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Genotypic analysis of virulence genes and antimicrobial profile of diarrheagenic *Escherichia coli* isolated from diseased lambs in Iran

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Abstract The aim of the present study was to determine the analysis of virulence genes and antimicrobial profile of diarrheagenic *Escherichia coli* isolated from diseased lambs. Two hundred ninety *E. coli* isolates were recovered from 300 rectal swabs of diarrheic lambs and were confirmed by biochemical tests. The pathotype determination was done according to the presence of genes including *f5*, *f41*, *LTI*, *STI*, *bfp*, *ipaH*, *stx₁*, *stx₂*, *eae*, *ehlyA*, *cnf₁*, *cnf₂*, *cdIII*, *cdIV*, and *f17* by PCR method. Sixty-six isolates (23.72%) possessed the *STI* gene and categorized into entrotoxigenic *E. coli* (ETEC). Nine isolates (3.1%) and five isolates (1.72%) were positive for the *cnf1* and *cnf2* genes which categorized into necrotoxic *E. coli* (NTEC). Hundred and seventeen isolates (40.34%) harbored *stx₁* and/or *stx₂* and classified as Shiga toxin-producing *E. coli* (STEC). Thirteen isolates (4.48%) were assigned to atypical entropathogenic *E. coli* (aEPEC) and possessed *eae* gene. Two isolates (0.68%) were positive for *ipaH* gene and were assigned to entroinvasive *E. coli* (EIEC). Statistical analysis showed a specific association between *eae* gene and STEC pathotype ($P < 0.0001$). The most prevalent resistance was observed against lincomycin (96.5%) and the lowest resistance was against kanamycine (56.02%), respectively. The high prevalence of STEC and ETEC indicates that diarrheic lambs represent an important reservoir for humans. ETEC may play an important role for frequent occurrence of diarrhea in lambs observed in this region. Due to high antibiotic resistance, appropriate control should be implemented in veterinary medicine to curb the development of novel resistant isolates.

Keyword *Escherichia coli* · Diarrheic lambs · Virulence genes · Antibiotic resistance

Introduction

Lamb diarrhea is a multifactor disease that can cause economic loss and is one of the most common reported diseases in lambs up to 3 months old (Aiello and Moses 2016). Since sheep are considered to be the lifeline agro-economy in many tropical regions around the world, identification, characterization, and treatment of the causal agents of this disease are of significant economic importance. An array of noninfectious and infectious agents has been identified as causal agents of diarrhea in lambs (Aiello and Moses 2016). Noninfectious factors include insufficient uptake of colostrum, poor sanitation, stress, overcrowding in the lamb pens, and cold water (Aiello and Moses 2016). Furthermore, various infection agents such as *Coronaviruses*, *Rotaviruses*, *Cryptosporidium* spp., *Clostridium perfrigens*, *Campylobacter* spp., *Escherichia coli*, and *Salmonlla* spp. have also been linked to this disease. Among these pathogenic agents, *E. coli* is the most common and important once (Muktar et al. 2015).

Pathogenic *E. coli* has been associated with two forms of enteric and septicemia infections, depending on the number of bacterium and the physiological condition of the affected hosts (Bihannic et al. 2014). Moreover, enteric pathogenic

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E. coli isolates often possess diverse virulence factors and are classified into several major pathotypes based on their pathogenesis (Beutin and Fach 2014). Enterotoxigenic *E. coli* (ETEC) isolates are the major cause of diarrhea in newborn farm animals (Cho and Yoon 2014). Fimbrial antigens (F5, F41) and enterotoxins (LT-I, LT-II, ST-I, and ST-II) are the most prominent virulence factors of ETEC (Duan et al. 2012). Shiga toxin-producing *E. coli* (STEC) and entero-pathogenic *E. coli* (EPEC) are frequently detected in small ruminants with or without diarrhea. STEC isolates carry genes encoding Shiga toxins and may possess other virulence genes for intimin and enterohemolysin. STEC strains, which also possess *eaeA* and *ehly* genes, are termed enterohemorrhagic *E. coli* (EHEC) (Askari Badouei et al. 2014). Two particular groups of virulence determinants, CNF1 and CNF2 and CDTs, have received attention because of their potential impact on animal and human health. These virulence factors have been isolated from healthy and diarrheic or septicemic calves and categorized to NTEC pathotype (Borriello et al. 2012).

