Molecular Characterization of Antibiotic Resistance Gene Pattern of *Staphylococcus aureus* and *Escherichia coli* in Mastitis Affected Dairy Cows

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ABSTRACT

Background: Antimicrobial resistance is one of the latest challenges facing the scientific community. Raising the drug resistance is caused mainly by indiscriminate usage of antibiotics in human and animal subjects and the spread of antibiotic resistance between the two has an emerging global threat. Hence, current study aimed to study the antimicrobial resistance pattern and molecular detection of antibiotic resistance genes in *Staphylococcus aureus* and *Escherichia coli* isolated from mastitis affected cows.

Methods: Milk samples from mastitis affected cows were subjected to antibiotic sensitivity test and screened for presence of *Staphylococcus aureus* and *Escherichia coli* using differential growth media. Molecular characterization of *Staphylococcus aureus* and *Escherichia coli* was done with the help of PCR by amplification of '*nuc'* and '*uspA'* gene respectively. MICs of Penicillin and Tetracycline were determined using microdilution method.

Result: Antibiotic sensitivity pattern for Penicillin G, Ampicillin, Amoxycillin, Cefotaxime, Ceftriaxone, Azithromycin, Ciprofloxacin, Gentamicin, Oxytetracycline, Tetracycline and Vancomycin were 74.19%, 100%, 93.50%, 61.29%, 29%, 35.48%, 9.70%, 9.70%, 70.96% and 70.96% respectively. More than 87.90% of the *S. aureus* and 50% of the *E. coli* isolated were resistant to β-lactam antibiotics while 75% of the *E.coli* and 65.70% of the *S. aureus* isolated were resistant to Tetracycline antibiotics. The MICs of Penicillin for *S.aureus* and *E.coli* are 26.88 µg/ml and 13.54 µg/ml respectively and the MICs of Tetracycline for *S. aureus* and *E. coli* are 243.75 µg/ml and 960.93 µg/ml respectively which is 8-9 folds higher than the standard MICs. From the present study, it can be inferred that bovine mastitis cases are highly resistant to antimicrobial drugs. Results further indicate that *Staphylococcus aureus* and *Escherichia coli* are both resistant to Penicillin and Tetracycline with very high MIC.

Key words: Antibiogram, Beta-lactam, PCR, Teteracycline.

INTRODUCTION

Antimicrobial resistance is one of the most formidable challenges facing the scientific community (Ventola, 2015; Bilal *et al.,* 2018). Owing to the indiscriminate usage of antibiotics in human and animal subjects, the problem of antibiotic resistance has only worsened and there isn't much forthcoming as a way of a solution to this growing concern (Malik and Bhattacharyya, 2019). Even though the causes for emergence of antibiotic resistance are multifarious, there is wealth of evidence to suggest that milk consumption from cows that have been treated with antibiotics and are still within the withdrawal time period contributes significantly to the development of antimicrobial resistance in human beings (Sharma *et al.,* 2018). Mastitis is one of the most commonly diagnosed infectious diseases in dairy cows and causes significant economic loss to the farmers (Singh *et al*., 2018; Chandrasekharan *et al*., 2014; Saini *et al*., 2012). Studies indicate that farmers across different parts of the country have incurred severe financial losses owing to outbreak of mastitis. (Sinha *et al*., 2014; Jingar *et al*., 2017). A wide range of organisms have been implicated in the occurrence of mastitis, chief among them being *Staphylococcus aureus* and *Escherichia coli* (Cremonesi *et al*., 2006; Clermont *et al*., 2000; Oliveira *et al*., 2000). Antibiotic therapy has been the mainstay in the treatment and control of mastitis. However, owing to their indiscriminate usage, emergence of resistance against a variety of antibiotics poses significant challenge for the clinicians and scientists alike (Kappeli *et al.,* 2019; Igbinosa *et al.,* 2016). Inappropriate usage of antimicrobials such as improper dose, drug or duration contributes the most to the increase in antimicrobial resistance without improving the outcome of treatment (Chandrasekaran *et al.,* 2014). In view of the need to reduce antibiotic resistance and develop appropriate strategies in

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antibiotic usage, the present study has been undertaken to determine the antibiotic sensitivity pattern, molecular detection of antibiotic resistance gene and MICs to help us in our choice of antibiotics and determine drug resistance pattern.

MATERIALS AND METHODS

Study area

The present study was carried out in the department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, TANUVAS, Chennai-600007. The samples were collected from the Large Animal Medicine Unit, Department of Clinics, Madras Veterinary College Hospital, TANUVAS, Chennai-600 007. The study was conducted between January 2018 and March 2019.

