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Antimicrobial Resistance of Entrotoxigenic and Non-Entrotoxigenic *Escherichia coli* Isolated From Diarrheic Calves in Iran

Hadi Pourtaghi,[1,*] and Hamid Reza Sodagari[2]

[1]Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, IR Iran
[2]Young Researchers and Elite Club, Karaj Branch, Islamic Azad University, Karaj, IR Iran

[*]*Corresponding author*: Hadi Pourtaghi, Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, IR Iran. Tel: +98-9122763975, E-mail: hadi.pourtaghi1@gmail.com

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Abstract

Background: Among diarrheagenic strains of *Escherichia coli*, entrotoxigenic *Escherichia coli* (ETEC) is most commonly associated with diarrhea in calves and lambs. Broad use of antimicrobials in agriculture selects for resistant bacteria that may enter the food chain, and potentially result in foodborne disease in humans that is less responsive to treatment with conventional antibiotics.
Objectives: This study was carried out to identify antimicrobial resistance in ETEC and non-ETEC isolated from diarrheic calves.
Materials and Methods: Disk diffusion methods and PCR were used to detect antimicrobial resistance. Antimicrobial susceptibility testing was performed by using the standards recommended by the clinical and laboratory standard institute (CLSI). Multiplex or monoplex PCR amplification was used to identify eight antibiotic-resistant genes, including *bla* SHV, *tet*(A), *Sul1, aac(3)*-IV, *ere(A)*, *catA1*, *cmlA*, *aadA1* and *qnr(A)*, which confer resistance to penicillin, tetracycline, sulfonamide, gentamicin, erythromycin, chloramphenicol, streptomycin, and fluoroquinolone, respectively.
Results: Antimicrobial resistance rates for ETEC isolates were detected against penicillin (100%), tetracycline (90.9%), erythromycin (90.9%), streptomycin (90.9%), sulfonamide (63.6%), chloramphenicol (63.6%), gentamicin (45.4%) and fluoroquinolone (36.3%). Furthermore, according to the results, antimicrobial resistance for non-ETEC isolates was detected against penicillin (100%), followed by erythromycin (97.6%), tetracycline (93%), streptomycin (91.8%), sulfonamide (73.2%), chloramphenicol (51.1%), fluoroquinolone (44.1%), and gentamicin (34.8%). In addition, the distribution of the resistant genes for ETEC isolates were *ere(A)* (100%), *catA1* (100%), *cmlA* (100%), *aadA1* (100%), *Sul1* (72.7%), *tet(A)* (54.5%), *aac(3)-IV* (54.5%), *bla SHV* (36.3%), and *qnr*(A) (9%). For non-ETEC isolates they were *ere(A)* (100%), *aadA1* (100%), *Sul1* (87.2%), *catA1* (67.4%), *cmlA* (67.4%), *tet*(A) (48.8%), *aac(3)-IV* (48.8%), *bla SHV* (41.8%), and *qnr(A)* (3.4%).
Conclusions: Among the eight antimicrobial agents examined in this investigation, the least resistance was observed against gentamicin and fluoroquinolone in both ETEC and non-ETEC isolates. Therefore, carrying out antimicrobial susceptibility tests before drug prescription seems necessary.

Keywords: Antimicrobial Resistance, Diarrheic Calves, Entrotoxigenic *Escherichia coli*

1. Background

Calf diarrhea remains the main cause of mortality in dairy calves (1). According to the USDA national animal health monitoring system report, diarrhea accounted for 62.1% of the deaths of calves (2). Calf mortality and treatment costs represent an enormous economic loss to the dairy industry, estimated to surpass 250 million USD annually in the United States (3). In Norway, this economic loss was estimated to be about 10 million USD in 2006 (4). According to previous studies, *Escherichia coli* (*E. coli*) is regarded as a major agent in the etiology of neonatal calf diarrhea; among diarrhoeagenic strains of *E. coli*, entrotoxigenic *Escherichia coli* (ETEC) is most commonly associated with diarrhea in calves and lambs (5, 6). Several investigations reported the high prevalence of calf diarrhea caused by ETEC around the world (7-9).

