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Molecular characterization of Enterotoxigenic *Escherichia coli* isolates harboring genetic elements mediating multiple-drug resistance

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ABSTRACT: Intensive antibiotics' use in the management of the disease in neonate calves, a major economic concern in bovine industry, is one of the contributors to high levels of antibiotic resistance of pathogenic bacteria. The objective of this study was to investigate the antibiotic resistance patterns and the frequency of integrons classes among Enterotoxigenic Escherichia coli (ETEC*)* strains isolated from neonatal calf diarrhea (NCD) in South of Iran. 412 recto-anal mucosal swabs from diarrheic calves were analyzed by biochemical fingerprinting and for virulence genes by polymerase chain reaction (PCR). The isolates were examined for their susceptibility to a panel of 8 antibacterial agents using the Kirby-Bauer disc diffusion method. Finally, the frequency of integron classes was detected in multi-drug resistant (MDR) strains by polymerase chain reaction restriction fragment length polymorphism (PCR- RFLP). 194 out of 412 (47.09%) diarrheic fecal samples harbored *E. coli* and 35 (18%) of them were identified as ETEC. The drug susceptibility test showed that all isolates were resistant to erythromycin, penicillin and trimethoprim/sulfamethoxazole and more than 80% were resistant to ampicillin and chloramphenicol. All isolates were MDR. 17 out of 35 (48.57%) isolates were identified possessed class 1 integron.
High prevalence of class 1 integron in ETEC isolates was mainly associated with multidrug resistance. Cefixime was the most effective antibiotic *in vitro*.

***Keywords*:** ETEC, Integron, MDR.

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INTRODUCTION

Neonatal calf diarrhea (NCD) leads to high mortality and morbidity in young calves (de Verdier et al., 2012). It is a disease characterized by varying degrees of diarrhea and dehydration (Ahmed et al**.,** 2009).

Escherichia coli strains normally colonizes the mammalian intestines, however there are some clones with potential to produce diarrheic infections in human or animals (Souto et al. 2017). Six pathotype of diarrheagenic *E. coli*, have been described, namely: enterotoxigenic *E. coli (*ETEC*),* enteropathogenic *E. coli (*EPEC*),* enterohemorrhagic (Shiga toxin-producing*) E.coli (*EHEC/STEC*),* enteroaggregative *E. coli (*EAEC*),* and enteroinvasive *E. coli (*EIEC*) and diffusely adherent E.coli DAEC)* (Umpiérrez, et al., 2016).

Among these *E. coli* pathogroups, *ETEC* strains are the most common cause of NCD which is one of the most difficult diseases to treat responding to only a few antimicrobial drugs

(Abraham et al., 2014). Its pathogenicity mechanism involves the attachment to the bovine intestinal microvilli via specific fimbrial adhesion factors conferring to ETEC the ability to attach receptors on the enterocytes and production of heat-stable enterotoxins (*STa*). The *STa* proteins are responsible for fluid hyper-secretion and diarrhea that are common causes of NCD (Oliver et al. 2016). Major virulence factors associated to calf ETEC isolates are the heat-stable enterotoxin (*STa*),as well as F5 (K99) and F41 fimbrial adhesins. The K99 fimbriae is plasmid-encoded, whereas F41 is chromosome-encoded. The plasmid harboring the K99 fimbrial gene also contains the gene for *STa* (Shams et al, 2012).

Antimicrobial agents in food-producing animals have been used either for animal treatment or for animal growing and fattening (Harada and Asai, 2010). This practice leads to the inevitable selection of antimicrobial resistance among pathogenic and commensal bacteria in the intestinal tracts of these animals, which could serve as important reservoirs for colonization and infection in human beings. Antibiotics have long been considered as the first line of defense to prevent pathogenic *E. coli* infections (Umpiérrez et al., 2016). The use of antimicrobials in the treatment of ETEC diarrhea is problematic, due to the rapid emergence and dissemination of antibiotic-resistant strains. Recent studies have shown that the level of antibiotic resistance among ETEC has steadily increased (Moredo et al. 2015). The rapid emergence of antibiotic resistance among bacteria is mainly due to horizontal transmission of antibiotic resistance genes via different types of mobile genetic elements, such as transposons, plasmids and integrons with which multi-drug resistance in *Enterotoxigenic Escherichia coli* is associated (Abraham et al., 2014). Integrons are capable of integrating or mobilizing single or multiple gene cassettes encoding antibiotic resistance determinants (El-Sokkary and Abdelmegeed., 2015).