Antimicrobial sensitivity testing

The milk samples were collected from mastitis affected cows following strict aseptic conditions. California Mastitis Test was used as a screening test for mastitis. The sensitivity of the isolates to various antibiotics was performed using disc diffusion method. The disc diffusion method was performed using the procedure followed by Pu *et al*. (2014).

Isolation and identification of *Staphylococcus aureus*

Bacteriological examination of the milk sample was carried out within 24 hrs of collection of the milk sample. Nutrient broth was prepared and 10 ml was transferred into sterile test tubes, autoclaved, cooled and stored. Milk swab was dipped in 10 ml of nutrient broth containing test tube, plugged with cotton and incubated at 37^{o}C for 18 hrs. The inoculum was streaked onto aseptically prepared Mannitol Salt Agar plates and incubated at 37^{o}C for 16-20 hrs. Growth of yellow coloured colonies indicated the presence of Staphylococcus organisms. The test was performed following the procedure of Kampf *et al.,* (1998) with certain modifications. The isolates were stored in 50% glycerol solution in eppendroff tubes for further use.

Isolation and identification of *Escherichia coli*

Nutrient broth was prepared and 10 ml of it transferred to sterile test tube, autoclaved, cooled and then stored. The milk swab was dipped into 10 ml nutrient broth containing test tube, plugged with cotton and incubated at 37^{o}C for 18 hrs. The inoculum was streaked onto Eosin Methylene Blue agar plates. Distinctive metallic green sheen on EMB indicates the presence of *E.coli*. The test was performed following the procedure of Horvath and Ropp (1974).

Biochemical tests

The isolated organisms were identified according to routine microbiological diagnostics, including cultural properties, catalase (Igbinosa *et al.,* 2016) and coagulase (Boerlin, 2003), utilization of mannitol and triple sugar iron agar and gram staining.

Antibiotic susceptibility

It was determined by the standardized agar diffusion test on Muller-Hinton agar (HiMedia) using the following disks (HiMedia): ampicillin (10mcg), amoxicillin + clavulanic acid (20/10mcg), azithromycin (15mcg), ciprofloxacin (5mcg), cefuroxime (30mcg), ceftriaxone (30mcg), gentamicin (10mcg), penicillin-G (10U), oxytetracycline (30mcg), tetracycline (30mcg), vancomycin (30mcg). Isolates were classified as susceptible and resistant based upon interpretative criteria developed by the Clinical and Laboratory Standards Institute (CLSI) (2005).

Minimum inhibitory concentration (MIC)

Microdilution method modified with resazurin dye as a redox indicator was utilized to determine the MIC value of resistant drugs. Inoculum was prepared by suspending overnight agar plate cultures of the isolates in sterile normal saline equivalent of 0.5 McFarland turbidity standards. The resazurin solution was prepared by dissolving 0.002g of resazurin powder (HiMedia) in 100mL of distilled water. The method was followed as reported by Sarkar *et al* (2007) with slight modifications.

Plates were prepared under aseptic conditions. A sterile 96 well plate was labeled. A volume of 25µL of the broth medium was pipetted into columns 2 to 9, 10 and 12 of every row. To all other wells in the same row and to the second column, a volume of 25µL of the highest concentration of the drug was added. Serial dilutions were done from the second well to the ninth well by pipetting out 25µL from each well. The last 25µL from the ninth well was discarded. 100µL of the inoculum diluted to 0.5 McFarland standard was added to all the wells other than columns 10 and 11 which acted as the negative control for the media and the drug dilutions; 100µL of un-inoculated broth was added to the tenth and the eleventh wells instead. After incubating the well plate for 24 hours at 35±2^{o}C, 5µL of 0.002% resazurin was added to all wells and was further incubated at 37^{o}C for 3 hours.

DNA extraction for PCR analysis

DNA extraction was done by methods suggested by Fang and Goran (2003). A bacterial suspension prepared from a loopful of colonies from an overnight agar culture was prepared in 100µL of Nuclease free water. The suspension was boiled at 100 for 5 minutes and was chilled immediately at -20^{o}C for 10 minutes. The clear supernatant obtained by centrifuging the chilled suspension at 10,000 rpm for 2 minutes served as the template for PCR reactions.

