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SHORT COMMUNICATION

Antibiotic Resistance Pattern of Mastitis Causing Escherichia Coli Toxinotypes

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A B S T R A C T

Mastitis is caused by many pathogens including *Staphylococcus, Streptococcus, Escherichia, Mycoplasma,* etc. Environmental mastitis is majorly caused by *E. coli*. Aim of the study was to detect toxigenic genes and antibiotic resistance of invasive *E. coli*. Milk samples (n= 150) were screened for mastitis by California mastitis test (CMT). The CMT positive samples (n=100) were processed. Eight isolates were identified as *E. coli.* Then multiplex PCR was performed for detection of toxigenic genes. All isolates were positive for toxigenic genes including *aggR* (100%) followed by *est* and *elt* (62%), *eae* (37.5%) and *Asp* (25%), respectively. Antibiotic susceptibility test was performed using different antibiotics. Isolates were resistant to Cefixime (37%) followed by Ampicillin, Ceftriaxone and Co-trimoxazole (25%), Amoxicillin, Tetracycline, Nalidixic acid and Cefotaxime (12%), respectively. It was concluded that *eae, elt, est, aggR* and *Asp* genes were present in *E. coli* isolates and highly resistant to Cefixime, minimum resistant against Gentamicin and Ciprofloxacin.

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INTRODUCTION

Mastitis is an inflammatory condition of the udder. Sub-clinical mastitis is the latent form and no clinical signs appear in this form of mastitis. In case of clinical mastitis, changes in the appearance of udder occur, systemic illness and chemical and physical changes also occur in milk. The common mastitogens are *Staphylococcus, Streptococcus, Escherichia, Pseudomonas, Salmonella, Corynebacterium, Mycoplasma,* etc. (Abdalhamed *et al*., 2018). One of the major pathogens of mastitis is *E. coli*. *Escherichia coli* has some pathogenic strains which include Shiga toxin producing *E. coli* (STEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Entero-invasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC) and Enterohaemorrhagic *E. coli* (EHEC). Toxigenic genes are responsible for virulence properties of these pathogenic strains (Vila *et al*., 2016). Emergence of the antibiotic resistance is a serious issue now-a-days. *Escherichia coli* have ESBL genes for production of beta lactamase enzymes against beta lactam antibiotics. For aminoglycosides, aminoglycoside modifying enzymes produce resistance. There are several genes that encode resistance for sulfonamide antibiotics including *sul 1, sul 2* and *sul3* while, in case of trimethoprim *dhfr, dhfr1* and *dhfr II* genes are responsible for increasing resistance against these antibiotics (Alizade, 2018).

The objective of the study was to detect toxigenic genes and antibiotic resistance of invasive *E. coli* isolates from cattle suffering from mastitis.

MATERIALS AND METHODS

Sample collection: A total of 150 samples of milk were collected from the cattle suspected for mastitis from Ravi Campus Pattoki, UVAS, Lahore and around areas in sterile containers. These samples were further proceeded for California mastitis test (CMT). Briefly, few milliliters of milk sample were placed in paddle and in equal volume reagent of CMT (1% KOH) was mixed properly. The samples showing gel formation were considered positive for mastitis.

Isolation and identification of *E. coli*: Samples positive for CMT were subjected for the isolation and identification of *E. coli* following Bergey's manual of Determinative Bacteriology (Brown, 1939). Isolates

identified as *E. coli* were subjected to pathogenicity testing by inoculating on Congo red agar media.

Detection of toxigenic genes of *E. coli*: DNA extraction was performed using kit (Gene All Exgene^{TM}). Multiplex PCR was performed for detection of toxigenic genes including *eae, stx, est, elt, ipaH, aggR* and *Asp* using specific primers (Table 1). Initial denaturation (95ºC for 10 minutes), denaturation (95ºC for 1 minute), annealing (52ºC for 1 minute), extension (72ºC for 1 minute) for 30 cycles and final extension at 72ºC for 10 minutes.

Antibiotic Susceptibility test by disc diffusion method: Isolates were subjected to antibiotic susceptibility test by disc diffusion method. Antibiotic discs of Amoxicillin, Cefixime, Ampicillin, Cefotaxime, Tetracycline, Ceftriaxone, Co-trimoxazole, Ciprofloxacin, Gentamicin and Nalidixic acid were placed on agar plates and incubated at 37 ºC for 24 hours. Zones of inhibition were measured in millimeter compared with CLSI standards (CLSI, 2018).