EIEC isolates, which are involved in invasive intestinal infections in humans and animals, contain ipaH sequences that encode determinants for entry into epithelial cells and dissemination from cell to cell (Clements et al. 2012).

Besides identification and determination of the prevalence of pathogenic strains, analysis of antibiotic resistance of *E. coli* strains is another important factor in the treatment and control of diarrheal diseases (World Health Organization 2014). In addition, development and persistence of antibiotic resistance in commensal and nonpathogenic bacteria is one of the worldwide concerns, due to their potential role as a reservoir of resistance genes capable of transferring genes to foodborne and other zoonotic pathogens (Szmolka and Nagy 2013).

Sheep are considered as the lifeline agro-economy in the southeast of Iran. Thus, the aims of the current study were to determine different pathotypes of *E. coli* isolated from diarrheic lambs and to characterize their antimicrobial resistance profile phenotypically.

Material and method

Sample collection and *E. coli* isolation

This study was carried out from Jan to Dec 2014. A total number of 300 fecal samples from diarrheic lambs were collected in southeast of Iran (Kerman province). Each sample belonged to one animal which was between 1 and 12 weeks old. All swab samples were placed into Amies medium (Becton Dickinson, BBL, and USA) immediately and were sent out to the laboratory in ice-cooled containers. For the initial enrichment, they were inoculated into 3-ml buffered peptone water (Merck, Germany) and were incubated at 37 °C for 5–6 h. Subsequently, the enriched samples were streaked on MacConkey agar (Merck, Germany) and were incubated at 37 °C for overnight. Biochemical confirmations were performed on suspected colonies using IMViC (indole, methyl-red-Voges-Proskauer, and citrate) tests (Markey et al. 2013) and finally confirmed *E. coli* isolates were subjected to antibiotic susceptibility tests and PCR assays.

Nine *E. coli* isolates were used as positive controls: 510 (*f5+*, *f41+*); H10407 (*LT-I+*, *ST-I+*); Sakaï (*stx₁+*, *stx2+*, *eaeA+*); 28C (*cdtIV+*, *cnf1+*); 1404 (*cdtIII+*, *cnf2+*, *f17A+*); 25KH9 (*f17a-A+*), S5 (*f17b-A+*); 31A (*f17c-A+*); and 85b (*ipaH+*). Laboratory nonpathogenic *E. coli* isolate MG1655 was used as a negative control. All the reference isolates were provided from the bacterial collection of Microbiology Department of Ecole Nationale Vétérinaire Toulouse, France.

PCR assay for detection of virulence genes

DNA extraction of overnight cultures of *E. coli* isolates and reference isolates were prepared by boiling. All isolates were subjected to several PCR protocols for the presence of the genes encoding F5 and F41 fimbriae (Shams et al. 2012); *cnf1* and *cnf 2* gene (Shahrani et al. 2014); *LT-I*, *ST-I*, *ipaH*, *stx₁*, *stx₂*, *eae*, *cdtIII*, *cdtIV*, and *bfp* genes (Sidhu et al. 2013); and F17 family: *f17a-A*, *f17b-A*, *f17c-A*, and *f17d-A* genes (Bihannic et al. 2014).

