEM (Ahmed et al., 2009).

CONCLUSION

This study spot highlights on prevalence of E.coli as a major bacterial etiology of diarrhea in newborn calves and reported that both O26 and O157 were the predominant *E.coli* serogroups. Interestingly, *E. coli* strains showed higher resistance to various antibiotic groups such as β-lactamases, tetracycline, and aminoglycosides. Although, high susceptiblity was observed to chloramphenicol, enrofloxacin and cefotaxime which provide insight role of these antibiotics in treatment in veterinary field. Furthermore, most of *E.coli* carried several antimicrobial resistance genes such as *bla*TEM, *tet*A, *aad*A2, *sul*1, and *drf*A. The present study supposed a great relationship between antibigram profile and antimicrobial resistance genes which could be explain the severity of this problem that facing veterinarians. Further studies are required to investigate the substantial role and possible relationship between antbiogram pattern and resistance of *E.coli* strains to overcome this problem in the future.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contribute equally in this study.

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