VETERINARSKI ARHIV 93 (5), 503-512, 2023 DOI: 10.24099/vet.arhiv.1814

Phenotypic and genotypic investigation of antimicrobial resistance and extended-spectrum beta-lactamase production among *Escherichia coli* isolated from bovine mastitis

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AFLAKIAN, F., N. MOHSENI, M. HAFIZ, H. NIKOUEIAN, M. A. BADOUEI, A. R. ZOMORODI: Phenotypic and genotypic investigation of antimicrobial resistance and extended-spectrum beta-lactamase production among *Escherichia coli* isolated from bovine mastitis. Vet. arhiv 93, 503-512 2023.

ABSTRACT

Escherichia coli has been one of the most highlighted pathogens isolated from clinical cases of mastitis. Unfortunately, the deficiency of appropriate antimicrobial stewardship and overuse of antimicrobial agents have increased antimicrobial resistance in animal isolates. The current study aimed to demonstrate the prevalence of extended-spectrum beta-lactamase (ESBL) production, resistance genes, and multi drug resistant (MDR) among *E. coli* isolates from cases of clinical mastitis. For this purpose, 40 *E. coli* isolates were collected from clinical mastitis milk samples from five farms in Mashhad, Iran. The antimicrobial susceptibility testing (AST) was applied to 15 antimicrobial agents of veterinary and human medicine interest. Also, ESBL production was evaluated using a double-disc synergy test (DDST). The distribution of 20 resistance genes was sought among *E. coli* isolates by six multiplex-PCR and three uniplex-PCR assays. The highest sensitivity was identified against imipenem and amikacin (100%). On the other hand, the highest resistance was observed for tetracycline and trimethoprim-sulfamethoxazole (70% - 72.5%), respectively. According to the AST and DDST tests, one isolate was confirmed as ESBL-producing and MDR. In addition, the most frequent resistance genes were *bla*TEM and *AmpC* (100% each). The *qnrA encoding resistance to quinolones* was similarly prevalent and detected in 50% of the isolates. In conclusion, at least three resistant genes were detected in 28 isolates (70%), but the majority of isolates were sensitive against most of the tested antibiotics. This fact might relate to the low expression of these genes within the isolates. The horizontal gene transfer of the present genes may confer resistance to other related bacterial species in humans or domestic animals.

Key words: *E. coli;* antimicrobial susceptibility; mastitis; ESBL; resistance genes

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ISSN 0372-5480
Printed in Croatia

Introduction

Mastitis is a significant disease of worldwide importance in dairy farming (MAĆEŠIĆ et al., 2012; SERDAL and FUNDA, 2021). *Escherichia coli*, a gram-negative opportunistic, environmental, rod-shaped bacterium, is known as one of the most highlighted pathogens isolated from clinical cases of mastitis [Cebron, 2020 #2](CEBRON et al., 2020; ZHANG et al., 2020). Mastitis causes significant economic losses, such as decreased milk production, increases in expenditure on veterinary services, and increases in mortality rates (CVETNIĆ et al., 2016; KNEŽEVIĆ et al., 2021; WANG et al., 2021). In addition, *E. coli* isolates from dairy products are indicated as a cause of intense foodborne diseases such as bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (SCHNUR et al., 2021). Currently, antimicrobial therapy is used for preventing and treating mastitis infections (BENIĆ et al., 2018; RUEGG, 2021). Unfortunately, the deficiency of appropriate antimicrobial stewardship and overuse of antimicrobial agents have caused increasing antimicrobial resistance (MAĆEŠIĆ et al., 2016; JEAMSRIPONG et al., 2021). Beta-lactams, sulphonamides, quinolones, aminoglycosides, tetracycline, and macrolides are extensively used as routine antimicrobial agents (CHENG and HAN, 2020). Due to their commensal nature and presence in the environment, enteric bacteria readily transfer resistance genes to other species of bacteria, and therefore, the World Health Organization (WHO) has noted the pivotal role of *E. coli* as a remarkable organism in the development of antimicrobial resistance (ROTH et al., 2019; CVETNIĆ et al., 2021). In particular, extended-spectrum β-lactamase-producing *E. coli* strains have been identified as a global concern for increasing resistance against β-lactam antibiotics (OMBARAK et al., 2021). Furthermore, the emergence and spread of Carbapenem resistance Enterobacteriaceae (CRE) has recently become a serious problem (PUMIPUNTU and PUMIPUNTU, 2020). As a result, the increase in multidrug-resistance (MDR) and extensive drug-resistance (XDR) has limited the success of antimicrobial therapy (LIU et al., 2020; VANSIA et al., 2021). There is a lack of any comprehensive survey to assess antimicrobial resistance among *E. coli* isolates from mastitis in Iran. Therefore, the current study aimed to demonstrate the prevalence of ESBL production, resistance genes, and MDR among *E. coli* isolates from clinical mastitis cases in this area.