Antimicrobial susceptibility test

Antibiotic resistance profiles of the isolates against ten selected antibacterial agents were determined by disc diffusion method according to the Clinical and Laboratory Standards Institute's guidelines (CLSI 2013). The following antimicrobial discs (Padtan-Teb, Tehran, Iran) were used in disc diffusion assay: lincomycin (L; 2 μg), cephalexin (CN; 30 μg), ciprofloxacin (CP; 5 μg), enrofloxacin (NFX; 5 μg), kanamycin (K; 30 μg), gentamycin (G; 10 μg), trimethoprim/sulfamethoxazole (SXT; 25 μg), oxytetracycline (T; 30 μg), penicillin G (P; 10 μg), and streptomycin (S; 10 μg).

Statistical analysis

The data were analyzed by using SPSS software (version 17. SPSS Inc., USA) and *P* value was calculated using chi-square and Fisher's exact tests to find any significant relationship. *P* value less than 0.05 was considered statistically significant.

Results

From 300 fecal samples, 290 *E. coli* isolates were isolated. Virulence gene analysis showed 66 isolates (23.72%)

possessed *STI* gene, which categorized into ETEC pathotype. Nine isolates (3.1%) were positive for *cnf1* gene and five isolates (1.72%) were positive for *cnf2* gene which categorized into NTEC pathotype. Hundred and seventeen isolates (40.34%) contained *stx₁* and/or *stx₂* in combination with the *eae* or/and *ehly* genes and classified as STEC pathotype. Thirteen isolates (4.48%) were assigned to EPEC pathotype and possessed *eae* gene. The majority of the EPEC isolates (13/290) encountered in the present study were aEPEC, since the *bfp* gene was not detected in these isolates. Two isolates (0.68%) were positive for *ipaH* gene that is an EIEC virulence gene. All the examined isolates were negative for *cnf2*, *bfp*, and *LT1* genes.

According to the results, stx₂ (34.48%), stx₁ (31.72%), and *eae* (24.13%) were the most prevalent virulence genes,

respectively. In addition, 25.86% of diarrheic samples were diagnosed as non-detected. In this study, the presence of $f17_{c-A}$ and $f17_{a-A}$ genes were 3.1 and 0.68% which distributed into NTEC and EIEC pathotypes, respectively.

Virulotyping analysis of the isolates showed all of the detected pathotypes were positive for at least two of the examined virulence genes. Twelve different combinations of the virulence genes were detected. Statistical analysis showed a specific association between *eae* gene and STEC pathotype ($P < 0.0001$). In addition, a specific association between *STa* gene and *F5* was found ($P < 0.0001$). In the current study, stx1/stx2 with the frequency of 28.2% was found as predominant gene profile (Table 1).

There were no significant differences ($P > 0.05$) in the presence of *ehly* gene in STEC, NTEC, and aEPEC pathotypes.