Antimicrobial therapy is the primary control approach for decreasing morbidity and mortality in animals infected with diarrhoeagenic bacteria (10). According to the previous studies, there are several reports related to the rates of antimicrobial resistance in isolates of animal origin worldwide (11-15). Broad use of antimicrobials in agriculture selects for resistant bacteria that may enter the food chain, and potentially result in foodborne disease in humans that is less responsive to treatment with conventional antibiotics (16). In addition to the human health concerns, antimicrobial-resistant pathogens also pose a severe and costly animal health problem, in that they may prolong illness and decrease productivity through higher morbidity and mortality (15).

2. Objectives

This study was carried out to identify antimicrobial resistance in ETEC and non-ETEC *E. coli* isolated from diarrheic calves, by PCR and the disk diffusion method.

3. Materials and Methods

3.1. Sample Collection

A total of 11 ETEC strains were detected from 97 *E. coli* isolated from diarrheic calves in 10 different farms of Alborz and Tehran provinces, between 2010 and 2014. The resistance of each isolate to eight antimicrobial agents was evaluated by PCR and disk diffusion.

3.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by using the disk diffusion method, and interpreted according to the standards recommended by the CLSI (2008) (17), for the following antimicrobial agents: penicillin (10 IU), tetracycline (30 μg), sulfonamide (5 μg), gentamicin (10 μg), erythromycin (15 μg), chloramphenicol (30 μg), streptomycin (10 μg), and fluoroquinolone (5 μg).

3.3. Antibiotic Resistant Gene Detection

DNA extraction was carried out using the boiling method. Overnight cultures in 2 mL nutrient broth were centrifuged for 5 minutes at 5,000 rpm. The bacterial pellet was re-suspended in 200 μL of distilled water and boiled for 10 minutes. Tubes were centrifuged again, and the supernatant was used as template DNA. Multiplex or monoplex PCR amplification was used to identify eight antibiotic resistant genes, including *aac(3)-IV* for gentamicin, *sul1* for sulfonamide, *bla SHV* for penicillin antibiotics, *ereA* for erythromycin, *cat1* and *cmlA* for chloramphenicol, and *qnr* for fluoroquinolone resistance genes (18-20). The primer sets were synthesized by Cinna-Gene, Iran. Each PCR assay was carried out with a 25 μL mixture containing 2.5 μL of 10 $\times$ PCR buffer, 2 mM $MgCl_2$, 250 μM dNTP, (Cinna-Gene, Iran), 0.5 mM of each primer set, 1 U of Taq DNA polymerase, and 3 μL of the DNA template. PCR amplification was conducted in a Swift Minipro thermal cycler (ESCO Micro Pte, Singapore). Polymerase chain reaction analysis of the *aac(3)-IV*, *sul1*, *bla SHV*, *ereA*, *cat1*, *cmlA*, and *qnr* genes were carried out using the primers, amplification cycles, and references described in Table 1. The specificity and optimal concentration of primers was determined experimentally. Positive and negative controls for each PCR were included. Amplicons were visualized by electrophoresis in 2% agarose gels prepared in a TBE buffer (89 mM tris, 89 mM boric acid, 2.5 mM EDTA), stained with ethidium bromide and visualized using UV illumination. A 100 bp DNA molecular marker (Fermentas, Lithuania) was used to determine the size of the amplicons.

The presence of *E. coli* virulence genes was examined by PCR using primers and PCR conditions, as documented by Pourtaghi et al. (21).

4. Results

The results indicated that among the 97 *E. coli* strains isolated from diarrheic calves, 11 (11.3%) isolates were ETEC due to presence of three virulence genes, including K99 (F5), F41, and STa, and 86 (88.6%) of them were non-ETEC. All the *E. coli* isolates were subjected to antimicrobial susceptibility testing and genomic detection for the presence of drug-resistant genes. Antibiotic resistance rates for ETEC isolates were detected against penicillin (100%), tetracycline (90.9%), erythromycin (90.9%), streptomycin (90.9%), sulfonamide (63.6%), chloramphenicol (63.6%), gentamicin (45.4%), and fluoroquinolone (36.3%) (Table 2). Furthermore, the results of antimicrobial susceptibility testing for non-ETEC isolates exhibited the highest level of resistance to penicillin (100%), followed by erythromycin (97.6%), tetracycline (93%), streptomycin (91.8%), sulfonamide (73.2%), chloramphenicol (51.1%), fluoroquinolone (44.1%), and gentamicin (34.8%) (Table 2). The distribution of the resistance genes was *bla SHV* (36.3%), *tet(A)* (54.5%), *Sul1* (72.7%), *aac(3)-IV* (54.5%), *ere(A)* (100%), *catA1* (100%), *cmlA* (100%), *aadA1* (100%), and *qnr(A)* (9%) for the ETEC isolates, and *bla SHV* (41.8%), *tet(A)* (48.8%), *Sul1* (87.2%), *aac(3)-IV* (48.8%), *ere(A)* (100%), *catA1* (67.4%), *cmlA* (67.4%), *aadA1* (100%), *qnr(A)* (3.4%) for the non-ETEC isolates (Table 3).