Materials and methods

Sample Collection. Forty *E. coli* isolates were collected from clinical mastitis milk samples from five industrial farms in Mashhad, Iran. All isolates were identified *as E. coli* by biochemical standard tests, earlier described by KAYASTHA et al. (2020), such as the following: gram staining, catalase, oxidase tests, and cultured on blood agar, MacConkey, Eosin methylene blue (EMB), then incubated overnight at 37 °C. The confirmation of the *E. coli* isolates was performed on the basis of polymerase chain reaction (PCR) using 16SrRNA primers (WANG et al., 2002). Finally, *E. coli* isolates were preserved at -20 °C in a brain/heart infusion broth containing 15% glycerol.

Antimicrobial Susceptibility Testing. The Kirby-Bauer disc diffusion method was performed to determine antimicrobial susceptibility testing (AST) as recommended by the Clinical & Laboratory Standards Institute (CLSI., 2021) for 15 antibiotic discs of veterinary and human medicine interest (Padtanteb, Iran), as follows: cefpodoxime (30 µg), cefotaxime (30µg), ceftazidime (30µg), cefoxitin (30µg), cefepime (30µg), amoxicillin-clavulanic acid (30/10µg), imipenem (10µg), norfloxacin (10µg), ciprofloxacin (5µg), nalidixic acid (30µg), gentamycin (10µg), amikacin (30µg), azithromycin (15µg), tetracycline (30µg), trimethoprim-sulphamethoxazole (1.25/23075µg). *Escherichia coli* ATCC 25922 was used as the positive control strain.

ESBL Detection. Evaluation of ESBL producing isolates was performed using a double-disc synergy test (DDST). As described earlier by SILAGO et al. (2021), the cephalosporin discs (cefotaxime, ceftazidime, cefoxitin, and cefepime) were placed around the amoxicillin-clavulanic acid disc at a 20mm distance. After overnight incubation at 37 °C, any deformation of the inhibition zone towards the

amoxicillin-clavulanic acid disc was reported to be positive. *Klebsiella pneumonia* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as positive and negative control strains, respectively.

Molecular Detection of Antimicrobial Resistance. The genomic DNA of all 40 *E. coli* isolates was extracted using the boiling method, as explained previously by AHMED and DABLOOL (2017). The distribution of 20 resistance genes was sought among the *E. coli* isolates by six multiplex-PCR and three uniplex-PCR. Each PCR reaction comprised: 12.5 µL of PCR 2× Master Mix (Parstous, Iran) containing Taq DNA Polymerase, reaction buffer, dNTPs mixture, protein stabilizer, and its convenience for use was optimized by adding sediment for electrophoresis and 2× solution of loading dye, 1 µL of each primer (2 µM), 2 µL of template DNA, and to nuclease-free water was added up to 25 µL final volume. The properties of the oligonucleotides primers are listed in Table 1.

