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Antimicrobial Resistance of *Escherichia coli* Isolates from Mastitic Milk and its Possible Relationship with Resistance and Virulence Genes

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ABSTRACT

Mastitis caused by *Escherichia coli* is a notable threat to dairy industry due to its high genetic variability, enormous number of environmental sources and increased resistance to antibiotics. The present study was aimed to determine the antimicrobial resistance of *E. coli* isolates from bovine mastitis and identification of antimicrobial resistance and virulence genes associated with them. Antimicrobial susceptibility testing was performed by disk diffusion method. Resistance and virulence genes were detected by PCR. The results showed that 100% of isolates were resistant to penicillin, 54% to ampicillin, 44% to tetracycline and 30% to streptomycin, while none of them was resistant to chloramphenicol. These *E. coli* isolates carried one or more antimicrobial resistance genes. Genes present with highest frequency were *tetA* (42%), *tetB* (28%) and *ampC* (26%). Fewer *E. coli* isolates carried *tetD* (10%), *tetE* (8%) and *tetG* (8%) genes. None of the isolates was positive for *bla2* resistance genes. PCR results of virulence genes confirmed that 66% of strains were carrying the *traT* gene, 26% the Shiga toxin gene and 16% the intimin (*eae*) gene, while all strains were negative for aerobactin gene. Conclusively, *E. coli* isolates were resistant to at least two or more antibiotics, irrespective of presence or absence of relevant resistance and virulence genes.

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Authors' Contribution
MI and YC conceived and designed the study, and supervised the work. AA carried out experimental work and wrote the manuscript.

Key words
Antimicrobial resistance, Bovine mastitis, *E. coli*, Resistance genes, Virulence genes.

INTRODUCTION

Bovine mastitis is one of the most important and costly diseases of the dairy industry (Hogeveen *et al*., 2011). *E. coli* is among the most prevalent bacteria in an environment with high degree of genetic variability, so it is difficult to control and eliminate from dairy herds. It is one of the most common opportunistic environmental pathogens, which causes bovine mastitis. Severity of intra-mammary infection depends on host characteristics (Burvenich *et al*., 2003). Antibiotic resistance cases are increasing remarkably due to their excessive use and high rates of antibiotic resistance transfer between different bacteria (Kahlmeter and Poulsen, 2012). Various studies were performed to find out the relationship between virulence factors of *E. coli* and its pathogenicity for causing mastitis, but no association was observed (Suojala *et al*., 2011; Wenz *et al*., 2006).

Antimicrobial resistant phenotypes and their genetic determinants may have an association with specific epidemiological features. To understand and control antimicrobial resistance, evaluation of antimicrobial resistance genes and virulence factors is important (Seyda *et al*., 2014). Antibiotic resistance and virulence genes could be located on the same chromosomal structures or plasmids, the evaluation of these virulence factors and antibiotic resistance genes can be helpful (Bean *et al*., 2004; Suojala, 2010).

The emergence of antimicrobial resistance among *E. coli* strains of animal origin is a critical public health issue worldwide (Copur-Cicek *et al*., 2014; Paterson, 2006). Several studies also explained that antimicrobial resistant *E. coli* infections in humans are often due to strains coming from animal sources (Altalhi *et al*., 2010; Lei *et al*., 2010; Rasheed *et al*., 2014). Antibiotics used for humans and animals are closely related, overuse of these drugs resulted into emergence of multidrug-resistant bacteria (Cantas *et al*., 2013; Walther *et al*., 2017). The present study was performed to investigate antimicrobial resistance and the frequency of resistance genes and virulence factors in *E. coli* isolates from bovine mastitis.

MATERIALS AND METHODS

Sample collection

In this study fifty *E. coli* strains isolated from mastitic

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milk were collected from Quality Milk Production Services, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA. These *E. coli* were picked randomly from the isolates collected in the last five years. *E. coli* cultures, stored in Luria-Bertani medium and glycerol, were sub-cultured on tryptic soy agar for further use in the current study.

Antimicrobial sensitivity testing

Antimicrobials were tested for susceptibility by the Kirby-Bauer disk diffusion method using Mueller-Hinton agar (Sigma-Aldrich, USA), following Clinical and Laboratory Standards Institute (CLSI) standard guidelines. The antimicrobial agents tested were ampicillin (10µg), chloramphenicol (30µg) penicillin (10µg), streptomycin (10µg) and tetracycline (30µg). The quality control strain used was *E. coli* American Type Culture Collection (ATCC) 25922. Plates were incubated for 18 to 24 h at 37°C and sensitivity was tested for *E. coli* isolates against all antimicrobial agents, and the results were inferred according to the criteria given by Clinical Laboratory Standards Institute guidelines (Patel *et al*., 2015).

