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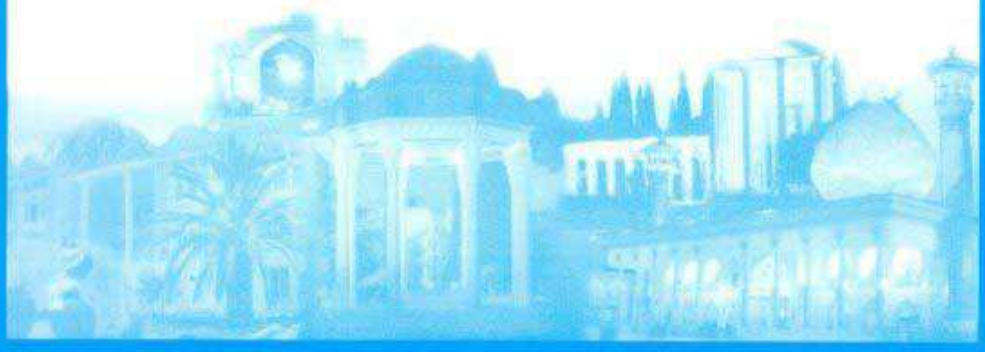

Short Paper

Molecular detection of virulence genes and multi-drug resistance patterns in *Escherichia coli* (STEC) in clinical bovine mastitis: Alborz province, Iran

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Summary

The aim of this study was to identify virulence genes and antimicrobial resistance of *Escherichia coli* isolated from bovine clinical mastitis in dairy herds in Iran. Sampling was done from 86 inflamed quarters of dairy cows in 8 commercial farms of Alborz province, Iran in summer 2015. Shiga toxin-producing *E. coli* (STEC) virulence genes were detected by multiplex PCR and multi-drug resistance profiles were confirmed using disk diffusion method. Among 60 *E. coli* isolated from examined samples, 13 (21.6%) of them were STEC. The results of PCR assay showed that *eaeA* gene was carried by 4 (30.8%) of STEC isolates. Although *stx1* in combination with *eaeA* gene was detected from 7 (53.8%) of STEC isolates, *stx1* and *stx2* genes were detected from only 1 (7.7%) of the examined samples. The result of the disk diffusion method showed that all *E. coli* isolates were resistant to penicillin, tylosin, oxytetracycline, erythromycin, ampicillin, streptomycin and neomycin. However all isolates were susceptible to enrofloxacin. Therefore, according to the results establishing a regular monitoring system for identification of cases with clinical mastitis and conducting antibiotic sensitivity tests are recommended.

Key words: Antimicrobial resistance, Clinical mastitis, *E. coli*, STEC, Virulence factors

Introduction

There are several reports related to the incidence of bovine mastitis caused by *Escherichia coli* around the world (Bradley and Green, 2001). According to the previous studies Shiga toxin-producing *E. coli* (STEC) strains are an important group for mastitis (Kobori *et al*., 2004; Guler and Gündüz, 2007). Identification of virulence factors of *E. coli* isolated from bovine clinical mastitis has been conducted in numerous previous investigations (Wenz *et al*., 2006). These studies demonstrated that Shiga toxins (*Stx1*, *Stx2*) and *eae* (intimin) are the most significant virulence genes in *E. coli* strains isolated from bovine clinical mastitis (Momtaz *et al*., 2012). Antibiotic therapy is the common treatment for bovine clinical mastitis. Widespread use of antimicrobials in farm animals has resulted in a considerable rise of antimicrobial-resistant strains of bacteria which can increase treatment cost and period (Sawant *et al*., 2007).

Therefore, the aim of this study was to identify virulence genes and antimicrobial resistance of *E. coli* isolated from bovine clinical mastitis in dairy herds in Iran.

Materials and Methods

Sample collection

Sampling was performed from 86 inflamed quarters of dairy cows in eight commercial farms of Alborz province, Iran in summer 2015.