5. Discussion

Several antibiotics are used on farms for treating animals and for prophylactic purposes. This broad use of antimicrobials selects resistant bacteria that may enter the food chain and have potentially adverse effects on consumers' health, as these bacteria are less responsive to treatment with conventional antibiotics (22). In this study 11 (11.3%) out of 97 *E. coli* isolates were ETEC, which was lower than the result of a previous investigation in Iran (28.4%) (23). The antibiotic susceptibility test for ETEC isolates indicated 100% antimicrobial resistance to penicillin, which is in agreement with the result reported by Shahrani et al. (23). These results demonstrated that penicillin is frequently used for farm animals in Iran. Although high antimicrobial resistance rates were found for tetracycline, erythromycin, streptomycin, sulfonamide, and chloramphenicol, lower rates were observed for gentamicin and

Table 1. Primers Used for Detection of Antibiotic Resistance Genes of Diarrheagenic *Escherichia coli*

Primers	Sequence	PCR Program[a]	Product Size, bp	Antibiotic	Reference
bla SHV		1	768	Penicillin	(20)
Forward	TCGCCTGTGTATTATCTCCC				
Reverse	CGCAGATAAATCACCACAATG				
tet(A)		2	577	Tetracycline	(19)
Forward	GGTTCACTCGAACGACGTCA				
Reverse	CTGTCCGACAAGTTGCATGA				
Sul1		3	822	Sulfonamide	(20)
Forward	TTCGGCATTCTGAATCTCAC				
Reverse	ATGATCTAACCCTCGGTCTC				
aac(3)-IV		4	286	Gentamicin	(20)
Forward	CTTCAGGATGGCAAGTTGGT				
Reverse	TCATCTCGTTCTCCGCTCAT				
ereA		5	419	Erythromycin	(20)
Forward	GCCGGTGCTCATGAACTTGAG				
Reverse	CGACTCTATTCGATCAGAGGC				
cat1		6	547	Chloramphenicol	(20)
Forward	AGTTGCTCAATGTACCTATAACC				
Reverse	TTGTAATTCATTAAGCATTCTGCC				
cmlA		6	698	Chloramphenicol	(20)
Forward	CCGCCACGGTGTTGTTGTTATC				
Reverse	CACCTTGCCTGCCCATCATTAG				
aadA1		7	447	Streptomycin	(19)
Forward	TATCCAGCTAAGCGCGAACT				
Reverse	ATTTGCCGACTACCTTGGTC				
Qnr		8	670	Fluoroquinolone	(18)
Forward	GGGTATGGATATTATTGATAAAG				
Reverse	CTAATCCGGCAGCACTATTTA				

[a] PCR programs: 1, 35 cycles × (94°C for 40 seconds, 52°C for 60 seconds, 72°C for 75 seconds); 2, 30 cycles × (94°C for 60 seconds, 56°C for 60 seconds, 72°C for 60 seconds); 3, 30 cycles × (94°C for 60 seconds, 58°C for 60 seconds, 72°C for 60 seconds); 4, 35 cycles × (92°C for 60 seconds, 53°C for 60 seconds, 72°C for 60 seconds); 5, 30 cycles × (94°C for 45 seconds, 52°C for 60 seconds, 72°C for 60 seconds); 6, 35 cycles × (94°C for 60 seconds, 55°C for 60 seconds, 72°C for 60 seconds); 7, 30 cycles × (94°C for 60 seconds, 58°C for 60 seconds, 72°C for 60 seconds); 8, 30 cycles × (94°C for 60 seconds, 50°C for 60 seconds, 72°C for 60 seconds).

fluoroquinolone, which were higher than the results obtained by Hariharan et al. (24). This means that these two kinds of antimicrobial agents are less applied in farm animal medicine in Iran, and could be more effective for treatment of ETEC infections.