Table I.- Oligonucleotide primers used to detect antimicrobial resistance genes and virulence genes.

Target gene	Primer sequence	Size (bp)	Reference
Resistance genes			
tetA	F: GGCCTCAATTTCCTGACG R: AAGCAGGATGTAGCCTGTGC	372	Guillaume *et al*. (2000)
tetB	F: GAGACGCAATCGAATTCGG R: TTTAGTGGCTATTCTTCCTGCC	228	Guillaume *et al*. (2000)
tetC	F: TGCTCAACGGCCTCAACC R: AGCAAGACGTAGCCCAGCG	397	Guillaume *et al*. (2000)
tetD	F: GGATATCTCACCGCATCTGC R: CATCCATCCGGAAGTGATAGC	436	Guillaume *et al*. (2000)
tetE	F: TCCATACGCGAGATGATCTCC R: CGATTACAGCTGTCAGGTGGG	442	Guillaume *et al*. (2000)
tetG	F: CAGCTTTCGGATTCTTACGG R: GATTGGTGAGGCTCGTTAGC	844	Gebreyes and Altier (2002)
cmlA	F: CCGCCACGGTGTTGTTGTTATC R: CACCTTGCCTGCCCATCATTAG	698	Gebreyes and Altier (2002)
strA	F: CTTGGTGATAACGGCAATTC R: CCAATCGCAGATAGAAGGC	548	Gebreyes and Altier (2002)
strB	F: ATCGTCAAGGGATTGAAACC R: GGATCGTAGAACATATTGGC	509	Gebreyes and Altier (2002)
aadA	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	525	Lanz *et al*. (2003)
ampC	F: TTCTATCAAMACTGGCARCC R: CCYTTTTATGTACCCAYGA	1048	Poirel *et al*. (1999)
bla1	F: TCGCCTGTGTATTATCTCCC R: CGCAGATAAATCACCACAATG	768	Van *et al*. (2008)
bla2	F: TGGCCAGAACTGACAGGCAAA R: TTTCTCCTGAACGTGGCTGGC	462	Van *et al*. (2008)
Virulence genes			
eae	F: ATATCCGTTTTAATGGCTATCT R: AATCTTCTGCGTACTGTGTTCA	425	Güler *et al*. (2008)
aer	F: TACCGGATTGTCATATGCAGACCGT R: AATATCTTCCTCCAGTCCGGAGAAG	602	Oliveira *et al*. (2012)
traT	F: GATGGCTGAACCGTGGTTATGCACA R: CGGGTCTGGTATTTATGC	307	Kaipainen *et al*. (2002)
stx1	F: ATAAATCGCCATTCGTTGACTACAG R: AACGCCCACTGAGATCATCGGCACT	180	Fitzmaurice (2003)
stx2	F: GTCTGAAACTGCTCCTCGCCAGTTA R: TCGCCAGTTATCTGACATTCTG	255	Fitzmaurice (2003)

DNA isolation

E. coli isolates were sub-cultured in Luria-Bertani broth (Merck, Germany) overnight. Genomic DNA was isolated from these cultures by Qiagen DNeasy Blood and Tissue Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The concentration of genomic DNA was determined using Nanodrop 2000c (Thermo Fischer Scientific Inc., Waltham, MA, USA) and stored at −20°C until further use.

PCR amplification

Oligonucleotide primers used for PCR amplification of resistance and virulence genes are given in Table I. PCR reaction mixtures were prepared in a total volume of 25 µL containing 1.5 µL $MgCl_2$, 2.5 µL PCR buffer, 200 µM dNTPs, 1 µM each primer, 5 U of *Taq* DNA polymerase, and 1 µL (50–200 ng/µL) of DNA. PCR reaction mixtures were thermally denatured for 5 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 60 s at 52-58 °C (annealing temperature varied according to primer pairs) and 1 min at 72 °C. A final extension step of 10 min at 72 °C was also performed. PCR products were analyzed by using 1.2 to 1.5% agarose gels, depending on the fragment size of PCR product.