DNA extraction

Overnight cultures of the bacteria in 2 ml nutrient broth were centrifuged for 5 min at 5,000 rpm. The bacterial pellet was re-suspended in 200 µL of distilled water and boiled for 10 min. Tubes were centrifuged again, and the supernatant was used as template DNA (Pourtaghi *et al*., 2013; Pourtaghi and Sodagari, 2016).

STEC detection

PCR was performed on the samples to detect the presence of the *stx1*, *stx2* and *eaeA* genes. The primer sets and related genes that encode virulence genes and PCR condition to amplification are described in Table 1. Amplification reactions were performed in a total volume of 25 µL containing 2.5 µL of 10 × PCR buffer, 0.5 mM $Mgcl_2$, 250 µM dNTP, 1 µM of each primer and 0.5 U Taq DNA polymerase. To determine molecular weight, 100 bp DNA ladder (Fermentas) was used.

Table 1: Primers used for PCR and DNA sequencing

Virulence factor	Primer sequence 5´-3´	Position in open reading frame	Size of product	References
Stx1	TTCGCTCTGCAATAGGTA TTCCCCAGTTCAATGTAAGAT	125-142 of A subunit 659-679 of A subunit	555*	Frank *et al*. 1998
Stx2	GTGCCTGTTACTGGTTTTTCTTC AGGGGTCGATATCTCTGTCC	30-53 of A subunit 128-147 of A subunit	118	Frank *et al*. 1998
eaeA	ATATCCGTTTTAATGGCTATCT AATCTTCTGCGTACTGTGTTCA	992-1013 of eaeA 1395-1416 of eaeA	425	Frank *et al*. 1998

* PCR condition: 35 × (94°C for 60 s, 50°C for 60 s, 72°C for 90 s)

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by using the disk diffusion method and interpreted according to the standards recommended by CLSI, for the following antimicrobial agents including ampicillin (10 µg), ceftifor (30 µg), colistin (10 µg), erythromycin (15 µg), florfenicol (30 µg), gentamicin (10 µg), lincospectin (15/200 µg), neomycin (30 µg), tetracyclin (30 µg), penicillin (10 IU), sulfamethoxazole/ trimethoprim (1.25/23.75 µg), streptomycin (10 µg), tylosin (30 µg), and enrofloxacin (5 µg). The results were interpreted in accordance with interpretive criteria provided by CLSI (2008). *Escherichia coli* ATCC 25922 was used as a quality control strain.

Results

As shown in Table 2 of the 60 *E. coli* isolated from examined samples, 13 (21.6%) of them were STEC. The results of PCR assay showed that *eaeA* gene was carried by 4 (30.8%) of the STEC isolates. Although *stx1* in combination with *eaeA* gene were detected from 7 (53.8%) of STEC isolates, *stx1* and *stx2* genes were detected from only 1 (7.7%) of the examined samples. Furthermore, the present study revealed that there was only 1 (7.7%) STEC isolate that possessed all three identified virulence genes (Table 2). Figure 1 shows the virulence genes of some STEC isolates. The result of the disk diffusion method showed that all *E. coli* isolates were resistant to penicillin, tylosin, oxytetracyclin, erythromycin, ampicillin, streptomycin and neomycin. However, all isolates indicated susceptibility to enrofloxacin (Table 3). Additionally, multi-drug resistance was found among *E. coli* isolates (Table 3). Sixteen and eight multi-drug resistance patterns were observed for *E. coli* and STEC isolates respectively. According to the results, the pattern number 15 in Table 3 demonstrated the highest rate of multi-drug resistance.

