

Journal of the Hellenic Veterinary Medical Society

Vol 71, No 2 (2020)



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

M. KOHANSAL, A. NAJAFI, R. VIDAL

doi: [10.12681/jhvms.24164](https://doi.org/10.12681/jhvms.24164)

Copyright © 2020, M. KOHANSAL, A. NAJAFI, R. VIDAL



This work is licensed under a [Creative Commons Attribution-NonCommercial 4.0](https://creativecommons.org/licenses/by-nc/4.0/).

To cite this article:

KOHANSAL, M., NAJAFI, A., & VIDAL, R. (2020). Molecular characterization of Enterotoxigenic Escherichia coli isolates harboring genetic elements mediating multiple-drug resistance. *Journal of the Hellenic Veterinary Medical Society*, 71(2), 2193–2200. <https://doi.org/10.12681/jhvms.24164>

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

M. Kohansal^{1,2}, A. Najafi³, R. Vidal⁴

¹Department of Medical Genetics, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran.

²Department of Biology, Payame Noor University, Tehran, Iran.

³Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran.

⁴Disciplinary Program of Microbiology, Institute of Biomedical Sciences, Faculty of Medicine, Universidad de Chile, Santiago, Chile

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.

Corresponding Author:

M. Kohansal, Department of medical genetics, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran., Department of biology, Payame Noor University, Tehran, Iran.

E-mail address: k_kohansal@yahoo.com

Date of initial submission: 09-09-2019

Date of revised submission: 30-01-2020

Date of acceptance: 26-03-2020

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 *differently 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

