

Detection of virulence genes and determination of the antimicrobial susceptibility of *Escherichia coli* isolates with mastitis in Mashhad, Iran – a short communication

Fateme Aflakian¹, Mehrnaz Rad¹, Himen Salimizand^{2,3}, Ali Nemati¹, and Abolfazl Rafati Zomorodi^{4,5*}

¹Department of Pathobiology, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

²Vaccine and Infectious Disease Organization Saskatoon, SK, Canada

³Department of Vaccinology and Immunotherapeutics, School of Public Health, University of Saskatchewan, Saskatoon, SK, Canada

⁴Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Science, Shiraz, Iran

⁵Student Research Committee, Shiraz University of Medical Science, Shiraz, Iran

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ABSTRACT

The purpose of this study was to determine the virulence genes and antimicrobial resistance patterns of *Escherichia coli* isolated from milk samples of cows with bovine mastitis. Forty-seven *E. coli* isolates from clinical mastitis milk samples, from five dairy farms in Northeast of Iran, were subjected to multiplex PCR to determine virulence genes *stx1*, *stx2*, *eaeA*, *hlyA*, *sta*, *F4*, *F17*, *fliC*, and *rfbE*. In addition, antimicrobial susceptibility was assessed by applying disk diffusion methods. The *eaeA* and *stx1* genes were most frequently detected in 42 (89.3%) and 34 (72.3%) isolates, respectively. However, the least frequent gene was *F4I* as it was found in only one isolate (2.1%). Furthermore, 9 out of 47 isolates were *hlyA* positive, and four isolates harbored the *sta* gene. The antimicrobial susceptibility demonstrated the highest resistance against lincomycin (100%) and neomycin (91.4%). Since these bacteria represent a high-risk pathogen on farms, the emergence of multiple antibiotic-resistant and pathogenic *E. coli* strains should be of great concern for public health.

Key words: *E. coli*, virulence genes; antimicrobial resistance; mastitis

Introduction

Mastitis causes a large amount of economic loss since it causes medical and veterinary expenses and through declining milk production, disposal of milk from cows treated with antibiotics, and ultimately the extirpation of dairy cattle in herds (ZAFARANE et al., 2017). *E. coli* is a Gram-

negative, opportunistic bacterium, which is commonly found in the environment of dairy cows, that infects the mammary glands through environmental contact (TODOROVIĆ et al., 2018). However, non-pathogenic *E. coli* strains are used in dairy products for several metabolic activities

*Corresponding author:

Abolfazl Rafati Zomorodi, Department of Bacteriology and Virology, Shiraz Medical School, Shiraz University Medical Science, Shiraz, Iran

(MLADENOVIĆ et al., 2018). Although therapy with antibiotics can significantly reduce *E. coli* mastitis, antimicrobial resistance has arisen due to the use of broad-spectrum antimicrobial agents for coliform mastitis treatment (FERNANDES et al., 2011). Antibiotic monitoring should be undertaken to detect and control microbial resistance. This study was carried out to assess the major virulence genes and antibiotic susceptibility profile of *E. coli* isolates from milk samples from dairy cattle with clinical mastitis in Mashhad, North-East Iran.

Materials and methods

Bacterial identification. In the present study, 47 *E. coli* isolates were collected from October 2015 to December 2016, from clinical mastitis milk samples from five different farms in Mashhad. Standard biochemical tests, as published by MLADENOVIĆ et al. (2018) were carried out in order to identify *E. coli* isolates. The isolates were then preserved at -20 °C in nutrient broth with 15% glycerol in the microbiology laboratory of the Veterinary Faculty, Ferdowsi University of Mashhad.