Table 2: Frequency of STEC virulence genes

Virulence gene(s)	Number (%)
stx1	0 (0)
stx2	0 (0)
eaeA	4 (30.8)
stx1 and *stx2*	1 (7.7)
stx1 and *eaeA*	7 (53.8)
stx2 and *eaeA*	0 (0)
stx1, *stx2* and *eaeA*	1 (7.7)

Discussion

In the present study, 13 (21.6%) of the *E. coli* isolates were STEC which was higher than the previous report in Iran (Mansouri-Najand and Khalili, 2007) but lower than

Table 3: Multi-drug resistance patterns in 60 *E. coli* isolated from clinical mastitis

Number	Antibiotics resistance patterns	Number (%) of multi-resistant isolates	
		All *E. coli* isolates	STEC isolates
1	P, Ty, Ot, E, Am, S, N, Sxt	2 (3.3%)	0 (0%)
2	P, Ty, Ot, E, Am, S, N, Sxt, Ls	1 (1.7%)	0 (0%)
3	P, Ty, Ot, E, Am, S, N, FF, Ls	3 (5%)	2 (15.4%)
4	P, Ty, Ot, E, Am, S, N, Gm, Ls	2 (3.4%)	0 (0%)
5	P, Ty, Ot, E, Am, S, N, Sxt, Cf, Cl	4 (6.7%)	1 (7.7%)
6	P, Ty, Ot, E, Am, S, N, FF, Ls, Cl	2 (3.3%)	1 (7.7%)
7	P, Ty, Ot, E, Am, S, N, FF, Ls, Cf	5 (8.3%)	0 (0%)
8	P, Ty, Ot, E, Am, S, N, Gm, FF, Cf	2 (3.3%)	0 (0%)
9	P, Ty, Ot, E, Am, S, N, Sxt, FF, Ls, Cf	3 (5%)	1 (7.7%)
10	P, Ty, Ot, E, Am, S, N, Sxt, Gm, FF, Cf	5 (8.3%)	1 (7.7%)
11	P, Ty, Ot, E, Am, S, N, Sxt, Gm, FF, Ls	3 (5%)	0 (0%)
12	P, Ty, Ot, E, Am, S, N, Sxt, Gm, Cf, Cl	4 (6.7%)	1 (7.7%)
13	P, Ty, Ot, E, Am, S, N, Gm, FF, Ls, Cl	2 (3.3%)	0 (0%)
14	P, Ty, Ot, E, Am, S, N, Sxt, Gm, FF, Ls, Cf	4 (6.7%)	0 (0%)
15	P, Ty, Ot, E, Am, S, N, Sxt, Gm, FF, Cf, Cl	16 (26.7%)	5 (38.4%)
16	P, Ty, Ot, E, Am, S, N, Gm, FF, Ls, Cf, Cl	2 (3.3%)	1 (7.7%)

P: Penicillin, Ty: Tylosin, Ot: Oxytetracyclin, E: Erythromycin, Am: Ampicillin, S: Streptomycin, N: Neomycin, Sxt: Sulfamethoxazole-trimethoprim, Gm: Gentamicin, FF: Florfenicol, Ls: Lincospectin, Cf: Ceftifor, and Cl: Colistin