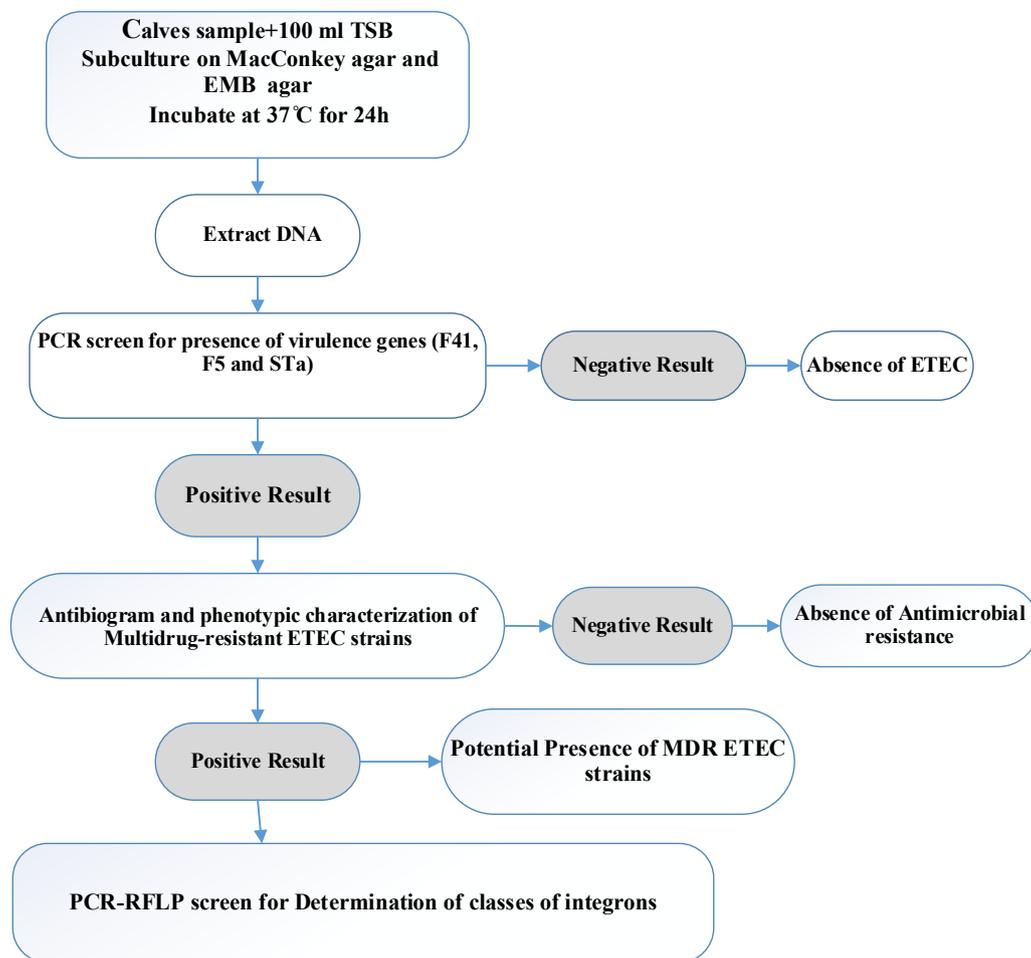


Figure 1. Preparation, screening and confirmation steps carried out for evaluating the multi drug resistant *ETEC* strains in samples

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 Figure 1.

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 Z230 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), enrofloxacin (ENR: 10 µg), trimethoprim/sulfamethoxazole (SXT: 30µg), chloramphenicol (CHL: 30 µg), penicillin (PCN: 10 µg), 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 Labo-

ratory 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 µL Taq 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	<i>F5, F41, STa</i>
CHL- ERY-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-ERY-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
CHL-ERY-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-ERY- ENR -SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-CFM-ERY-SXT-PCN-TET	+	1	<i>F5, F41, STa</i>
ERY-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-ERY-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-CFM-ERY-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-ERY-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-ERY- ENR-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-ERY-PCN-SXT	+	1	<i>F5, F41, STa</i>
AMP-CHL-ERY-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>
AMP-CHL-ERY-NFX-PCN-SXT-TET	+	1	<i>F5, F41, STa</i>

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 = enrofloxacin, 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
enrofloxacin	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 enrofloxacin 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).

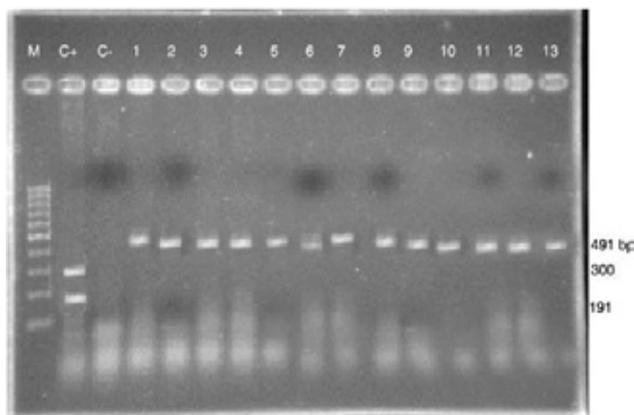


Figure 2. PCR-RFLP of integrase gene products. Lanes C -,C+: negative and positive control (A 491 bp PCR product for *intI2* possessed a restriction site for *RsaI*, which was digested into 327 bp and 119 bp)

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 class 1 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 enrofloxacin (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.