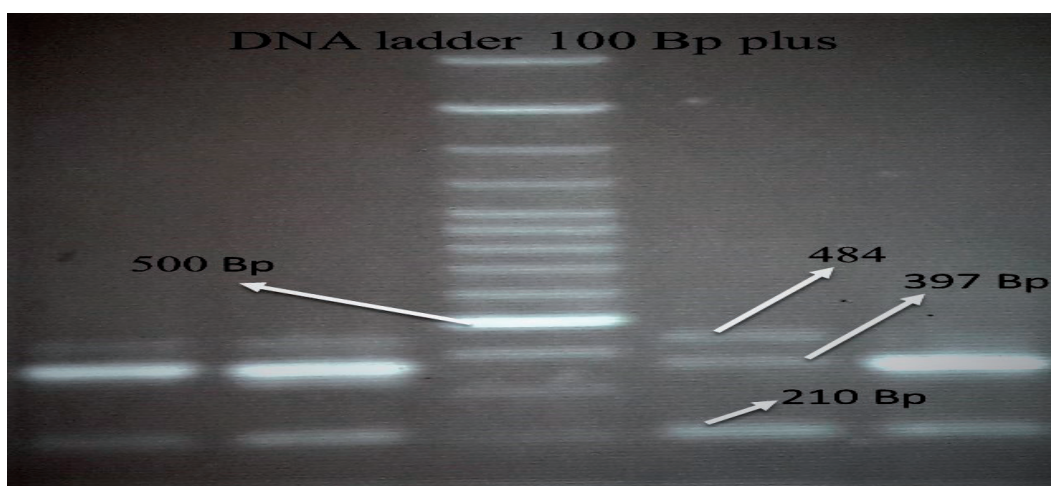


Fig. 1. The detection of *stx1*, *stx2*, *eaeA* and *hlyA* genes by multiplex PCR. *stx1* amplicon size, 210bp; *stx2* amplicon size, 484bp; *eaeA* amplicon size, 397 bp; *hlyA* amplicon size 166 bp.

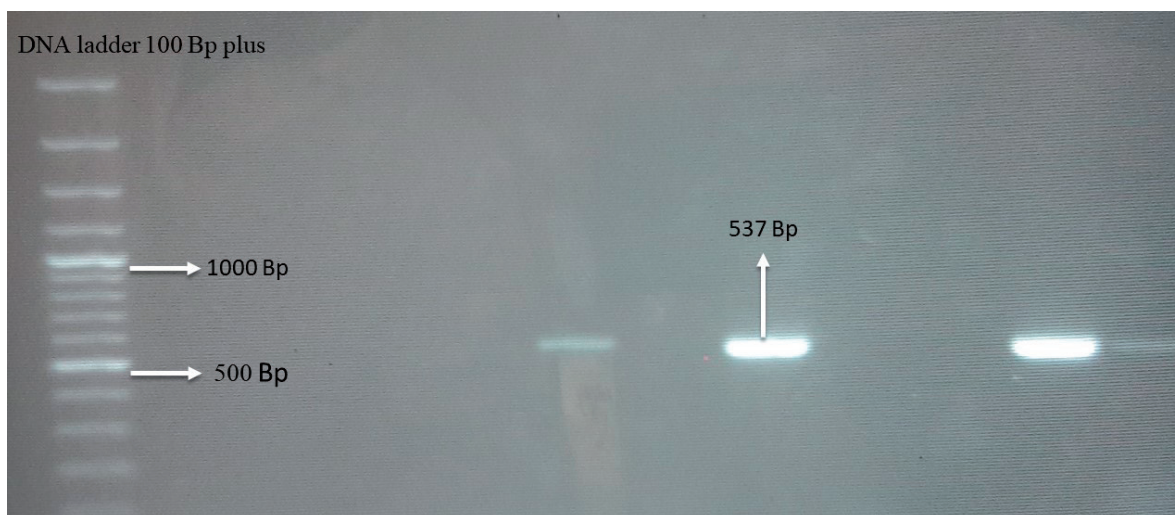


Fig. 2. The detection of *F17* gene by monoplex PCR.

Antimicrobial Susceptibility Testing. The evaluation of antimicrobial susceptibility was carried out with the Kirby-Bauer disk diffusion method (HUDZICKI, 2009) for 10 antibiotic disks (MAST, United Kingdom) as follows: linco-spectin (15/200 µg), gentamicin (10 µg), lincomycin (2 µg), ampicillin (10 µg), oxytetracycline (30 µg), enrofloxacin (5 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg), streptomycin (10 µg), and neomycin (30 µg).

Detection of the Virulence Genes. The multiplex PCR assay described by FRATAMICO et al. (2000) was performed to assess nine virulence genes, including: *stx1* and *stx2*, *eaeA*, *hlyA*, *sta*, F4, F17, *fliC* and *rfbE*. In this order, genomic DNA was extracted by the boiling method (AHMED et al., 2014). Further information regarding primers is given in Table 1 (BERTIN et al., 1996; DONG et al., 2015; FRANCK et al., 1998).