PCR analysis

Polymerase chain reaction analysis of the *mecA, tetK, tetM, blaSHV* and *tetB* genes were carried out using the primers and the respective amplifications program. Reaction was performed in a final volume of 25µL of mixture containing 12.5µL of Master Mix (AmpliconTaqPol), 1µL each of the forward and reverse primer, 3µL of the template DNA and 7.5µL of nuclease free water. The products were analyzed by electrophoresis through 1.2% agarose gel. The PCR was carried out using appropriate protocol (Garibyan and Avashia, 2014). The primer sequences for identification of

Staphylococcus aureus and *Escherichia coli* and their resistant genes are depicted in Table 1, 3, 4. The cyclic conditions of Polymerase Chain Reaction for *uspA* and *nuc* gene is depicted in Table 2 and for amplification of resistance gene in Table 5.

RESULTS AND DISCUSSION

Isolation, identification and molecular characterization

The phenotypic and biochemical analysis shows 100% and 94% prevalence respectively for *S. aureus* and the genotypic prevalence of *S. aureus* was found to be 12.9%. The prevalence of *E. coli* was found to be 12.9% based on the phenotypic test and 100% on biochemical test. The genotypic analysis reports 50% prevalence for *E. coli*. The results were found to be similar to earlier studies conducted by Chandrasekaran *et al.,* (2014). The results accumulated from the tests are depicted in Table 6.

Antibiotic sensitivity testing

The antimicrobial susceptibility pattern of *S. aureus* and *E. coli* isolates from mastitis specimens against antimicrobial agents of different classes were found to be highly variable. Antibiotic Sensitivity Pattern showed that the isolates were resistant to Ampicillin, Amoxicillin and Penicillin-G, Tetracycline and Oxytetracycline, Vancomycin, intermediately sensitive to Azithromycin and sensitive to Gentamicin, Enrofloxacin and Ciprofloxacin. There is abundant evidence to corroborate the emergence of resistance of penicillin and tetracycline for both *Staphylococcus aureus* and *Escherichia coli* (Foster, 2017; Ragbelti *et al*., 2016; Arabzadeh *et al*., 2018). The emergence of resistance can be ascribed to indiscriminate antibiotic usage and non-compliance to the recommended dose regimens. The antibiotic sensitivity pattern is depicted in Fig 1 and 2.

Table 1: Primer sequences flanking the genes used for identification of *E. coli* and *S. aureus.*

Gene	Primer sequence	Amplicon size
uspA	(F) CCG ATA CGC TGC CAA TCA GT	884bp
	(R) ACG CAG ACC GTA GGC CAG AT	
Nuc	(F) GTGCTGGCATATGTATCGCAATTGT	181bp
	(R) TACGCCCTTATCTGTTTGTGATGC	

Table 2: Cyclic conditions for Polymerase Chain Reaction of *uspA* and *nuc* gene.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycle
uspA	94°C/5m	94°C/1m	55°C/1m	72°C/2m	72°C/5m	30
nuc	94°C/5m	94°C/30s	54°C/30s	72°C/30s	72°C/10m	30

Table 3: Primer sequences of *E. coli* antibiotic resistance genes.

Antibiotic group	Gene	Sequence (5′-3′)	Amplicon size
Tetracycline	*tet(B)*	(F) CCT CAG CTT CTC AAC GCG TG	634bp
		(R) GCA CCT TGC TGA TGA CTC TT	
Beta-lactam	bla_{SHV}	(F) TCG CCT GTG TAT TAT CTC CC	768bp
		(R) CGC AGA TAA ATC ACC ACA ATG	

Table 4: Primer sequences of *Staphylococcus aureus* antibiotic resistance genes.

Antibiotic group	Gene	Sequence (5′-3′)	Amplicon size
Tetracycline	*tet(K)*	(F) GTA GCG ACA ATA GGT AAT AGT	360bp
		(R) GTA GTG ACA ATA AAC CTC CTA	
	tet(M)	(F) AGT GGA GCG ATT ACA GAA	158bp
		(R) CAT ATG TCC TGG CGT GTC TA	
Beta-lactam	*mec(A)*	(F) AAA ATC GAT GGT AAA GGT TGG C	532bp
		(R) AGT TCT GCA GTA CCG GAT TTG C	

Table 5: PCR cyclic conditions for the amplification of resistance genes.

Name of gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension
mecA	94°C/5m	94°C/1m	55°C/1m	72°C/2m	72°C/5m
tetK	95°C/5m	95°C/60s	55°C/60s	72°C/90s	72°C/5m
tetM	94°C/5m	94°C/60s	50°C/60s	72°C/90s	72°C/5m
bla_{SHV}	94°C/10m	94°C/40s	60°C/40s	72°C/1m	72°C/7m
tetB	95°C/5m	95°C/30s	55°C/30s	72°C/30s	72°C/2m

Table 6: The results of biochemical and genotypic confirmation tests.