Table 1 Specific primers used for PCR amplifications of target gens

Gene	Primer sequence (5′–3′)	Product size (bp)	Reference
f5	TATTATCTTAGG TGGTATGG	314	Shams et al. (2014)
f41	GGTATCCTTTAGCAGCAGTATTTC GCATCAGCGGCAGTATCT	380	Shams et al. (2014)
ST1	GTCCCTAGCTCAGTATTATCACCT ATTTTTMTTTCTGTATTRTCTT	190	Sidhu et al. (2013)
LT1	CACCCGGTACARGCAGGATT GGCGACAGATTATACCGTGC	450	Sidhu et al. (2013)
Stx1	CGGTCTCTATATTCCCTGTT AGAGCGATGTTACGGTTTG	388	Sidhu et al. (2013)
Stx2	TTGCCCCCAGAGTGGATG TGGGTTTTTCTTCGGTATC	807	Sidhu et al. (2013)
eae	GACATTCTG GTTGACTCTCTT TGCGGCACAACAGGCGGCGA	629	Sidhu et al. (2013)
ehly	CGGTCGCCGCACCAGGATTC CAATGCAGATGCAGATACCG	432	Sidhu et al. (2013)
ipaH	CAGAGATGTCGTTGCAGCAG GTTCCTTGACCGCCTTTCCGATACCGTC	600	Sidhu et al. (2013)
cnf1	GCCGGTCAGCCACCCTCTGAGAGTAC GGGGGAAGTACAGAAGAATTA	1111	Shahrani et al. (2014)
cnf2	TTGCCGTCCACTCTCACCAGT TATCATACGGCAGGAGGAAGCACC	1240	Shahrani et al. (2014)
cdtIII	GTCACAATAGACAATAATTTTCCG GAAAATAAATGGAATATAAATGTCCG	555	Sidhu et al. (2013)
cdtIV	TTTGTGTCGGTGCAGCAGGGAAAA CCTGATGGTTCAGGAGGCTGGTTC	350	Sidhu et al. (2013)
f17A	TTGCTGCAGAATCTATACCT GCAGAAAATTCAATTTATCCTTGG	537	Bihannic et al. (2014)
f17a-A	CTGATAAGCGATGGTGTAATTAAC CTGATAAGCGATGGTGTAATTAAC	321	Bihannic et al. (2014)
f17b-A	GCTGGAAGGGTGCAATACGCCTG CTGATAAGCGATGGTGTAATTAAC	323	Bihannic et al. (2014)
f17c-A	CAACTAACGGGATGTACAGTTTC CTGATAAGCGATGGTGTAATTAAC	416	Bihannic et al. (2014)
f17d-A	GCAGGAACCGCTCCCTTGGC CTGATAAGCGATGGTGTAATTAAC	239	Bihannic et al. (2014)
bfp	GATAGTCATAACCTTAATATTGCA AAT GGT GCT TGC GCT TGC TGC GCC GCT TTA TCC AAC CTG GTA	324	Sidhu et al. (2013)

Details of detected combination patterns of examined virulence genes in relation to different pathotypes are shown in Table 2.

Antibiogram of the isolates against 10 antibiotics showed that all of the 290 isolates were resistant to two or more examined antibacterials. The most prevalent resistance was recorded against lincomycin (96.5%) and oxytetracycline (92.75%). The lowest resistance was observed against trimethoprim/sulfamethoxazole (46.89%) and kanamycine (56.02%), respectively. Results of antibiotic susceptibility tests showed that *E. coli* isolates could be classified in 12 different groups according to antibiotic resistance patterns. Sixty-seven isolates (23.1%) were resistant to all of the tested antibiotic, which were the most prevalent antibiotic resistance pattern followed by CN, NFX, G, SXT, T, L, S, and P (17.2%) and CN, NFX, K, T, L, and P (13.1%).

In this study, 38 (13.1%) of STEC isolates, 13 (4.4%) of ETEC isolates, one (0.34%) of aEPEC, one (0.34%) of NTEC, and one (0.34%) of EIEC isolates were resistance to all ten used antibiotics. Whereas, only one isolate (0.34%) of each abovementioned pathotypes were resistance to lincomycin, cephalexin, and enrofloxacin which was the less prevalent pattern among isolates.

Resistance to all antibiotics (pattern 1) in STEC isolates had significant differences ($P < 0.05$) in comparison to the other pathotypes. On the other hand, there were no any significant differences ($P > 0.9999$) between ETEC, aEPEC, NTEC, and EIEC in all antibiotic resistance patterns. Prevalence of 12 detected antibiotic resistance patterns in each abovementioned pathotype are presented in Table 3.

Table 2 Virulotyping of diarrheagenic *E. coli* isolated from diseased lambs

Combination of genes	Pathotypes	Total (%)
ST1, f5	ETEC	33 (11.37)
ST1, f5, f41		36 (12.41)
eae, f41	aEPEC	8 (2.75)
eae, f41, ehly		5 (1.72)
eae, stx1	STEC	17 (5.86)
eae, stx2		25 (8.62)
eae, stx1, stx2		15 (5.17)
stx1, stx2, ehly		27 (9.31)
stx1, stx2		33 (11.37)
cnf1, f17c-A, cd III, cnf2, ehly, cdIV	NTEC	9 (3.1)
		5 (1.72)
ipaH, f17a-A	EIEC	2 (0.68)
Non-detected		75 (25.86)
Total		290