PCR was also performed to detect the virulence genes encoding intimin (*eaeA*), outer membrane protein (*traT*), aerobactin (*aer*) and Shiga toxin (*stx1* and *stx2*). PCR was carried out in a total volume of 25 µL containing 1× PCR buffer, 1.5 mM of $MgCl_2$, 250 µM of each of dNTPs, 0.5 µM of each of the virulence gene-specific primers, 1.5 U of *Taq* DNA polymerase (Sigma) and 2 µL of template DNA. The thermal PCR profile included initial denaturation at 95°C for 30 s, followed by 35 cycles of 94 °C for 30 s, 50-60 °C for 45 s (annealing temperature varied according to primer pairs) and 70 °C for 90 s. A final extension step of 10 min at 72 °C was also performed (Güler *et al*., 2008). The PCR products were analyzed by 1.5% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Antimicrobial susceptibility testing

Antimicrobial susceptibility of *E. coli* strains, isolated from randomly collected mastitic milk samples, was evaluated to five commonly used antimicrobials, as shown in Figure 1. Percentage of multidrug resistant isolates, which are resistant to two or more antimicrobials, was high. In fact, all *E. coli* isolates were resistant to two or more antimicrobials under study.

All *E. coli* isolates were completely resistant to penicillin as shown in Figure 2. It has been reported that *E. coli* is intrinsically resistant to some of the antibiotics including penicillin (Greenway and England, 1999). If we compare the resistance patterns of these commonly used antibiotics to a study conducted by Srinivasan *et al*. (2007) almost ten years ago in the same area, we can see a remarkable difference. Ampicillin resistance has decreased fundamentally from 98.4% to 54% in this study. Similarly, streptomycin resistance has also decreased from 40.3% to 30%. However, tetracycline resistance in *E. coli* isolates increased from 24.8% to 44%. None of the isolates was resistant to chloramphenicol. The reason for this can be attributed to the fact that it is not commonly used in the herd. Chloramphenicol is not allowed to be used as veterinary medicine in the United States since the 1980s (Gilmore, 1986). Tetracycline resistance found in 44% of *E. coli* isolates in this study is higher than reported by San Martín *et al*. (2002) and Lanz *et al*. (2003). According to these studies, 20.6% and 20% of *E. coli* isolates from bovine mastitis were resistant to tetracycline, respectively. In some other studies, tetracycline resistance of *E. coli* isolates from bovine mastitis was found to be 33.2% (Erskine *et al*., 2002) and 62% (Lehtolainen *et al*., 2003). Ampicillin resistance, which is 54% in this study, is high as compared to some previously reported studies, where 15.7% (Makovec and Ruegg, 2003) and 21.9% (Erskine *et al*., 2002) of *E. coli* isolates from bovine mastitis appeared as ampicillin resistant. Various factors contribute to the difference in resistance pattern in this study and previously reported studies. They include differences in sensitivity of assays, microbial culture conditions and identification, sampling conditions and area of sampling, initial exposure to antibiotics, etc. However, antibiotic administration for treatment of gram negative mastitis is not recommended (Suojala *et al*., 2013).

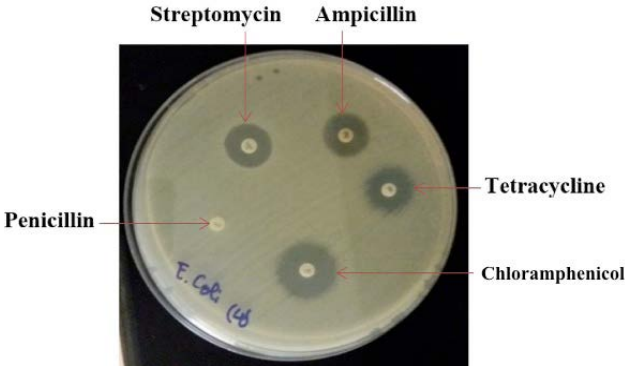

Fig. 1. Antimicrobial sensitivity of *E. coli* against ampicillin, chloramphenicol, penicillin, streptomycin and tetracycline, as assessed by Kirby-Bauer disk diffusion method.

Table II.- Percentage of occurrence of antimicrobial resistance genes in *E. coli* isolates from mastitic milk.

Gene	*tetA*	*tetB*	*tetC*	*tetD*	*tetE*	*tetG*	*cmlA*	*strA*	*strB*	*aadA*	*ampC*	*bla1*	*bla2*
Occurrence (n=50)	21	14	3	5	4	4	1	12	2	6	13	2	0
Percentage	42%	28%	6%	10%	8%	8%	2%	24%	4%	12%	26%	4%	0

Antimicrobial resistance genes

Antimicrobial resistance genes were found in various combinations among different *E. coli* isolates. Most of *E. coli* (78%) isolates were resistant to more than one antibiotic under study. Similarly, they were positive for more than one antimicrobial resistance genes as mentioned in Table II. Results indicated that 31 of *E. coli* isolates contained at least two antimicrobial resistance genes, 16 carried three of the genes tested and five isolates carried four antimicrobial resistance genes; however, combinations were different.