REFERENCES

- Abraham Sam, Darren J. Trott, David Jordan, David M. Gordon, Mitchell D. Groves, John M. Fairbrother, Matthew G. Smith, Ren Zhang, and Toni A. Chapman (2014) Phylogenetic and molecular insights into the evolution of multidrug-resistant porcine enterotoxigenic *Escherichia coli* in Australia. *Int J Antimicrob Agents*, 44: 105-111.
- Acha S J, Kühn I, Jonsson P, Mbazima, G, Katouli M, Möllby R (2004) Studies on calf diarrhoea in Mozambique: prevalence of bacterial pathogens. *J Appl Microbiol*, 45(1), 27.
- Acres S D (1985) Enterotoxigenic *Escherichia coli* infections in newborn calves: a review. *J. Dairy Sci.* 68(1), 229-256.
- Ahmed AM, Younis E E, Osman SA, Ishida Y, El-khodery SA, Shimamoto T (2009) Genetic analysis of antimicrobial resistance in *Escherichia coli* isolated from diarrheic neonatal calves. *Vet Microbiol*, 136, 397-402. doi: 10.1016/j.vetmic.2008.11.021.
- Atlas RM (2010) Handbook of microbiological media. 4th ed. Boca Raton, USA: CRC Press.
- Azam H, Ghezeljeh S M, Mahmoud S (2015) Prevalence of class 1 and 2 integrons among the multidrug resistant uropathogenic strains of *Escherichia coli*. *Asian Biomed*, 9:49-54. doi: 10.5372/1905-7415.0901.367.
- Backx A, Smith HE, Essen van A, Veldman KT, Mevius DJ (2006) Characterization of integrons in *E. coli* isolates of Dutch veal calves and dairy cows. *ISVEE*, 11:724-730.
- Bakhshi B, Najibi S, Sepehri-Seresht S (2014) Molecular characterization of enterohemorrhagic *Escherichia coli* isolates from cattle. *J Vet Med Sci*, 76:1195-1199. doi:10.1292/jvms.13-0237.
- Barigye R, Ablesh G, Lisa MP, Lynn PS, Darlene F (2012) Krogh, and Susan Olet. Prevalence and antimicrobial susceptibility of virulent and avirulent multidrug-resistant *Escherichia coli* isolated from diarrheic neonatal calves. *Am J Vet Res*, 12: 1944-1950.
- Bass L, Liebert CA, Lee MD, Summers AO, White DG, Thayer SG, Maurer J J (1999) Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrob Agents Ch.* 43:2925-9.
- Bewick V, Cheek L, Ball J (2003) Statistics review 8: Qualitative data—tests of association. *Critical care* (Crit Care), 30:8:46. doi: 10.1186/cc2428
- Boerlin P, Travis R, Gyles CL, Reid-Smith R, Lim NJ, Nicholson V, McEwen SA, Friendship R, Archambault M (2005) Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *AEM*. 71: 6753-6761.
- Cameron A, McAllister TA (2016) Antimicrobial usage and resistance in beef production. *J Anim Sci Biotechnol*. 2016 Dec;7:68.
- de Verdier K, Nyman A, Greko C, Bengtsson B (2012) Antimicrobial resistance and virulence factors in *Escherichia coli* from Swedish dairy calves. *Acta Vet Scand*. 54: 2.
- Diaz-Mejia J J, Amabile-Cuevas CF, Rosas I, Souza V (2008) An analysis of the evolutionary relationships of integron integrases, with emphasis on the prevalence of class 1 integrons in *Escherichia coli* isolates from clinical and environmental origins. *Microbiology*, 154: 94-102. doi: 10.1099/mic.0.2007/008649-0.
- Diekema DJ, BootsMiller BJ, Vaughn TE, Woolson RF, Yankey JW, Ernst EJ, Flach SD, Ward MM, Francisus CL, Pfaller MA, Doebbeling BN 2004 Antimicrobial resistance trends and outbreak frequency in United States hospitals. *Clin Infect Dis*: 38: 78-85.
- Du X, Shen Z, Wu B, Xia S, Shen J (2005) Characterization of class 1 integrons-mediated antibiotic resistance among calf pathogenic *Escherichia coli*. *FEMS Microbiol Lett*. 2005;245:295-8. doi: 10.1016/j.femsle.03.021.
- El-Seedy F R, Abed A H, Yanni H A, El-Rahman S A (2016) Prevalence of