An integron is mainly composed of an integrase enzyme (*IntI)* that is responsible for gene cassette integration, a recombination site (*attI*) which is the target of the enzyme, and a promoter that is located upstream of the integration site (Díaz-Mejía et al., 2008).

Based on the sequences of integrase genes (Yu et al., 2004), there are at least eight classes of integrons (Nield et al., 2001) which could be distinguished by their respective integrase (*int*) genes (White et al. 2001). Class 1 and 2 of integrons are the most frequently detected in many bacterial species that carry different arrangements of gene determinants related to antibiotic resistance (El-Sokkary and Abdelmegeed., 2015). Significant association of class 1 integrons, most commonly found in clinical isolates of Gram-negative bacteria, with MDR has been shown (Yu et al., 2004). The distribution of integron in multi-drug resistant *Escherichia coli* strains has been previously studied in many different countries among them Germany (Friedrich et al., 2010), India (Mathai et al. 2004), USA (Diekema et al., 2004), Spain (Oteo et al., 2005) and Sudan (Ibrahim et al., 2013).

However, no studies on the prevalence of integron classes in MDR isolates of ETEC in diarrheic calves have been published in Iran to date. Consequently, the goal of this study was to investigate the genotypic screening of virulence genes in ETEC from diarrhoeic calves and to assess the occurrence of multi-drug resistant, dissemination of different classes of integrons in MDR isolates.

MATERIALS AND METHODS

Sampling and clinical signs of the studied population

A total of 412 fresh fecal samples were collected with rectal swabs from 412 untreated diarrheic neonatal calves, within the age of up to 30 days old. The calves were characterized as suffering from NCD by sudden onset of profuse yellow/white diarrhea, leading to rapid and severe dehydration. The fecal swabs were collected within a period of one year, from