Antimicrobial resistance genes *tetA, tetB* and *ampC* were most frequent and they were found in 42%, 28%, and 26% of isolates, respectively. Fewer *E. coli* isolates carried *tetD* (10%), *tetE* (8%) and *tetG* (8%) genes. *bla2* genes were not found in any of the *E. coli* isolates under study.

In total, 20% of *E. coli* isolates resistant to tetracycline were not positive for any *tet* gene, suggesting that other genes conferring tetracycline resistance might have been responsible for the observed resistance. Also, 26% of *E. coli* isolates resistant to ampicillin and streptomycin did not carry any of the tested antimicrobial resistance genes, suggesting that other mechanisms or genes might have contributed to the observed resistance phenotype. None of the *E. coli* strain was resistant to chloramphenicol, but *cmlA* gene was detected in one of the sample. There was no association of specific pattern seen that could be linked with penicillin resistance.

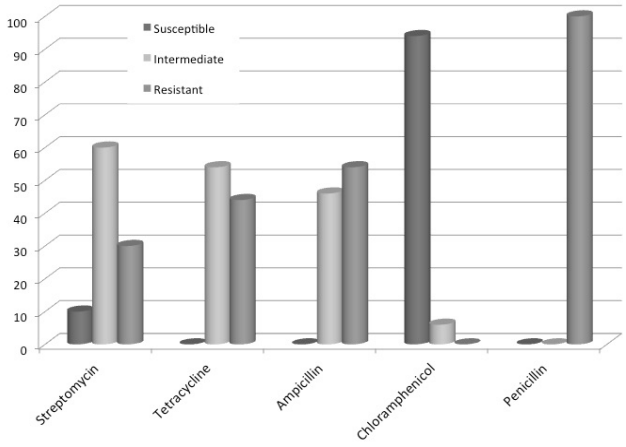

Fig. 2. Percentage of antimicrobial resistance of *E. coli* to five antibiotics.

In the present study, the strong associations observed among various antimicrobial resistance genes raise the possibility that a selective pressure due to the use of one antimicrobial during livestock production may result in the dissemination of strains carrying resistance genes for other antimicrobials. Resistance to aminoglycosides, beta-lactams, chloramphenicol, sulfonamides, tetracycline and trimethoprim has been acquired by *E. coli* strains from other microorganisms (Lietzau *et al*., 2006).

In general, antibiotic resistance profiles of *E. coli* isolates demonstrated that a large percentage of isolates were resistant to the majority of antibiotics tested. Most of *E. coli* isolates were found to be resistant to two or more antibiotics tested, but did not essentially contain resistant gene responsible for the conferred resistance.

Virulence genes

PCR results of virulence genes showed that 33 (66%) strains were carrying the *traT* gene, 11 (22%) the *stx1* gene, 4 (2%) the *stx2* gene, 8 (16%) the intimin (*eae*) gene, while all strains were negative for *aer* gene as shown in Table III. In another recently reported study, the percentage of eae gene was found to be 14.8%; however, stx1 and stx2 genes were not detected in any of pathogenic E. coli isolates (Dong *et al*., 2017). The isolates were jointly evaluated for antibiotic susceptibility and virulence properties. However, in some cases there was no significant difference in virulence genes between antibiotic-resistant and antibiotic-susceptible strains.

Table III.- Percentage of occurrence of virulence genes in *E. coli* isolates from mastitic milk.

Gene	*eae*	*aer*	*traT*	*stx1*	*stx2*
Occurrence (n=50)	8	0	33	11	2
Percentage	16%	0%	66%	22%	4%

The distribution of resistance genes and virulence genes was variable and could not be accounted fully for antimicrobial resistance as there were many isolates showing susceptibility to a particular antibiotic and carrying the resistance gene for that as well. However, in a few cases antimicrobial resistance was associated with

the identified resistance genes. Increased incidence of antimicrobial resistance and growing demand of animal based proteins for human consumption is very challenging. The above-mentioned concerns made it more difficult to control dairy animal diseases due to limited availability of suitable antibiotics (Ganda *et al*., 2016).

CONCLUSION

The antimicrobial resistance of *E. coli* isolates from bovine mastitis was found to be higher against commonly used antibiotics in dairy industry, which include ampicillin, streptomycin, and tetracycline. Various resistance and virulence genes were also detected in these isolates, but no relationship was established on the basis of results obtained. The discovery of underlying dynamics of these antimicrobial resistant pathogens may lead to better control and prevention of bovine mastitis.

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Animal rights statement

Not applicable.

Statement of conflict of interest

Authors declare no conflict of interest.

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