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

Primer's name	Gene	Primer sequence (5´-3´)	Product size, bp	Annealing Temperature, °C	Reference
TEM-F	*bla*TEM	TCG CCG CAT ACA CTA TTC TCA GAA TGA	445bp	61 °C	(OMBARAK et al., 2021)
TEM-R		ACG CTC ACC GGC TCC AGA TTT AT			
CTX-M-F	*bla*CTX-M	ATG TGC AGC ACC AGT AAA GTG ATG GC	593bp		
CTX-M-R	*bla*SHV	TGG GTA AAG TAA GTG ACC AGA ATC	747bp		
SHV-F		AGC GGATG CGT TAT ATT CGC CTG TG			
SHV-F		TGC TTT GTT ATT CGG GCC AA			
NDM-F	*bla*NDM	GGTTTGGCGATCTGGTTTTC	621bp	52 °C	(POIREL et al., 2011)
NDM-R		CGGAATGGCTCATCACGATC			
OXA-F	*bla*OXA-48	GCGTGGTTAAGGATGAACAC	438bp		
OXA-R		CATCAAGTTCAACCCAACCG			
KPC-F	*bla*KPC	CGTCTAGTTCTGCTGTCTTG	798bp		
KPC-R		CTTGTCATCCTTGTTAGGCG			
IMP-F	*bla*IMP	GGAATAGAGTGGCTTAAYTCTC	232bp	52 °C	(POIREL et al., 2011)
IMP-R		GGTTTAAYAAAACAACCACC			
VIM-F	*bla*VIM	GATGGTGTTTGGTCGCATA	390bp		
VIM-R		CGAATGCGCAGCACCAG			
AAC(6')-Ib-F	*aac(6')-Ib*	TATGAGTGGCTAAATCGAT	395bp	54 °C	(LIU et al., 2020)
AAC(6')-Ib-R		CCCGCTTTCTCGTAGCA			
APH(3')-Ia-F	*aph(3')-Ia*	CGAGCATCAAATGAAACTGC	623bp		
APH(3')-Ia-R		GCGTTGCCAATGATGTTACAG			
AAC(3)-IIa-F	*aacC2*	ATGCATACGCGGAAGGC	822bp	49 °C	(LIU et al., 2020)
AAC(3)-IIa-R		TGCTGGCACGATCGGAG			
ant(2")-Ia-F	*aadB*	ATCTGCCGCTCTGGAT	404bp		
ant(2")-Ia-R		CGAGCCTGTAGGACT			

Table 1. Sequence of oligonucleotides used as primers in the multiplex PCR and monoplex PCR (continued)

Primer's name	Gene	Primer sequence (5´-3´)	Product size, bp	Annealing Temperature, °C	Reference
tet(A)-F tet(A)-R tet(B)-F tet(B)-R	*tet(A)* *tet(B)*	GTGAAACCCAACATACCCC GAAGGCAAGCAGGATGTAG CCTTATCATGCCAGTCTTGC ACTGCCGTTTTTTCGCC	888bp 774bp	50 °C	(OMBARAK et al., 2021)
qnrA-F qnrA-R qnrB-F qnrB-F qnrS-F qnrS-R	*qnrA* *qnrB* *qnrS*	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	516bp 469bp 417bp	57 °C	(VANSIA et al., 2021)
ant(3″)Ia-F ant(3″)Ia-R	*aadA1*	ATGAGGGAAGCGGTGATCG TTATTTGCCGACTACCTTGGTG	792bp	52 °C	(PUMIPUNTU and PUMIPUNTU, 2020)
armA-F armA-R	*armA*	ATTCTGCCTATCCTAATTGG ACCTATACTTTATCGTCGTC	315bp	55 °C	(LIU et al., 2020)e>
AmpC-F AmpC-R	*AmpC*	GATCGTTCTGCCGCTGTG GGGCAGCAAATGTGGAGCAA	271bp	57 °C	(LIU et al., 2020)e>
E16S-a E16S-b	*E. coli* 16S rRNA	CCCCCTGGACGAAGACTGAC ACCGCTGGCAACAAAGGATA	401bp	58 °C	(KAYASTHA et al., 2020)

Results

Antimicrobial Susceptibility & ESBL Production. The results revealed the highest activity for imipenem, amikacin (100% each), and cefoxitin (97.5). On the other hand, the lowest activity was observed for tetracycline and trimethoprim-sulfamethoxazole (70% - 72.5%), respectively. However, there was only one ESBL positive isolate in the phenotypic test. In addition, the ESBL positive isolate was considered an MDR isolate; it was resistant against at least one antibiotic in three antibiotic classes (β-lactam, quinolone, macrolide, and tetracycline). All AST results are presented in Fig. 1.