- Salmonella and *E. coli* in neonatal diarrheic calves. *BUJAS*. 31;5:45-51. doi:10.1016/j.bjbas.2015.11.010.
- El-Sokkary M M A, Abdelmegeed ES (2015) Characterisation of class 1 integron among *Escherichia coli* isolated from Mansoura University Hospitals in Egypt. *Adv Microbiol*.5: 269-277. doi: 10.4236/aim.2015.54025.
- Friedrich T, Sven Rahmann S, Weigel W, Rabsch W, Fruth A, Ron E, Gunzer F, Dandekar T, Hacker J, Müller T, Dobrindt U (2010) High-throughput microarray technology in diagnostics of enterobacteria based on genomewide probe selection and regression analysis. *BMC Genomic*, 11: 591-612.
- Guerra B, Junker E, Schroeter A, Malorny B, Lehmann S, Helmuth R (2003) Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *J Antimicrob Chemother*. 52:489-92. doi: 10.1093/jac/dkg362.
- Gulsun S, Oguzoglu N, Inan A, Ceran N (2005) The virulence factors and antibiotic sensitivities of *Escherichia coli* isolated from recurrent urinary tract infections. *Saudi Med J*. :26:1755-8.
- Gular L, Gündüz K, Ok Ü (2008) Virulence factors and antimicrobial susceptibility of *Escherichia coli* isolated from calves in Turkey. *Zoonoses Public Health*. 55:249-57.
- Harada K, Asai T (2010) Role of antimicrobial selective pressure and secondary factors on antimicrobial resistance prevalence in *Escherichia coli* from food-producing animals in Japan. *J Biomed Biotechnol*. 180682. doi: 10.1155/2010/180682.
- Hariharan, H, Coles, M, Poole D, Page R (2004) Antibiotic resistance among enterotoxigenic *Escherichia coli* from piglets and calves from piglets and calves with diarrhea. *Can Vet J*. 45:605.
- Ibrahim ME, Magzoub M A, Bilal NE, Hamid M E (2013) Distribution of Class I integrons and their effect on the prevalence of multi-drug resistant *Escherichia coli* clinical isolates from Sudan. *Saudi Med J*, 34: 240-7.
- Kang S, Lee D, Shin S, Ahn J, Yoo H (2005) Changes in patterns of antimicrobial susceptibility and class 1 integron carriage among *Escherichia coli* isolates. *J Vet Sci*. 6:201-5
- Karczmarczyk M, Walsh C, Slowey R, Leonard N, Fanning S (2011) Molecular characterization of multidrug-resistant *Escherichia coli* isolates from Irish cattle farms. *Appl Environ Microbiol*. 77(20):7121-7. doi: 10.1128/AEM.00601-11.
- Kargar M, Mohammadipour Z, Doosti A, Lorzadeh S, Japoni-Nejad A (2014) High prevalence of class 1 to 3 integrons among multidrug-resistant diarrheagenic *Escherichia coli* in southwest of Iran. *Osong Public Health Res*, 5: 193-198.
- Keyes K, Hudson C, Maurer J J, Thayer S, White DG, Lee M D (2000) Detection of Florfenicol Resistance Genes in *Escherichia coli* Isolated from Sick Chickens. *Antimicrob agents ch*, 44: 421-424.
- Kohansal, M., & Asad, A. G. (2018). Molecular analysis of Shiga toxin-producing *Escherichia coli* O157: H7 and non-O157 strains isolated from calves. *Onderstepoort Journal of Veterinary Research*, 85(1), 1-7.
- Koneman EW, Allen SD, Janda W M, Schreckenberger P C, Microbiology (1997) In *Color Atlas and Textbook of Diagnostic Microbiology*; 5th edn., pp 983–1057 Philadelphia, PA: Lippincott Williams & Wilkins.
- Lindblom G.B, Åhrén C, Changalucha J, Gabone R, Kaijser B, Nilsson LÅ, Sjögren E, Svennerholm AM, Temu M (1995) *Campylobacter jejuni*/coli and Enterotoxigenic *Escherichia coli* (ETEC) in Faeces from Children and Adults in Tanzania. *Scand J Infect Dis*, 27: pp.589-593.