These results also exhibited that antimicrobial resistance is widespread among potentially diarrheagenic *E. coli* strains. The results of antimicrobial susceptibility testing for non-ETEC isolates exhibited the highest level of resistance to penicillin (100%), which was similar to the reported result for ETEC isolates. Moreover, the frequency of resistance was also high in erythromycin (97%), tetracycline (93%), and streptomycin (91.8%), with rates approximately similar to the previous results reported by Boerlin et al. (25) and Shahrani et al. (23). Furthermore, lower rates were found against sulfonamide (73.2%), chloramphenicol (51.1%), fluoroquinolone (44.1%), and gentamicin (34.8%). The results for the antimicrobial resistant genes indicated that *ere(A)* and *aadA1* genes were detected in all ETEC and non-ETEC isolates, while the distribution of the *tet(A)*, *aac(3)-IV*, *catA1*, *cmlA* and *qnr(A)* genes were found to be higher in ETEC isolates, although the distribution of

Table 2. Antimicrobial Susceptibility Test for Entrotoxigenic *Escherichia coli* and Non-Entrotoxigenic *Escherichia coli* Isolates[a]

Antimicrobial Agent	ETEC	Non-ETEC
Penicillin	11 (100)	86 (100)
Tetracycline	10 (90.9)	80 (93)
Sulfonamide	7 (63.6)	63 (73.2)
Gentamicin	5 (45.4)	30 (34.8)
Erythromycin	10 (90.9)	84 (97.6)
Chloramphenicol	7 (63.6)	44 (51.1)
Streptomycin	10 (90.9)	79 (91.8)
Fluoroquinolone	4 (36.3)	38 (44.1)

Abbreviation: ETEC, entrotoxigenic *Escherichia coli*.
[a]Values are expressed as No. (%).

Table 3. Antimicrobial Resistance Genes of Entrotoxigenic *Escherichia coli* and Non-Entrotoxigenic *Escherichia coli* Isolates[a]

Antimicrobial Resistance Gene	ETEC	Non-ETEC
blaSHV	4 (36.3)	36 (41.8)
tet(A)	6 (54.5)	42 (48.8)
Sul1	8 (72.7)	75 (87.2)
aac(3)-IV	6 (54.5)	42 (48.8)
ere(A)	11 (100)	86 (100)
catA1	11 (100)	58 (67.4)
cmlA	11 (100)	58 (67.4)
aadA1	11 (100)	86 (100)
qnr(A)	1 (9)	3 (3.4)

Abbreviation: ETEC, entrotoxigenic *Escherichia coli*.
[a]Values are expressed as No. (%).

bla SHV, *Sul1* genes were reported as higher in non-ETEC isolates. Higher resistance to penicillin, tetracycline, and fluoroquinolone in the disk diffusion method compared to PCR can be due to the interference of other antimicrobial resistant genes (26). Moreover, other resistance mechanisms not involving resistance genes, such as point mutations, can be another reason for this issue (27). Previous investigations indicated different results related to the prevalence of resistant genes in farm animals (23, 25, 28) which can be due to variations in antimicrobial usage in each geographical area.

According to the present study's results, a high percentage of antimicrobial resistance in ETEC and non-ETEC isolates indicates that unlimited access to antimicrobial agents in Iran, as well as a low rate of antibiotic sensitivity tests for selection of suitable drugs, can lead to veterinary public health hazards. Therefore, the use of antimicrobial agents regarded as critically or highly important for use in humans should be judiciously or minimally used in food animals, to preserve the efficiency of these antimicrobial agents for treatment of infection in humans (29). Furthermore, among the eight examined antimicrobial agents in this investigation, the least resistance was observed against gentamicin and fluoroquinolone, in both ETEC and non-ETEC isolates. Therefore, carrying out antimicrobial susceptibility tests before drug prescription seems necessary.

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Footnotes

Authors' Contribution: Study concept and design and acquisition of data: Hadi Pourtaghi; analysis and interpretation of data: Hadi Pourtaghi and Hamid Reza Sodagari; drafting of the manuscript: Hamid Reza Sodagari; critical revision of the manuscript for important intellectual content: Hadi Pourtaghi; statistical analysis: Hamid Reza Sodagari; administrative, technical, and material support: Hadi Pourtaghi; study supervision: Hadi Pourtaghi.

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