Table 1. Sequences of the oligonucleotides used as primers in multiplex PCR and monoplex PCR

PCR name	Primer name	Direction	Primer sequence (5'-3')	Product size (bp)	Reference
PCR1	<i>F41</i>	Forward	GCATCAGCGGCAGTATCT	380	8
		Reverse	GTCCCTAGCTCAGTATTATCACCT		
	<i>Sta</i>	Forward	GCTAATGTTGGCAATTTTTATTTCTGTA	190	8
PCR2	<i>Stx1</i>	Reverse	AGGATTACAACAAAGTTCACAGCAGTAA	210	9
		Forward	TGTAACCTGGAAAGGTGGAGTATACA		
	Reverse	GCTATTCTGAGTCAACGAAAATAAC			
	<i>Stx2</i>	Forward	GTTTTTCTTCGGTATCCTATTCC	484	9
		Reverse	GATGCATCTCTGGTCATTGTATTAC		
<i>eaeA</i>	Forward	ATTACCATCCACACAGACGGT	397	9	
Reverse	ACAGCGTGGTTGGATCAACCT				
<i>hlyA</i>	Forward	ACGATGTGGTTTATTCTGGA	166	9	
	Reverse	CTTCACGTCACCATACATAT			
PCR3	<i>F17</i>	Forward	GCAGAAAATTCAATTTATCCTTGG	537	10
Reverse		CTGATAAGCGATGGTGTAAATTAAC			
PCR4	<i>rfbE</i>	Forward	GGATGACAAATATCTGCGCTGC	213	9
		Reverse	GGTGATTCTTAATTCCTCTCTTTCC		
<i>fliC-H7</i>	Forward	Reverse	GCGCTGTCGAGTTCTATCGAGC	625	9
		Reverse	CAACGGTGACTTTATCGCCATTCC		

Results

Antimicrobial susceptibility testing. The interpretation of the antimicrobial susceptibility test was performed according to EUCAST 2018 guidelines. The disc diffusion method revealed the highest resistance against lincomycin and neomycin

in 47 (100%) and 43 (91.4%) isolates, respectively. Also, the least resistance was demonstrated for gentamycin and ampicillin in zero and one (2.13%) isolate, respectively. The results of resistotyping are listed in Table 2.

Table 2. Susceptibility testing of *E. coli* isolates (n=47). Results presented as percentages.

Antibiotic disks	LSP	NFX	AMP	STR	TET	GEN	OTE	TMP	NEO	LIN
Susceptible	76.6	87.2	82.9	76.5	68	100	80.8	85.1	0	0
Resistant	23.4	12.8	17.1	23.4	31.9	0	19.1	14.8	100	100

Abbreviations: LSP, linco-spectin; NFX, enrofloxacin; AMP, ampicillin; STR, streptomycin; TET, tetracycline; GEN, gentamycin; OTE, oxytetracycline; TMP, Trimethoprim/sulfamethoxazole; NEO, neomycin; LIN, lincomycin.

The frequency of virulence genes. The most frequent genes, among all the isolates, as determined by PCR, were *eaeA* 42 (89.3%) and *stx1* 34 (72.3%). The least frequent gene was *F41*, in one isolate (2.1%). The frequencies of *stx2*, *hlyA*, *F17* and *sta* genes in *E. coli* isolates were found to be in 26 (55.3%), 9 (19.1%), 6 (12.7%) and 4 isolates (8.5%), respectively. In addition, 12 (25.5%) and four (8.5%) isolates carried only *rfbE* and *fliC*, respectively.

Discussion

In the present paper we investigated the presence of virulence genes among pathogenic *E. coli* strains that were collected from dairy cattle with clinical mastitis. There were non-pathogenic *E. coli* strains used in the processing of dairy products, so these commensal strains are capable to turn into the wide strains by transferring virulence genes (BOK et al., 2015). In the current study, the prevalence of *stx1* and *stx2* genes were (72.3%) and (55.3%), respectively. The results of our study were in accordance with previous research that reported the prevalence of *stx1* at the rate of 35.7% (CENGIZ et al., 2014). However, ZAFARANE et al. (2017) and IWERIEBOR et al. (2015) observed greater distribution of *stx2* than *stx1* in their studies.