The results of the AST were interpreted according to the (CLSI., 2021).

Prevalence of Resistance Genes. The gel electrophoresis of the PCR amplicon demonstrated the most frequent rates of resistance gene for *bla*TEM and *AmpC* at 100%, followed by *qnrA* which showed 50% frequency among the isolates. Also, no positive isolates were observed harboring the *bla*SHV, *bla*OXA-48, *bla*NDM, *bla*IMP, *bla*VIM, *tetA*, and *aadB* genes. The results of the prevalence of resistant genes are illustrated in Fig. 2.

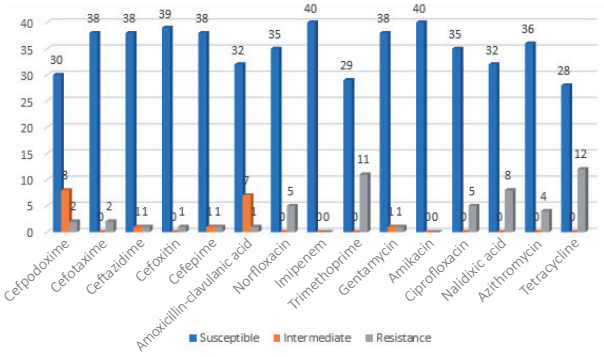

Fig 1. The results of antimicrobial susceptibility testing of *E. coli* isolates from clinical mastitis (n=40)

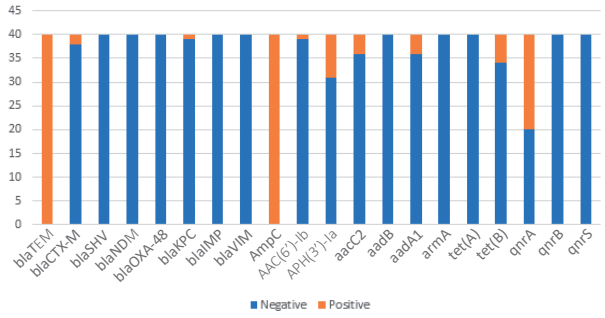

Fig 2. The prevalence of resistance genes among *E. coli* isolates from clinical mastitis (n=40)

Discussion

Mastitis is one of the most frequent diseases that results in economic problems around the world. For instance, it has been reported that dairy farmers in the EU and US lose nearly 2 billion dollars every year due to bovine mastitis (TURK et al., 2017; GRUDLEWSKA-BUDA et al., 2021; MIMOUNE et al., 2021). Additionally, antimicrobial therapy has reduced antimicrobial effectiveness in animals (BUROVIĆ, 2020; SAED and IBRAHIM, 2020). Therefore, AST surveys and genotypic characterization of resistance genes are necessary to monitor resistant rates in different geographical settings. In the current study, AST determined the highest sensitivity against imipenem and amikacin (100%) followed by gentamycin (97.5%). Although these facts are in line with earlier reports (YU et al., 2020; GRUDLEWSKA-BUDA et al., 2021), other publications found a higher rate of resistance for amikacin and gentamycin (YANG et al., 2018; USHKALOV et al., 2021). Our observation of resistance rates to tetracycline and trimethoprim of 30% and 27.5%, respectively, concurs well with a previous study conducted by YU et al. (2020). Even though these results differ from some other published studies, they found tetracycline resistance in the range of 55% - 100% (SAIDANI et al., 2018; RAMASAMY et al., 2021; USHKALOV et al., 2021). Our experiment yielded resistance of nalidixic acid (20%), and ciprofloxacin, and norfloxacin (12.5%) which was confirmed by prior literature (GRUDLEWSKA-BUDA et al., 2021; USHKALOV et al., 2021). Bacteria can become resistant to antibiotics using various resistance mechanisms, including limiting uptake, altering target sites, destruction of the agent, and active drug efflux (ARZANLOU et al., 2017). For example, β-lactam antibiotics (penicillin & cephalosporins) are hydrolyzed by Extended-spectrum β-lactamase (ESBL) or AmpC β-lactamase enzymes (HANDA et al., 2013; ALI et al., 2017). In contrast to earlier research (SHARIF et al., 2017; YANG et al., 2018), in this study there was only one ESBL-producing *E. coli* isolate that was confirmed by the double-disc synergy test. Similarly, FILIOUSSIS et al. (2020) identified only one ESBL producer of *E. coli,* similar to our study. Although our findings showed only one ESBL-producing *E. coli*, all isolates carried *the bla*TEM gene, which shows the widespread occurrence of some ESBL-encoding genes. This is similar to other studies that reported *bla*TEM as a dominant β-lactamase gene (YANG et al., 2018; YU et al., 2020). However, all isolates were negative for *bla*CTX-M and *bla*SHV, except for two that harbored *bla*CTX-M. Our findings of the prevalence of *bla*CTX-M and *bla*SHV were different from other reports (YANG et al., 2018; YU et al., 2020; RAMASAMY et al., 2021). The phenotype-genotype contradiction might be related to the low expression of the genes, mutation, and unstable tandem gene amplification (URMI et al., 2020). Whole-genome sequencing-based analysis could help to understand the association of genotype and resistance phenotype.