- Levin BR, Lipsitch M, Perrot V, Schrag S, Antia R, Simonsen L, Moore Walker N, Stewart FM, (1997) The population genetics of antibiotic resistance. *Clin Infect Dis*. 24 Suppl 1:S9-16.
- Rusheeba M, Shah MI, Wani SA, Pandit F, Ahmad Dar P, Iqbal Mir M (2015) Prevalence, serodiversity and antibiogram of enterotoxigenic *Escherichia coli* (ETEC) in diarrhoeic calves and lambs of Kashmir valley (J&K), India. *IJANS*. 7: 477-481.
- Mathai, E, Grape M, Kronval G (2004) Integrons and multidrug resistance among *Escherichia coli* causing community-acquired urinary tract infection in southern India. *Apmis*, 112: 159-164.
- Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *CMI*. 18:268-81.
- Maynard C, Fairbrother JM, Bekal S, Sanschagrín F, Levesque RC, Brousseau R, Masson L, Larivière S, Harel J (2003) Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149: K91 isolates obtained over a 23-year period from pigs. *Antimicrob agents Ch*. Oct 1;47(10):3214-21.
- Moredo FA, Pineyro PE, Márquez GC, Sanz M, Colello R, Etcheverría A, Padola NL, Quiroga MA, Perfumo CJ, Galli L, Leotta GA (2015) Enterotoxigenic *Escherichia coli* subclinical infection in pigs: bacteriological and genotypic characterization and antimicrobial resistance profiles. *Foodborne pathogens dis*. Aug 1;12:704-11.
- Nield B S, Holmes A J, Gillings MR, Recchia GD, Mabbutt BC, Nevalainen K H, Stokes HW (2001) Recovery of new integron classes from environmental DNA. *FEMS Microbiol Lett*, 195: 59-65.
- Orden J A, Cid D, Ruiz-Santa-Quiteria JA, García S, Martínez S, De la Fuente R (2002). Verotoxin-producing *Escherichia coli* (VTEC), enteropathogenic *E. coli* (EPEC) and necrotoxicogenic *E. coli* (NTEC) isolated from healthy cattle in Spain. *J Appl Microbiol*, 93: 29-35.
- Oteo J, Lázaro E, de Abajo FJ, Baquero F, Campos J, members of EARSS S. Antimicrobial-resistant invasive *Escherichia coli*, Spain. *Emerg Infect Dis*, 2005 Apr;11(4):546.
- Prapasawat W, Intarapuk A, Chompook P, Nakajima C, Suzuki Y, Suthienkul O (2017) Antimicrobial resistance, integron, virulence gene, and multilocus sequence typing of *Escherichia coli* isolates from postweaning piglets with and without. *Se Asian J Trop Med*, 48: pp.1042-1055.
- Prism G. 2005. 5. GraphPad Software. San Diego, CA, USA.
- Pourtaghi-Shotorban H, Dahpalehvan V, Shaghayegh A, Badiie A (2011) Molecular detection of diarrheagenic enterotoxigenic *Escherichia coli* (ETEC) from calves fecal samples in Alborz province. 2011. *J.et.Clin. Res* 2: 33-38
- Rijavec M, Erjavec MS, Avguštin JA, Reissbrodt R, Fruth A, Krizan-Hergouth V, *Zgur-Bertok D* (2006) High prevalence of multidrug resistance and random distribution of mobile genetic elements among uropathogenic *Escherichia coli* (UPEC) of the four major phylogenetic groups. *Curr Microbiol*. Aug 1;53:158-62.
- Sahm D F, Thornsberrry C, Mayfield DC, Jones M E, Karlowsky JA (2001) Multidrug-Resistant Urinary Tract Isolates of *Escherichia coli*: Prevalence and Patient Demographics in the United States in 2000. *Antimicrob agents ch*, 45: 1402-1406.
- Shams Z, Tahamtan Y, Pourbakhsh A, Hosseini MH, Kargar M, Hayati M (2012) Detection of enterotoxigenic K99 (F5) and F41 from fecal sample of calves by molecular and serological methods. *Comp Clin Path*. 2012 Aug 1;21:475-8.