Statistical analysis: Data of antibiogram assay was analyzed with the help of one-way analysis of variance by SPSS (version 20.0).

RESULTS AND DISCUSSION

Out of 150 milk samples screened for mastitis by CMT, 100 were positive for mastitis and showed gel formation. Out of 100 positive samples, eight (8%) *E. coli* isolates were identified. All eight (100%) *E. coli* isolates showed positive result for congo red binding activity and produced red colored colonies on the Congo red agar which showed the presence of invasive *E. coli*.

Multiplex PCR was performed using specific primers for toxigenic genes (Table 1). Isolates showed the presence of toxigenic genes including *aggR* (100%) followed by *est* and *elt* (62%), *eae* (37.5%) and *Asp* (25%), respectively (Figure 1).

The isolates were sensitive to Ciprofloxacin and Gentamicin (100%) followed by Tetracycline and Amoxicillin (87%), Ceftriaxone, Co-trimoxazole and Cefotaxime (75%), Nalidixic acid (62%), Ampicillin and Cefixime (50%), respectively.

The isolates were intermediate to Ampicillin and Nalidixic acid (25%) followed by Cefixime and Cefotaxime (12%), respectively (Table 2). The isolates were resistant to Cefixime (37%) followed by Ampicillin, Ceftriaxone and Co-trimoxazole (25%), Amoxicillin, Tetracycline, Nalidixic acid and Cefotaxime (12%), respectively.

Present study revealed the low percentage of *E. coli* isolates (8%) indicating organized conditions of farm. A study conducted by Mpatswenumugabo *et al*. (2017) correlates to present study as they reported the low percentage of *E. coli* (1.5%). This low percentage was because of good milking practices and use of teat dips before milking. While, another study by Preethirani *et al*. (2015) showed high percentage (9.8) of *E. coli* isolates from an unorganized dairy farm. These results were not in accordance to present study.

Current study concluded that all isolates were positive for Congo red binding activity which indicated the presence of toxigenic genes in *E. coli.* Similarly, a study reported by Osman *et al*. (2018) on pathogenicity of *E. coli* reported 95% of the isolates positive for Congo red binding activity due to the presence of the virulence markers.

In this study toxigenic genes were targeted. Total eight (100%) isolates showed the presence of *aggR* gene, followed by five (62%) *est* gene, five (62%) *elt* gene, three (37.5%) *eae* gene and two (25%) *Asp* gene, respectively. Bako *et al.* (2017) detected *est, elt* and *aggR* genes which was in accordance to the present study. High prevalence of these genes indicated the improper management of the effluents and spread of *E. coli* from humans to animals through environment and vice versa.

The isolates of *E. coli* were resistant to Cefixime (37%), Ampicillin (25%), Ceftriaxone (25%), Co-trimoxazole (25%), Amoxicillin (12%), Tetracycline (12%), Nalidixic acid (12%) and Cefotaxime (12%), respectively. Yassin *et al.* (2017) observed resistance against Gentamicin (8.2%), Cefotaxime (4.9%), Ceftriaxone (4.9%), Amoxicillin (8.2%), Ampicillin (23%), Nalidicxic acid (21.3%) and Tetracycline (19.7%). These results were in accordance to the results of present study in which low resistance aganist antibiotics was observed. While, other study revealed high resistance against Ampicillin, Amoxicillin, Nalidicxic acid, Tetracycline, Cefotaxime and Cephalosporins (Ali *et al.*, 2016). Low resistance indicated less useage of antibiotics while high resistance indicated frequent use of antibiotics in treatment of mastitis.