Regarding the carbapenemase resistance genes, including *bla*KPC, *bla*NDM, *bla*IMP, *bla*VIM, and *bla*OXA-48, no positive isolate was detected in this study. *A previous study by YADAV et al. (2021) reported the incidence of carbapenemase genes among E. coli isolates from mastitis milk samples as follows: one positive isolate for blaNDM and blaKPC, and no positive isolates for blaOXA-48*. Acetyltransferase, phosphotransferase, and adenyltransferase enzymes are responsible for aminoglycosides modification (AME) (CHRISTAKI et al., 2020). The frequency of AME genes was observed for *aph (3)-Ia* (22.5%), *aadA1*, and *aaC2* (10%), and was lower than that observed by YU et al. (2020) in China. Interestingly, in both studies, the isolates were susceptible to most aminoglycoside antibiotics. Besides the mutation in genes encoding the V domain of the 23SrRNA, several *erm* genes encode enzymes that methylate 23SrRNA (CHRISTAKI et al., 2020). In the current study, the *ermA* gene was not present in the isolates, which was consistent with the previous observation by SARITHA et al. (2021).

Harboring of transferable plasmid-mediated quinolone resistance causes a rise in quinolone resistance bacteria due to *Qnr*, a pentapeptide repeat structure protein, which reduces the binding of fluoroquinolones to DNA gyrase (VAN DER PUTTEN et al., 2018). Whilst *qnrA* was present among 50% of the isolates, *qnrB* and *qnrS* were not detected. We found a much higher value for *qnrA* than those reported by ISMAIL and ABUTARBUSH (2020). Our observation may indicate the widespread dissemination of a particular *qnr* encoding plasmid in the region, that carries this gene.

The tetracycline-resistant bacteria implement several strategies, such as efflux pump, ribosomal preservation, and enzymatic degradation; this mostly occurs through *tet* gene families (JAHANTIGH et al., 2020). The striking incidence of *tet* genes within *E. coli* was reported in previous studies (FARIDAH et al., 2020). According to the results, the frequency of *tetA* and *tetB* were 0% and 15%, respectively. Other studies demonstrated *tetA* and *tetB* in a range of 23% - 75%, which is higher than the amount we observed in this study (PYATOV et al., 2017; ISMAIL and ABUTARBUSH, 2020). There are several reasons for the differences observed in the results of surveys that might affect the results, including: geographical discrepancies, sample size, the ages of the cattle, the season of sampling, variations of serotypes, and the application of disparate antibiotics for therapy. Interestingly, in the present work, the only ESBL producer isolate carried *bla*TEM, *bla*CTX-M, *AmpC*, *aac (6)-Ib*, *aph (3)-Ia*, *aadA1,* and *aaC2* simultaneously. In addition, this isolate was resistant to cefpodoxime, cefepime, ceftazidime, cefotaxime, cefoxitin, amoxicillin/clavulanic acid, norfloxacin, nalidixic acid ciprofloxacin, tetracycline, azithromycin, and trimethoprim, so it should be considered a potentially important resistant clone.