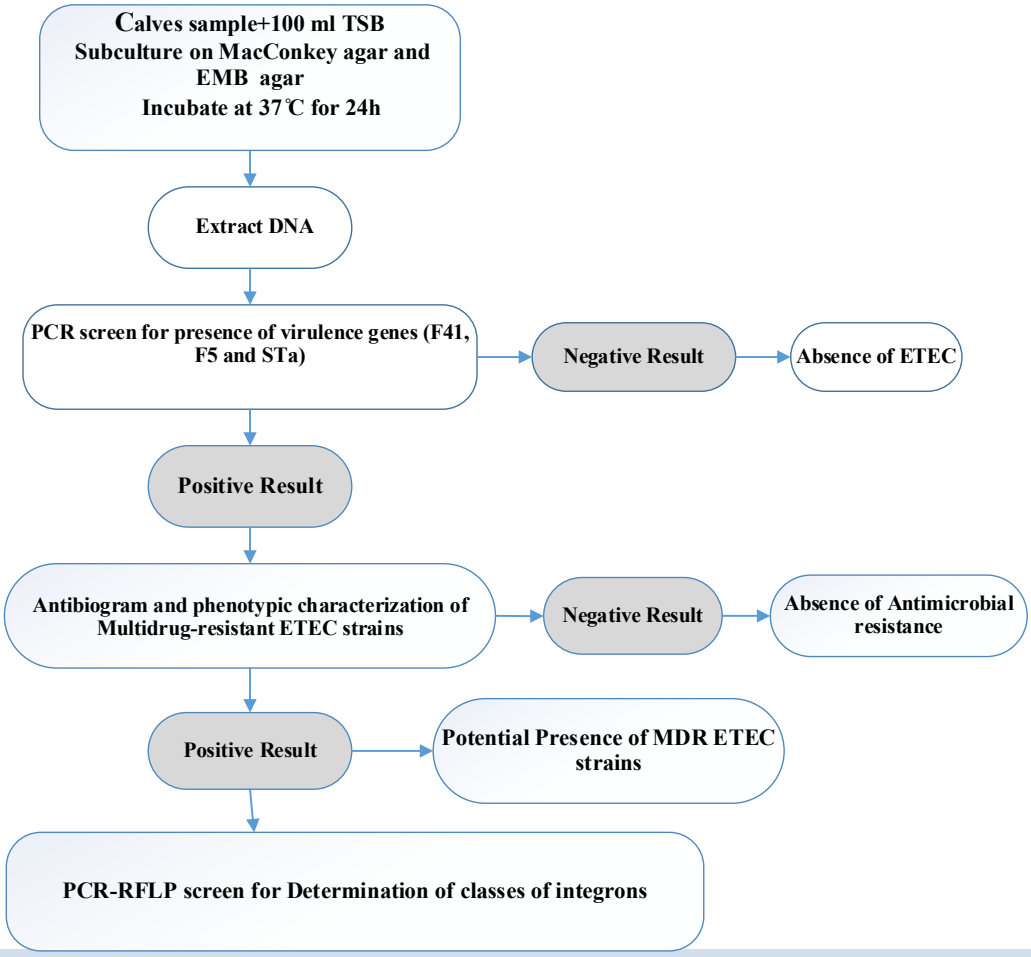

Figure 1. Preparation, screening and confirmation steps carried out for evaluating the multi drug resistant *ETEC* strains in samples

October 2016 to October 2017 from 25 farms belonging to six geographic areas of Fars province, Iran. The methods used in this study are summarized in Figure1.

Cultural and biochemical tests

A recto-anal mucosal swab sample from each diarrheic calf was collected and transferred to distinct tubes containing Tryptic Soy Broth (TSB) (Merk, Germany). The samples were transported within 6 hours to the main laboratory inside an ice box and subsequently incubated at 37°C for 18 hours. Then, the samples were subcultured on MacConkey agar and Eosin Methylene Blue (EMB) agar plates, at 37°C for 24–48 hours. The isolates were confirmed as *E. coli* using standard biochemical tests i.e. indole test (+ve) (Ehrlich's reagent) (Merk, Germany), oxidase test (−ve) (TMPD reagent) (Merk, Germany), urease (−ve) (Phenol red indicator) (Merk, Germany), Simon's citrate (−ve) (Bromothymol Blue, Reagent) and hydrogen sulfide (−ve) [(SO4 (NH4) Fe indicator] (Merk, Germany) (Atlas, 2010). The biochemically confirmed *E. coli* colonies were subjected to DNA analysis.

Bacterial DNA preparation for PCR

1ml of overnight mTSB culture from *E. coli* strains was employed to obtain template DNA for PCR. Bacterial cultures were pelleted at 3,000 rpm for 5 min (Hermle Z23o MA centrifuge) and then the SinaPure ™ DNA was used, as previously described by the provider (Sinaclon kit, Iran-Cat No.: EX6011). Extraction was performed according to the manufacturer's instructions. Briefly, bacterial pellet were lysed using lysis buffer then a precipitation solution was applied for 5 seconds, centrifuged, the supernatant recovered and precipitation solution was added. The sample mixture was then passed through a spin column, followed by two washes with wash buffer. The DNA was eluted in a volume of 200 µl of elution buffer, which was passed through the same column twice.

PRESENCE OF THE VIRULENCE GENES

Isolates were analyzed for 3 different genes encoding the virulence factors of *E. coli K99* (*F41*, *F5* and *STa*) by Multiplex PCR. Sizes of PCR products are shown in Table I. PCR was carried out in a 25-µl

reaction volume containing 10×PCR buffer (2.5 µl), MgCl2 (1.25 µl), dNTP (0.5 µl), primers (1 µl), DNA template (1 µl), Taq DNA polymerase (0.25 µl) with distilled water to reach 25 µL. The PCR protocol was as follows: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, and 1 cycle of 72°C for 7 min. DNA extracts from *E. coli* HB101 and RCCT 86 strains were used as negative and positive controls, respectively.

Antimicrobial susceptibility and multi drug resistance

Kirby–Bauer disk diffusion method was used to characterize the antibiotic sensitivity phenotypes of ETEC strains with standards and interpretive criteria of Clinical and Laboratory Standards Institute (CLSI) (Wayne 2012a). The following antibiotics (all obtained from Difco Laboratories, MI, U.S.A.) were used: ampicillin (AMP: 10 µg), tetracycline (TET: 30 µg), erythromycin (ERY: 25 µg), enrofloxacine (ENR: 10 µg), trimethoprim/sulfamethoxazole (SXT: 30µg), chloramphenicol (CHL: 30 µg), penicillin (PCN: 10 µ g), cefixime (CFM: 5µg).The inhibition zones were measured to the nearest millimeter and according to CLSI guidelines, where available (Wayne 2012b), the bacterial isolates were classified as: intermediate (I), resistant (R) or susceptible (S). Due to the small number of isolates with intermediate susceptibility, they were considered susceptible, for practical purposes. As multi-drug resistance phenotype was defined the simultaneous resistance to three or more categories of antibiotic agents (Magiorakos et al., 2012). *E. coli,* ATCC 25922 (beta-lactamase negative, sensitive to all these drugs), recommended by the Clinical and Laboratory Standards Institute (CLSI) was used for a quality control. Quality control result was within specified range as published in M100-S22 (Wayne 2012b).