Table 1: Specific primers for toxigenic genes of *E. coli*

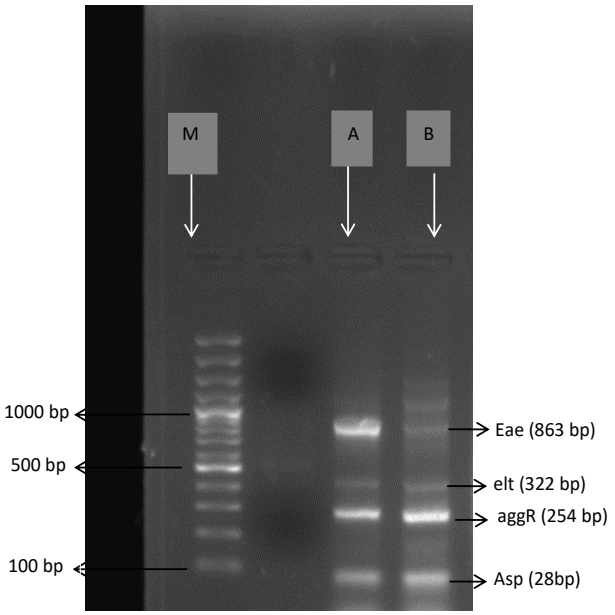
Primer set	Sequence	Target	Amplicon size (bp)
Sk1 f	5'-CCCGAATTCGGCACAAGCATAAGC-3'	Eae	863
SK2 r	3'-CCCGGATCCGTCTCGCCAGTATTCG-5'		
VTcom-u f	5'-GAGCGAAATAATTTATATGTG-3'	Stx	518
VTcom-d r	3'-TGATGATGGCAATTCAGTAT-5'		
AL 65 f	5'-TTAATAGCACCCGGTACAAGCA-3'	Est	147
AL12 r	3'-CCTGACTCTTCAAAAGAGAAAAT-5'		
LTL f	5'-TCTCTATGTGCATACGGAGC-3'	Elt	322
LTR r	3'-CCATACTGATTGCCGCAAT-5'		
ipa III f	5'-GTTCCTTGACCGCCTTTCCGATACCGTC-3'	IpaH	619
ipa IV r	3'-GCCGGTCAGCCACCCTCTGAGAGTAC-5'		
aggRks1 f	5'- GTATACACAAAAGAAGGAAGC-3'	AggR	254
aggRks2 r	3'- ACAGAATCGTCAGCATCAGC-5'		
aspU f	5'-GCCTTTGCGGGTGGTAGC-3'	Asp	28
aspU r	3'-AACCCATTCGGTTAGAGCA-5'		

bp: base pairs, SK1 and SK2: target eae gene, VTcom-u and VTcom-d: target stx gene, AL 65 and AL 12: target est gene, LTL and LTR: target elt gene, ipa III and ipa IV: target ipaH gene, aggRks1 and aggRks2: target aggR gene, asp U: target Asp gene, f: forward primer, r: reverse primer.

Table 2: Antibiotic susceptibility pattern of *E. coli*

Sr. No.	Antibiotic	Concentration (µg)	Antibiotic susceptibility pattern			CLSI standard of ZOI in mm		
			S n(%)	I n(%)	R n(%)	S n(%)	I n (%)	R n (%)
1	Ciprofloxacin	5	8 (100%)	0(0)	0(0)	>=21	16-20	<=15
2	Ampicillin	10	4 (50%)	2(25%)	2(25%)	>=17	14-16	<=13
3	Amoxicillin	30	7(87%)	0(0)	1(12%)	>=17	14-16	<=13
4	Cefixime	5	4(50%)	1(12%)	3(37%)	>=19	16-18	<=15
5	Gentamicin	10	8(100%)	0(0)	0(0)	>=15	13-14	<=12
6	Tetracycline	30	7(87%)	0(0)	1(12%)	>=15	12-14	<=11
7	Nalidixic acid	30	5 (62%)	2(25%)	1(12%)	>=19	14-18	<=13
8	Ceftriaxone	30	6 (75%)	0(0)	2(25%)	>=23	20-22	<=19
9	Co-trimoxazole	10	6(75%)	0(0)	2(25%)	>=16	11-15	<=10
10	Cefotaxime	30	6(75%)	1(12%)	1(12%)	>=28	26-27	<=25

S: Sensitive, R: resistant, I: intermediate, mm: millimeter, ZOI: Zone of inhibition.



M: DNA ladder (100bp), A and B: toxigenic gene amplicons

Fig. 1: Representative gel of toxigenic gene amplicons of invasive *Escherichia coli* on 1.5% agarose gel.

Conclusions: It was concluded that toxigenic genes including *eae*, *elt*, *est*, *aggR* and *Asp* were present in *E. coli* isolated from cattle suffering from mastitis. Maximum resistance was observed against Cefixime and minimum against Gentamicin and Ciprofloxacin.

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Authors contribution: SM, MAA, MR, AAA and MA conceived and designed study. SM executed the experiments. SM, MAA, TY and ZFH analyzed the data. SM and MAA prepared the manuscript. The manuscript is critically revised by all authors and approved final version of manuscript.

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