Conclusions

In conclusion, resistant genes were detected in most isolates, but the majority of isolates were sensitive to most of the antibiotics tested. This fact might relate to the low expression of these genes in the tested isolates; since the isolates were stored at -20 °C for a while, this might affect gene expression. So, increasing resistant gene expression in clinical conditions, and horizontal transfer of these genes leads to the development of resistance values. Also, the presence of one superbug in the mastitis isolates implies the necessity of implementing stewardship strategies in the region to prohibit the spread of dangerous clones. Considerable from the current results might carbapenems are not utilized in animal husbandry practice in Mashhad. However, the low sample number was a limitation in this survey. Therefore, further experiments are suggested for monitoring CRE with higher sample numbers in this area.

Conflict of Interest

None to declare.

Acknowledgments

The authors thank Mrs. Somayeh Bagherzadeh for her technical assistance. The authors would also like to thank Ferdowsi University of Mashhad (FUM) for providing the facilities required to conduct this study.

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Received: 27 November 2021
Accepted: 11 February 2022

AFLAKIAN, F., N. MOHSENI, M. HAFIZ, H. NIKOUEIAN, M. A. BADOUEI, A. R. ZOMORODI: Fenotipsko i genotipsko istraživanje otpornosti na antimikrobne lijekove i proizvodnja beta-laktamaze proširenog spektra kod bakterije *Escherichia coli* izolirane iz krava s mastitisom. Vet. arhiv 93, 503-512 2023.

SAŽETAK

Escherichia coli jedan je od najistaknutijih patogena izoliranih kod kliničkih slučajeva mastitisa. Nažalost, izostanak odgovarajućeg upravljanja antimikrobnim lijekovima i njihova prekomjerna upotreba povećali su otpornost varijanti izoliranih iz životinja prema tim lijekovima. Cilj je ovog istraživanja bio prikazati prevalenciju proizvodnje beta-laktamaze proširenog spektra (ESBL), gensku rezistenciju i višestruku rezistentnciju na lijekove (MDR) među izolatima *E. coli* dobivenim od krava s kliničkim mastitisom. U tu je svrhu prikupljeno 40 izolata *E. coli* iz uzoraka mlijeka krava s pet farmi u Mashhadu u Iranu. Istraživanje osjetljivosti na antimikrobne lijekove (AST) otkrilo je 15 antimikrobnih tvari važnih za veterinarsku i humanu medicinu. Proizvodnja ESBL-a pritom je procijenjena upotrebom testa sinergije s dvostrukim diskom (engl. *double-disc synergy test*, DDST). Raspodjela 20 gena rezistancije među izolatima *E. coli* pretražena je putem šest multiplex-PCR testa i tri uniplex-PCR testa. Najveća je osjetljivost pronađena na imipenem i amikacin (100%), dok je najveća rezistencija uočena kod tetraciklina (70%) i trimetoprim-sulfametoksazola (72,5%). Prema testovima AST i DDST, jedan je izolat potvrđen kao proizvod ESBL-a i MDR. Najveća (100 %) je stopa gena rezistencije uočena za gene *bla*TEM i *AmpC*. Gen *qnrA* koji kodira rezistenciju na kinolone imao je također visoku prevalenciju i otkriven je u 50% izolata. Zaključeno je da su najmanje tri gena rezistencije otkrivena u 28 izolata (70%). Većina se izolata pokazala osjetljivima na antibiotike testirane ovim istraživanjem. Ta spoznaja može biti povezana s niskom ekspresijom gena rezistencije u izolatima. Horizontalni prijenos ovih gena može uzrokovati rezistenciju prema drugim srodnim bakterijskim vrstama u ljudi i kod domaćih životinja.

Ključne riječi: *E. coli*; osjetljivost na antimikrobne lijekove; mastitis; ESBL; genska rezistancija