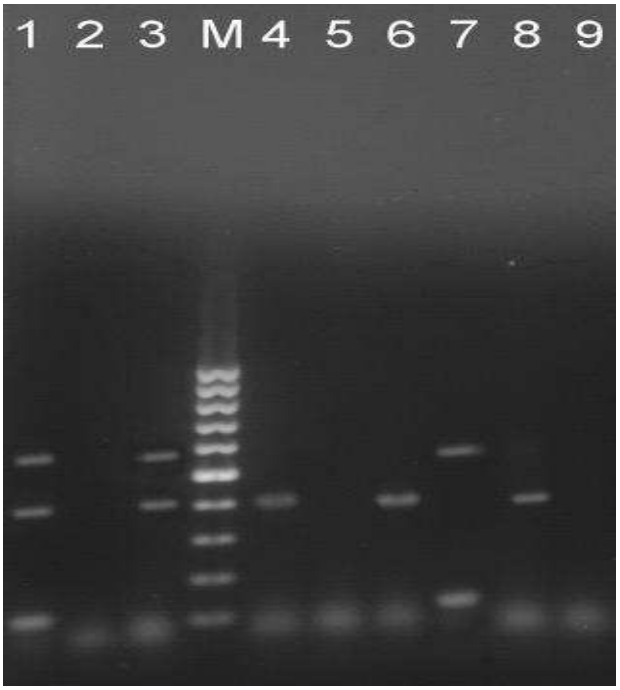

Fig. 1: Agarose (1%) gel electrophoresis of STEC PCR products of virulence factors genes. Lane M: 100 bp DNA marker. Lane 1: Positive isolate for *stx1, eaeA* and *stx2* (555, 425 and 118 bp), Lanes 2, 5 and 9: Negative control and negative isolates, Lane 3: Positive isolate for *stx1* and *eaeA*, Lanes 4, 6 and 8: Positive isolates for *eaeA*, and Lane 7: Positive isolate for *eaeA* and *stx2*

those found in Belgium (Vivegnis *et al*., 1999) and another investigation in Iran (Momtaz *et al*., 2012). According to the PCR assay the *eaeA* gene was carried by 4 (30.8%) of the STEC isolates. Higher result (33.3%) was reported in previous study in Iran (Momtaz *et al*., 2010). Numerous previous investigations have proved that there is a direct relationship between the presence of *eaeA* gene and the capacity of STEC to cause severe human disease, especially HUS (Beutin *et al*., 2004). Furthermore, the results indicated that *stx1* and *stx2* genes were detected from only 1 (7.7%) of the examined samples which was much lower than that found in the study conducted by Momtaz (2010). Another investigation on mastitis milk samples demonstrated that *stx1* gene was the predominant virulence factor with a prevalence of 31% and 35%, respectively (Seyda *et al*., 2014), while according to our results this virulence gene was carried by none of the STEC isolates. The significant difference between the reports was associated with the origin of the strains (Seyda *et al*., 2014). Moreover, the result of the disk diffusion method showed that all *E. coli* isolates were resistant to penicillin, tylosin, oxytetracyclin, erythromycin, ampicillin, streptomycin and neomycin. However in contrast with Seyda *et al*.'s study (2014), all isolates indicated susceptibility to enrofloxacin. It reveals that enrofloxacin is less applied in farm animal medicine in Iran and can be more effective for treatment of clinical mastitis caused by *E. coli*. Similar to our study, resistance to some of the above antibiotics has also been frequently reported in several previous investigations. However, the results of these studies showed lower resistance rate than our findings (Stephan *et al*., 2008; Momtaz *et al*., 2012; Dubravka *et al*., 2015). Multi-drug resistance was found among all *E. coli* isolates (Table 3). In addition to the present study, multiple antibiotic resistances have been indicated in numerous previous researches around the world (Rangel and Marin, 2009; Spnu *et al*., 2012). The high rate of antimicrobial resistance identified in this study can be explained by the widespread use of common antimicrobials in dairy farms for treatment of clinical mastitis. Our findings, similar to previous investigations indicated that cattle with clinical mastitis are recognized as the main reservoir of STEC. Therefore, establishing a regular monitoring system for identification of cases with clinical mastitis, restriction of widespread use of common antibiotics as well as conducting antibiotic sensitivity tests is recommended for reducing the prevalence of resistant strains of STEC in industrial dairy herds.

Conflict of interest

No conflict of interests is declared.

References

Beutin, L; Krause, G; Zimmermann, S; Kaulfuss, S and Gleier, K (2004). Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. J. Clin. Microbiol., 42: 1099-1108.

Bradley, AJ and Green, MJ (2001). Etiology of clinical mastitis in six somerset dairy herds. Vet. Rec., 148: 683-686.

CLSI (2008). *Performance and standards for antimicrobial disk diffusion and dilution susceptibility tests for bacteria isolated from animals; approved standards*. 3rd Ed., Vol. 28, M31-A3. Clinical and Laboratory Standard Institute, Wayne, Pennsylvania, USA.