Presence of integrons

To detect integron in confirmed ETEC isolates, a PCR protocol was performed. The base sequences and sizes of the amplified products for the specific oligonucleotide primers are shown in Table 1. The PCR reaction mixture included 1 µL of each primer, 0.25 µLTaq polymerase, 1.25 µL MgCl2, 1 µL dNTPs, 10×PCR buffer (2.5 µl), 1 µL of template DNA and nuclease free water to complete the reaction volume (25 µl). PCR amplification was performed in thermo-cycler (Eppendorf Mastercycler, Germany) under the following conditions: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec, and 1 cycle of 72°C for 7 min. DNA extracts from *E. coli* 96K062 and ATCC 25922 strains were used as positive and negative controls, respectively.

Restriction Fragments Length Polymorphism (RFLP)

Determination of classes of integrons was done by RFLP analysis of integrase PCR products. The restriction reaction was performed using RsaI (Thermo Fisher) restriction enzyme (White et al., 2001). Based on manufacturer's recommendations, the PCR product (7 µl) was digested by the addition of 19 µl of double-distilled water, 3 µl of 10X buffer, and 1 µl of restriction enzyme, and incubated at 37°C for 1 hour. The digested PCR products were fractionated by gel electrophoresis (3 % agarose gel). Table 2 shows the size and number of generated fragments..

Table 1. Primers and PCR conditions used in this study.

Primers	Oligonucleotide sequence(5'-3')	Product size (bp)	References
F5(K99)	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314	(Shams et al, 2012)
F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAG TATTATCACCT	380	(Shams et al, 2012)
STa	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	190	(Shams et al, 2012)
Intg	TGCGGGTYAARGATBTKGATTT * CARCACATGCGTRTARAT	491	(White et al, 2001)

R=A or G , Y = C or T, B=C or G or T, K=G or T *

Table 2. RFLP classification of integrase PCR products.

product PCR	Enzyme	No. of fragments	Fragment size(s) (bp)	References
IntI1	RsaI	1	491	(White et al, 2001)
IntI2	RsaI	2	300,191	(White et al, 2001)
IntI3	RsaI	2	119,327	(White et al, 2001)

Data analysis

The frequencies of resistance to particular antimicrobials in integron-positive and -negative isolates were compared with Fisher's exact test. The level for statistical significance was <0.05.

Statistical calculations were made with GraphPad Prism5 for Windows Edition (GraphPad Software, SanDiego, CA, USA).

RESULTS

ETEC isolates

194 out of 412 isolates collected fecal samples from diarrheic calves (47%) were identified as *E. coli* . Multiplex PCR identified two fibrial genes (*F5* and *F41*) previously associated with colonization of the bovine intestinal epithelium and a *STa* toxin gene which lead to fluid secretion. 35 out of 194 *E. coli* isolates examined by PCR (18%) were tested positive for *f5, f41* and *sta* (*E. coli* K99 strains) (Table 3).

Table 3. Overview of the integron-positive *ETEC* strains.

Resistance phenotypes	MDR	Integron class	Virulence gene
CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
CHL- ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-ERY- ENR -SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-CFM-ERY-SXT-PCN-TET	+	1	*F5*, *F41*, *STa*
ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-CFM-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY- ENR-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-ERY-PCN-SXT	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY-NFX-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*

F5, F41, Sta: isolates carrying *F5, F41* and *STa* genes. MDR: multidrug-resistance isolates, S: Antibiotic susceptible isolates,R: Antibiotic resistant isolates.
AMP= ampicillin, TET = tetracycline, ERY= erythromycin, ENR = enrofeloxacin, SXT = trimethoprim/sulfamethoxazole, CHL =chloramphenicol, PCN = penicillin, CFM= cefixime.