Intimin was encoded by the *eaeA* gene which produces a bacterial outer-membrane protein associated with the intimate connection of the bacteria to the gut mucosa of the host (BEAN et al., 2004). In our study, 42 isolates (89.3%) were *eaeA* positive, in contrast to previous studies that recorded the *eaeA* gene in 3.8% - 66% of isolates (TAVAKOLI and POURTAGHI, 2017; ZAFARANE et al., 2017). In this study, nine isolates (19.1%) were observed as *hlyA* positive. This finding was in accordance with another survey in Iran, which reported 87.3 % of *hlyA* positive

isolates from fecal samples of diarrheic children, sheep, and cattle (BADOUEI et al., 2016). The fimbriae *F41* and *F17*-related adhesins were the most common in *E. coli* isolates from diarrheic calves (KIM et al., 2016). The incidences of *F41* and *F17* were 2.1% and 12.7% in the current study, and these results were close to previous studies (GÜLER and GÜNDÜZ, 2007; MEMON et al., 2016). According to these reports, it seems that two *F17* and *F47* genes do not play any important role in mastitis infections. In this study, *fliC* was detected in four isolates (8.5%), while in another survey this gene was found in 49% *E. coli* isolates from raw milk samples (CAINE et al., 2014). The differences in the presence of virulence genes in *E. coli* depend on sampling strategy, seasonal variations, geographic area, hygiene on farms and the number of cows included for sampling.

The resistance rate to enrofloxacin and ceftriaxone was similar to the report by IWERIEBOR et al. (2015), but the observation of resistance to ampicillin in *E. coli* isolates was dissimilar to another study since those authors observed a high resistance rate in samples from raw milk in mastitis cases (FERNANDES et al., 2011; ZAFARANE et al., 2017). Resistance to tetracycline and oxytetracycline was detected in 31.9% and 19.1% of isolates, respectively, but it was determined that the resistance rate was not as high as in previous studies since there 80-90% isolates were resistance to these antibiotics (IWERIEBOR et al., 2015; KEANE, 2016). Also, in earlier investigations a higher resistance rate to cotrimoxazole was recorded (FERNANDES et al., 2011; ZAFARANE et al., 2017).

The differences between studies might be related to variations in geographic region, the ages of the cows, different serotypes, and the use of different antibiotics for therapy on the farms.

Thus, organizing an orderly monitoring system for recognition of cases of clinical mastitis, restriction of the widespread use of common antibiotics, and assessing antibiotic susceptibility are suggested to decrease the prevalence of resistant strains in industrial dairy herds.

There is a possibility that virulence genes and antibiotic-resistance genes exist on the same plasmids, so transfer of these genes could happen together. Therefore, the strains which had a resistant gene, had more than one virulence gene, although in this survey, antibiotic resistance was not assessed at the genomic level, genomic resistance determinants should be analyzed in the future.

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Conflict of interest

None to declare.

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SAŽETAK

Cilj je istraživanja bio odrediti gene virulencije i antimikrobnu rezistencije bakterije *Escherichia coli* izolirane iz uzoraka mlijeka krava s mastitisom. Ukupno 47 izolata bakterije *E. coli* iz uzoraka mlijeka krava s kliničkim mastitisom, s pet mliječnih farmi u sjeveroistočnom Iranu, podvrgnuto je protokolu multipleks PCR-a kako bi se odredili geni virulencije *stx1*, *stx2*, *eaeA*, *hlyA*, *sta*, *F4*, *F17*, *fliC* i *rfbE*. Antimikrobna je osjetljivost procijenjena primjenom disk-difuzijske metode. Najčešće određeni geni jesu gen *eaeA*, u 42 izolata (89,3 %) i gen *stx1*, u 34 izolata (72,3 %). Najrjeđi gen bio je *F41*, koji je pronađen u jednom izolatu (2,1 %). Nadalje, 9 od 47 izolata bilo je *hlyA* pozitivno, a četiri su izolata sadržavala gen *sta*. Procjena antimikrobne je osjetljivosti pokazala je najveću rezistenciju na linkomicin (100 %) i neomicin (91,4 %). Nalazi upućuju da se radi o visokorizičnim patogenima na farmama krava, stoga bi pojava višestruko rezistentnih i patogenih sojeva *E. coli* trebala izazvati veliku javnozdravstvenu zabrinutost.

Ključne riječi: *E. coli*; virulencija gena; antimikrobna rezistencija; mastitis