- Shahrani M, Dehkordi FS, Momtaz H (2014) Characterization of *Escherichia coli* virulence genes, pathotypes and antibiotic resistance properties in diarrheic calves in Iran. *Biological Research*. 2014 Dec;47:28.
- Shaheen BW, Oyarzabal OA, Boothe DM (2010) The role of class 1 and 2 integrons in mediating antimicrobial resistance among canine and feline clinical *E. coli* isolates from the US. *Vet Microbiol*. Aug 26;144:363-70.
- Souto MS, Coura FM, Dorneles EM, Styne AP, Alves TM, Santana JA, Pauletti RB, Guedes RM, Viott AM, Heinemann MB, Lage AP (2017) Antimicrobial susceptibility and phylotyping profile of pathogenic *Escherichia coli* and *Salmonella enterica* isolates from calves and pigs in Minas Gerais, Brazil. *Trop anim health pro*. Jan 1;49:13-23.
- Tahamtan Y, Fozzi MA, Hosseini MH, Mostafaei T (2014) Molecular characterization of class 1 integrons and antibiotic resistance to *E. coli* from cattle, sheep and goats. *OJVR*. 18:701-9.
- Umpiérrez A, Acquistapace S, Fernández S, Oliver M, Acuña P, Reolón E, Zunino P (2016) Prevalence of *Escherichia coli* adhesion-related genes in neonatal calf diarrhea in Uruguay. *J Infect Dev Ctries*, 10, 472-477.
- van den Bogaard AE, Stobberingh EE (2000) Epidemiology of resistance to antibiotics: links between animals and humans. *Int J of antimicrob ag*, 14, 327-335.(2013):
- Van Meervenne E, Boon N, Verstraete K, Devlieghere F, De Reu K, Herman L, Buvens G, Piérard D, Van Coillie E (2013) Integron characterization and typing of Shiga toxin-producing *Escherichia coli* isolates in Belgium. *J medical microbiol*. 62(5), pp.712-719.
- Ventola CL (2015) The antibiotic resistance crisis: part 1: causes and threats. *IJPT*. 40(4):277-283.
- Wani SA, Hussain I, Beg SA, Rather MA, Kabli ZA, Mir MA, Nishikawa Y (2013) Diarrhoeagenic *Escherichia coli* and salmonellae in calves and lambs in Kashmir: absence, prevalence and antibiogram. *Rev sci tech Off int Epiz*. Dec 1;32:1-7.
- Wayne PA. (2012)a. Clinical and laboratory standards . Performance standards for antimicrobial disk susceptibility tests; approved standard. *Approve Standard M02-A11*. CLSI, 11th ed. PA, USA.
- Wayne PA. (2012)b. Clinical and laboratory standards institute methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *Approve Standard M07-A9*, CLSI, 9th ed, PA, USA.
- White PA, McIver CJ, Rawlinson WD (2001) Integrons and gene cassettes in theenterobacteriaceae. *Antimicrob agents and chemotherapy*. AAC. 1;45:2658-61.
- Yadegari Z, Brujeni GN, Ghorbanpour R, Moosakhani F, Lotfollahzadeh S (2019) Molecular characterization of enterotoxigenic *Escherichia coli* isolated from neonatal calves diarrhea. *Vet Res Forum*, 10: 73
- Younis EE, Ahmed AM, El-Khodery SA, Osman SA, El-Naker YF (2009) Molecular screening and risk factors of enterotoxigenic *Escherichia coli* and *Salmonella* spp. in diarrheic neonatal calves in Egypt. *Res Vet Sci* 87(3):373-379
- Yu HS, Lee JC, Kang HY, Jeong YS, Lee EY, Choi CH, Tae SH, Lee YC, Seol SY, Cho DT (2004) Prevalence of *dfg* genes associated with integrons and dissemination of *dfgA17* among urinary isolates of *Escherichia coli* in Korea. *J Antimicrob Chemoth*, 53:pp.445-450.
- Zhang W, Zhao M, Ruesch L, Omot A, Francis D (2007) Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. *Vet Microbiol* 123:145–152