Dubravka, M; Bojana, P; Maja, V; Dalibor, T and Vladimir, P (2015). Investigation of biofilm formation and phylogenetic typing of *Escherichia coli* stains isolated from milk of cows with mastitis. Acta Vet. Beograd., 65: 202-216.

Frank, SM; Bosworth, BT and Moon, HW (1998). Multiplex PCR for enterotoxigenic, attaching and effacing, and Shiga toxin-producing *Escherichia coli* strains from calves. J. Clin. Microbiol., 36: 1795-1797.

Guler, L and Gündüz, K (2007). Virulence properties of *Escherichia coli* isolated from clinical bovine mastitis. Turk. J. Vet. Anim. Sci., 31: 361-365.

Kobori, D; Rigobelo, EC; Macedo, C; Marin, JM and Avila, FA (2004). Virulence properties of Shiga toxin-producing *Escherichia coli* isolated from cases of bovine mastitis in Brazil. Rev. Elev. Med. Vet. Pays. Trop., 57: 15-20.

Mansouri-Najand, L and Khalili, M (2007). Detection of Shiga-like toxigenic *Escherichia coli* from raw milk cheeses produced in Kerman-Iran. Veterinarski. Arhiv., 77: 515-522.

Momtaz, H (2010). Investigation of virulence factors in *Escherichia coli* isolated from clinical and subclinical

bovine mastitis. Bulg. J. Vet. Med., 13: 122-126.

Momtaz, H; Dehkordi, SF; Taktaz, T; Rezvani, A and Yarali, S (2012). Shiga toxin-producing *Escherichia coli* isolated from bovine mastitic milk: serogroups, virulence factors, and antibiotic resistance properties. Sci. World J., 2012: 618709.

Pourtaghi, H; Dahpahlavan, V and Momtaz, H (2013). Virulence genes in *Escherichia coli* isolated from calves with diarrhoea in Iran. Com. Clin. Pathol., 12: 513-515.

Pourtaghi, H and Sodagari, HR (2016). Antimicrobial resistance of entrotoxigenic and non-entrotoxigenic *Escherichia coli* isolated from diarrheic calves in Iran. Int. J. Enteric. Pathog., 4: e34557.

Rangel, P and Marin, JM (2009). Analysis of *Escherichia coli* isolated from bovine mastitic milk. Pesq. Vet. Bras., 29: 363-368.

Sawant, AA; Hegde, NV; Straley, BA; Donaldson, SC; Love, BC; Knabel, SJ and Jayarao, BM (2007). Antimicrobial-resistant enteric bacteria from dairy cattle. Appl. Environ. Microbiol., 73: 156-163.

Seyda, C; Gökçen, D and Ünlü, SM (2014). Detection of several virulence properties, antibiotic resistance and phylogenetic relationship in *E. coli* isolated from cow mastitis. Acta Vet. Beograd., 64: 413-425.

Spnu, M; Kobolkuti, L; Cadar, D; Niculae, M; Bianu, G; Popescu, S and Lukacs, L (2012). Changes in antibiotic resistance indices of animal *Escherichia coli* strains with number of isolates. Ann. Rom. Soc. Cell. Biol., 17: 361-366.

Stephan, R; Schumacher, S; Corti, S; Krause, G; Danuser, J and Beutin, L (2008). Prevalence and characteristics of Shiga toxin producing *Escherichia coli* in Swiss raw milk cheeses collected at producer level. J. Dairy. Sci., 91: 2561-2565.

Vivegnis, J; Lioui, EM; Leclercq, A; Lambert, B and Decallonne, J (1993). Detection of Shiga-like toxin producing *Escherichia coli* from raw milk cheeses produced in Wallonia. Biotechnol. Agron. Soc. Environ., 3: 159-164.

Wenz, JR; Barrington, GM; Garry, FB; Ellis, RP and Magnuson, RJ (2006). *Escherichia coli* isolates' serotypes, genotypes, and virulence genes and clinical coliform mastitis severity. J. Dairy. Sci., 89: 3408-3412.