Table 4. Association between integrons and antibiotics resistance in 35 multidrug-resistant isolates
Results are shown as the percentage resistant (% R), with the number of isolates (n) in parentheses

Antibiotic	Resistance integron PCR-RFLP positive		Resistance integron -PCR-RFLP negative		Total Resistance		Associatio with integron
	No.	%	No.	%	No.	%	
Ampicillin	13	37.1	18	51.4	31	88.5	0.312
cefixim	2	5.7	2	5.7	4	11.4	1.000
Chloramphenicol	13	37.1	16	45.7	29	82.8	0.401
trimethoprim/ sulfamethoxazole	17	48.5	18	51.5	35	100	1.000
enrofeloxacin	16	45.7	9	25.7	25	71.4	0.003*
erythromycin	17	48.5	18	51.5	35	100	1.000
Penicillin	17	48.5	18	51.5	35	100	1.000
tetracycline	16	45.7	10	28.5	26	74.2	0.017*

*, Correlation is significant at the 0.05 level.

Antibiotic resistance profiles

Table 4 shows the antimicrobial resistance profile among the 35 isolates of ETEC analyzed by the Kirby– Bauer disk diffusion method. Among the drugs under the study, all ETEC isolates were resistant to erythromycin, trimethoprim/sulfamethoxazole and penicillin. Susceptibility testing of ETEC revealed that 31 of 35 isolates (88.5%) were resistant to ampicillin, 29 (82.8%) to chloramphenicol, 26 (74.2%) to tetracycline, 25(71.4%) were resistant to enrofloxacine and 4 (11.4%) to cefixime .

PCR-RFLP analysis

17 out of 35 isolates (48.75%) were integron positive (Table 4) and RFLP analysis revealed that 100% of them contained class 1 integron (Figure2).

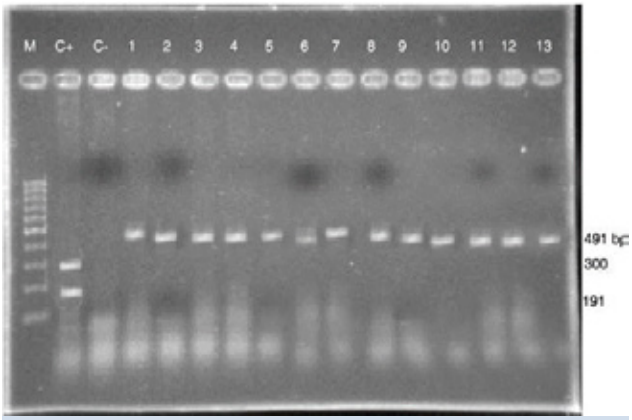

Figure 2. PCR-RFLP of integrase gene products. LanesC -,C+: negative and positive control (A 491 bp PCR product for *intI2* possessed a restriction site for *Ras1*, which was digested into 327 bp and 119 bp)
Lane1-13: PCR product of conserve of integrase; Lane2: RsaI treated of amplified products represent class1 integrons; M: molecular marker (100 base pair Ladder)

DISCUSSION

In pathogen detection systems, *E. coli* pathotypes, remarkably EHEC and ETEC isolates, had been identified by phenotypic methods for many years, including O- and H- serotyping and bacteriophage typing. These traditional techniques are generally time consuming and not always accurate. Therefore, they have been replaced by molecular-based techniques that have made it possible to more accurately assign the isolates to each pathotype based on their virulence-associated genes (Prapasawat et al. 2017). Accordingly, the K99, F41 fimbriae, and STa toxin genes were used in the present study for molecular identification of isolates. In the present study, among 194 *E. coli* strains from diarrheic calves, 35(18%) were positive for ETEC. The prevalence of ETEC coincided with the findings of another study in Tanzania (Lindblom et al. 1995); however, is higher than the 10.36% previously described by Younis *et al*., (2009) and the 5.8 % described by Yadegari *et al*. (2019). Conversely, it was lower than the 70%, 57.6% and 40% reported by El-Seedy *et al*.,(2016), Zhang et al. (2007) and Acha *et al*. (2004), respectively.

Most of the epidemiological studies in Iran have revealed that the prevalence of ETEC infection in diarrhoeic calves ranges between 5.3% and 28% (Shams et al., 2012; Pourtaghi-Shotorban et al., 2011; Shahrani et al., 2014). The differences of the prevalence rates of ETEC in diarrheic calves among different studies can be accounted to geographical locations and hygienic measures as well as management practice (El-Seedy et al., 2016).