In this table: *ETEC* entrotoxigenic *E. coli*, *aEPEC* atypical entropathogenic *E. coli*, *STEC* Shiga toxin-producing *E. coli*, *NTEC* necrotoxic *E. coli*, *EIEC* entroinvasive *E. coli*

Discussion

Lambs are considered as the lifeline agro-economy in the southeast of Iran. Cases of neonatal diarrhea are commonly associated with more than one of infectious agents, and the cause of most outbreaks is multifactor. In the present study, *E. coli* was the most prevalent isolate of all the bacterial agents in diarrheic lambs. This finding is in agreement with the previous studies which considered *E. coli* as the most important cause of neonatal diarrhea of animals (Aiello and Moses 2016).

The prevalence rate of pathogenic *E. coli* (71.66%) in our study was significantly higher than previous studies (Turkyilmaz et al. 2013). The majority of the EPEC isolates (13/290, 4.48%) encountered in the present study were aEPEC, since the *bfp* gene was not detected in these isolates. This is in agreement with the study of Chandran and Mazumder (2014) that showed humans are the only living reservoir of tEPEC, with the exception of a few isolates from dogs (Chandran and Mazumder 2013).

In the present study, the frequency of STEC isolates in lambs was 40.34%. Previously, STEC isolates were reported in 32% of *E. coli* isolates from diarrheic lambs in India (Bandyopadhyay et al. 2011) that is almost similar to the findings of this study. The moderately high proportion of STEC in the diarrheic lambs implicated that these animals are important reservoir of STEC. Detection of *stx2* in higher proportion in the present study may be a grave concern for the animal handlers as stx2 was reported to be intricately associated with dreadful human diseases like HUS (Bandyopadhyay et al. 2011).

Different combinations of virulence factors may be detected in pathogenic *E. coli* isolated from symptomatic animals. Accordingly, different gene combinations were found in our investigation *E. coli* with at least one virulence factor and different expression frequencies were isolated from diarrheic calves, kids, and lambs (Staji et al. 2015; Osman et al. 2013). Literature review showed that lambs could be the natural reservoirs for particular STEC isolates that mainly harbor *stx1/ehly* gene profile (Askari Badoueia et al. 2015). Whereas, in the current study, *stx1/stx2* with the frequency of 28.2% was found as predominant gene profile.

Among newborn small ruminants, ETEC is one of the most important pathogen that causes diarrhea (Pourtaghi and Sodagari 2016). In our study, about 23.72% isolates were harboring specific genes for ETEC. The latest study from Turkey and India showed that 11.2 and 44% of the fecal isolates from lamb were ETEC. In this study, most of ETEC isolates from lambs were *f41*+ or *f5*+ and produce ST1. The possible explanation for this association is that both of virulence factors are generally encoded in the same plasmid. In the present study, the absence of *LT-I* in the isolated isolates is not surprising since *LT-I* is

Table 3 Detected antibiotic resistance patterns of *E. coli* pathotypes isolated from diarrheic lamb

Pattern		Pathotype							
		ETEC	aEPEC	STEC	NTEC	EIEC	None	Percentage	Total
1	CN, CP, NFX, K, G, SXT, T, L, S, P	13	1	38	1	1	13	23.16	67
2	CN, CP, NFX, G, SXT, T, L, S, P	10	1	14	1	0	24	17.24	50
3	CN, CP, NFX, K, G, T, L, S, P	9	2	6	3	0	13	11.37	33
4	CN, CP, NFX, G, T, L, S, P	2	1	15	1	0	1	6.89	20
5	CN, CP, G, SXT, T, L, S, P	1	1	5	1	0	1	3.1	9
6	CN, NFX, K, G, SXT, P	2	1	5	1	0	1	3.44	10
7	CN, CP, G, T, L, S, P	12	1	13	1	0	3	10.34	30
8	CN, NFX, K, T, L, P	9	1	12	1	1	14	13.1	38
9	CN, G, T, S, P	2	1	2	1	0	1	2.41	7
10	CN, NFX, L	1	1	1	1	0	1	1.72	5
11	K, T, L, S	7	1	5	1	0	1	5.17	15
12	NFX, L	1	1	1	1	0	2	2.06	6
Total		69	13	117	14	2	75	100	290