Organism	No. of samples tested	Culture (MSA/EMB) positives	Catalase test positives	Coagulase test positives	TSI agar slant test	*Nuc/uspA* gene amplification
S. aureus	31	31	29/31	24/31	29/31	4/31
E. coli	31	4	3/4	0/4	4/4	2/4

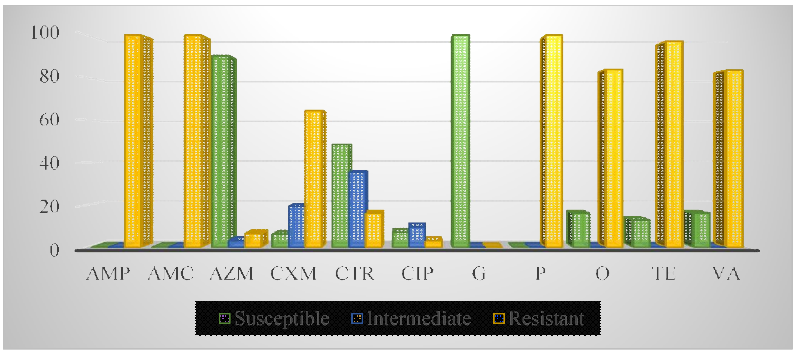

Fig 1: Antibiotic sensitivity profile of *Staphylococcus aureus*.

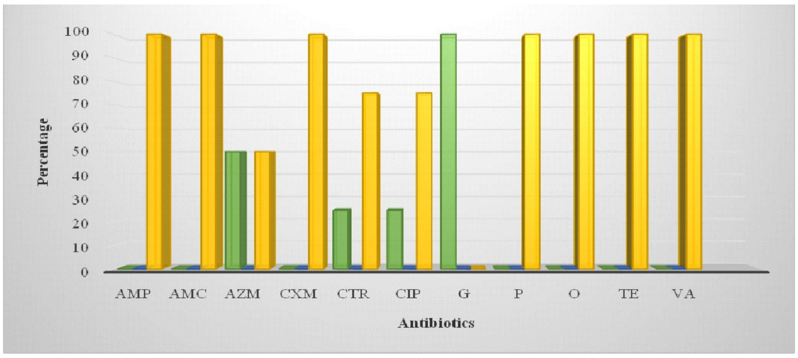

Fig 2: Antibiotic sensitivity pattern of *Escherichia coli.*

Minimum inhibitory concentration

The MIC values of Penicillin against *S. aureus* and *E. coli* were found to be 26.90µg/ml and 13.54µg/ml respectively and the MIC values of Tetracycline against *S. aureus* and *E. coli* were 243.75µg/ml and 960.93µg/ml respectively. The data generated has been depicted in Table 7.

Polymerase chain reaction

The genotypic analysis of the antibiotic resistance in isolated microorganisms has brought forward the prevalence pattern of resistance genes in them. The prevalence resistance genes in *S. aureus* was 51.67% tet (K), 67.7%, tet (M) for tetracycline and 87.09% mec (A) for penicillin. In *E. coli* isolates the prevalence of resistance genes were recoded to be 50% for bla_{SHV} and 75% for tet (B). Tetracyclines exert their antimicrobial effect by binding to the 30S ribosome and thereby preventing protein sysnthesis. Bacterial resistance to tetracycline is most commonly mediated by energy-dependent pumping of tetracycline out of the bacterial cell (Karami *et al.,* 2006). The most common resistance mechanism of *Escherichia coli* to tetracycline is through energy dependent efflux pump which is encoded by *tet*A, *tet*B, *tet*C, *tet*D and *tet*G, with *tet*A and *tet*Bgenes

Table 7: Minimum inhibitory concentration (MIC) of penicillin and tetracycline in *S. aureus* and *E. coli.*

Organisms	Penicillin G	Tetracycline
S. aureus	26.90 µg/ml	243.75 µg/ml
E. coli	13.54 µg/ml	960.93 µg/ml

Table 8: Prevalence of genes in S. aureus.