In this study, high incidence of multi-drug resistant strains was detected among the ETEC isolates from diarrheic calves. Multidrug resistance (MDR) level among ETEC strains isolated from diarrhoeic calves varies in different countries. In previous studies conducted in Spain, 15% (Orden et al. 2002) and in another study from Australia, 87% of the isolates had MDR (Barigye et al., 2010). In addition, MDR was 10.4% in Egypt (Ahmed *et al*. 2009). Moreover, in our study MDR ETEC isolates were highly resistant to penicillin (100%) and high-level of resistance to penicillin among ETEC strains isolated from diarrhoeic calves has also been documented in Australia and Iran (Barigye et al., 2010; Shahrani et al., 2014).

In the present study, most of ETEC isolates were resistant to tetracycline (71.1%). High resistance of ETEC strains to this antibiotic has been reported in previous studies in Thailand (Prapasawat., 2017), Canada (Maynard *et al*., 2003, Boerlin *et al*., 2005), Iran (Shahrani *et al*., 2014)), where resistance to TET was 96%, 93%, 96%, and 100% respectively. A high percentage (88.5%) of ETEC isolates exhibited resistance to ampicillin nearly similar to the survey of Rusheeba *et al*., (86%) in India (Rusheeba *et al*., 2015), yet higher than other study in Sweden (31%) (de Verdier *et al*., 2012) whilst lower than that reported in Thailand (100%) by Prapasawat (2017).

These various levels of resistance to different classes of antibiotics among ETEC suggest a direct relationship between the percentages of resistance in different parts of the world and the prevalence of antibiotic consumption in each country (Kargar et al., 2014). Class 1 integrons are found embedded in transposons and conjugative plasmids, allowing their rapid dissemination via lateral gene transfer. As a con-

sequence, class 1 integrons have spread to nearly all species of Gram-negative pathogens.

Some reports indicate that the presence of class 1 integrons among intestinal bacteria such as *E.coli* is associated with MDR (Shahrani et al., 2014). Class 1 integrons that seems to be the most frequent of these genetic elements among commensals and pathogens isolated from livestock as well as among isolates cultured from clinical cases (Kohansal and Asad., 2018).

Previous studies have illustrated the wide distribution of class1 integrons in *E. coli* isolated from animals; 63% of isolates from chickens (Bass et al. 1999), 82% also from chickens (Keyes et al. 2000), 64% of swine diarrhea isolates (Kang et al. 2005), and 59% from calf diarrhea isolates (Du et al. 2005). In this study, the frequency of integrons was estimated as 48.75%. Only integron class 1 was detected. Other researches revealed the prevalence of class 1 integron in ETEC isolates as: 10.4% in Egypt (Ahmed et al., 2009), 60% in Canada (Maynard et al., 2003), 33% in Thailand (Prapasawat *et al*., 2017) and 68.6% in Australia (Abraham et al., 2014). These reports together with our findings emphasize the worldwide distribution of class 1 integrons among intestinal bacteria in food-producing animals. In these isolates class 1 integrons were associated with a variety of resistance gene cassettes, which encode resistance to different antibiotics.

We also detected a significant relationship between class 1 integrons and resistance to tetracycline and enrofloxacine (Table 4). Resistance to these antibiotics are probably attributable to embedded resistance gene cassettes within the integrons. Previous studies demonstrated that the presence of integrons is closely related to gene cassettes encoding_resistance to quinolones and β-lactam antibiotics (Prapasawat *et al*., 2017; Kargar *et al*., 2014).

CONCLUSION

Antibiotic resistance is common in pathogenic *E. coli* isolated from calves of Fars province experiencing problems with neonatal diarrhea. Our data demonstrate the presence of multiple drug resistance and class 1 integrons that can be easily dispersed among other bacteria, resulting in the rapid spread of antibiotic resistance genes. Clonal spread could not be the only reason for class 1 integron prevalence in different sources and the bigger player in this prevalence is probably the horizontal transfer with conjugative plasmids. Cefixime can serve as the drug of choice for treatment of multi resistant ETEC in calves with NCD. Therefore, it is advised to stop routine antimicrobial treatment, and test for antibiotic susceptibility as well as the sequential analysis of class 1 integrons in ETEC of fecal samples.

CONFLICTS OF INTEREST

The authors declare having no conflict of interests with this study.

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