In this table: lincomycin (L; 2 μg), cephalexin (CN; 30 μg), ciprofloxacin (CP; 5 μg), enrofloxacin (NFX; 5 μg), kanamycin (K; 30 μg), gentamycin (G; 10 μg), trimethoprim/sulfamethoxazole (SXT; 25 μg), oxytetracycline (T; 30 μg), penicillin G (P; 10 μg) and streptomycin (S; 10 μg)

ETEC entrotoxigenic *E. coli*, *aEPEC* atypical entropathogenic *E. coli*, *STEC* Shiga toxin-producing *E. coli*, *NTEC* necrotoxic *E. coli*, *EIEC* entroinvasive *E. coli*

considered atypical in ruminant isolates (Turkyilmaz et al. 2013).

NTEC was reported in 4.8% of isolates in our study that is lower than the prevalence 3.49% in diarrheic calves in Iran (Shahrani et al. 2014). NTEC is detected in both diarrheic and non-diarrheic animals; thus, there is no clear evidence for its causative role in lamb diarrhea (Bekal et al. 2015). Moreover, the combination pattern of *cnf2*, *cdIII*, and *F17* were found in diarrheic lambs in this study, while this combination pattern was only reported in diarrheic calves previously (Valat et al. 2014). Only two isolates of this report was classified into EIEC pathotype. Whereas, epidemiologic significance of EIEC is less known in lambs (Kolenda et al. 2015).

In the present study, 75 *E. coli* isolates that were isolated from diarrheic samples had no any virulence factors. One possible reason for this finding is that maybe these isolates were nonpathogenic *E. coli* and diarrhea caused by some other infectious agents.

Diarrhea associated with *E. coli* infections is often treated with antibiotics; however, therapy may be unsuccessful due to resistant isolates in animals (Shahrani et al. 2014). Different patterns of antibiotic-resistant have been reported in bovine and ovine *E. coli* isolates (Ayaz et al. 2015; Goncuoglu et al. 2010). In our study, all isolates were found to be multidrug resistant. Whereas, Goncuoglu et al. (2010) have shown that 68% of the *E. coli* O157:H7 isolates belonging to cattle and sheep were susceptible to all antibiotics tested.

Our isolates were significantly resistant to the antibiotics lincomycin, tetracyclines, and streptomycin having prophylactic and therapeutic usages in lamb diarrhea. Irregular consumption of antibiotics and nourishment of lambs with antibiotic resistance-contaminated milk are the main risk factors that augment the selection of resistant isolates (Duse et al. 2015). Horizontal transferring of resistant bacteria and genes to environment, foods, and other hosts is completely probable (Yamamoto et al. 2013).

Conclusion

The moderately high prevalence of STEC and ETEC found in the diarrheic lambs indicates that these animal species represent an important reservoir of STEC and ETEC infection for humans in this part of the globe. Presence of *eae* gene in STEC isolates indicated that these isolates could be more virulent for humans. The study also indicated that ETEC may play a significant role for frequent occurrence of diarrhea in lambs observed in this region. According to the results, phenotypic antibiotic resistance were detected in all pathotyes isolated from lamb diarrhea. Appropriate control should be implemented in veterinary medicine to curb the development of novel resistant strains. Further molecular epidemiologic studies are needed to find the origin and means of transmission of antibiotic resistance genes as a first step to limit their

distribution, particularly among pathogenic bacteria that threaten human health.

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