Gene	Prevalence
Tet(K)	51.61%
Tet(M)	67.70%
Mec(A)	87.09%

Table 9: Prevalence of genes in *E. coli.*

Gene	Prevalence
bla_{shv}	50%
Tet(B)	75%

(Olowe *et al.,* 2013). The resistance of *Escherichia coli* to tetracycline may be attributed to the presence of *tet*B gene which was detected in the current study with the help of PCR with a prevalence rate of 75%. *Staphylococcus aureus* acquires resistance to tetracycline by active efflux, resulting

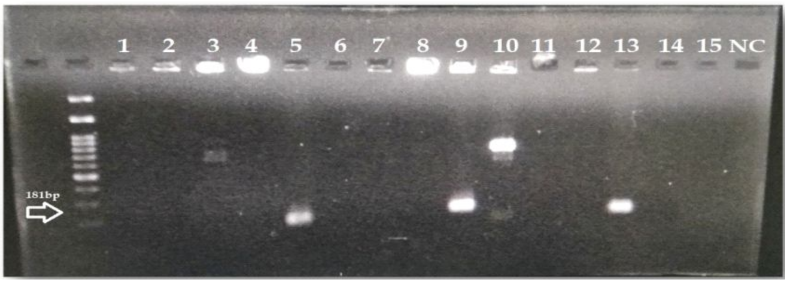

Fig 3: Agarose gel electrophoresis of *usp*A gene PCR product for confirmation of *E. coli*.

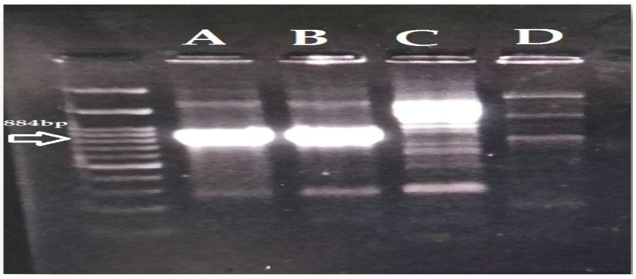

Fig 4: Agarose gel electrophosed *nuc* gene PCR.

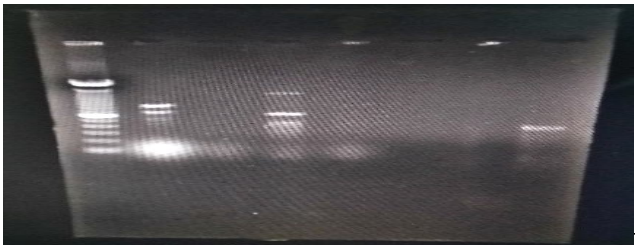

Fig 5: Agarose gel electrophoresed bla_{SHV} gene product.

Fig 6: Agarose gel electrophoresed *tet*(K) gene product.

from the acquisition of the plasmid-located *tetK* and *tetM* genes (Emaneini *et al.,* 2013). In the current study, presence of *tet*K and *tet*M gene were detected by using PCR with a prevalence rate of 51.61% and 67.7% respectively. The resistance of *Staphylococcus aureus* to tetracycline can be ascribed to the efflux pump encoded by *tet*K and *tet*M genes. In *Staphylococcus aureus*, biosynthesis of the peptidoglycan is accomplished by the membrane-bound enzymes known as penicillin-binding proteins (PBPs) (Sauvage *et al.,* 2008). Staphylococcal resistance to penicillin is usually conferred by the acquisition of a non-native gene encoding a penicillin-binding protein (PBP2a), with significantly lower affinity for β-lactams. (Peacock *et al.,* 2015) High-level resistance to methicillin is caused by the *mecA* gene, which encodes an alternative penicillin-binding protein, PBP 2a (Weilders *et al.,* 2002). Detection of *mecA* gene in the *Staphylococcus aureus* isolates leads us to presume that some of the resistance of *Staphylococcus aureus* to Penicillin G may be owing to the alternative penicillin binding protein, PBP 2a synthesis. Each bla gene of extended-spectrum β-lactamase-producing *E. coli* was further subtyped to be $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}104}$, $bla_{TEM\text{-}1}$ and $bla_{SHV\text{-}12}$ which are responsible for conferring resistance to Beta lactam antibiotics (Ombarak *et al.*, 2018). In the present study, bla_{SHV} gene was detected in *Escherichia coli* with a prevalence rate of 50% using PCR technique. The resistance of *Escherichia coli* to penicillin may be attributed to the presence of bla_{SHV} gene which confers resistance by production of beta lactamase enzyme.The results of the PCR are depicted in Table 8 and 9, Fig 3, 4, 5 and 6.

CONCLUSION

From the current study, it can be concluded that mastitis in dairy cows are highly resistant to various antimicrobial drugs. Present study further indicates *Staphylococcus aureus* and *Escherichia coli* are both resistant to Penicillin and Tetracycline with very high MIC. There is a need to carry out further studies in this direction to better understand the resistance pattern of these antibiotics and also arrive at a dosage regimen.

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