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Multidrug-resistant Bacterial Isolates in Bovine Subclinical Mastitis from Southern Rajasthan

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ABSTRACT

Background: In the present study two hundred milk samples were collected from cows and buffaloes with no history of clinical mastitis in the ongoing lactation, from the Sirohi district of Southern Rajasthan.

Methods: The pooled sample (collected from each quarter) and examined for the status of subclinical mastitis by Modified California mastitis test and Somatic cell count respectively. Positive samples were further investigated for isolation and identification of the major mastitis-causing pathogens: *S. aureus*, predominant Streptococcal species and *E. coli* for assessing antimicrobial resistance models in southern Rajasthan.

Result: The results of the current study indicate high levels of multi-drug antibiotic resistance among bacteria that commonly cause mastitis, particularly ampicillin, penicillin, tetracycline, erythromycin and methicillin. However, the highest sensitivity was conferred to ceftriaxone, gentamicin, and co-trimoxazole, suggestive of judicious use of these antibiotics in the treatment of bovine mastitis. Concurrent implementation of gradient PCR indicated the presence of mecA and blaZ genes in 51.9% and 81.4% of *S. aureus* isolates, respectively. Meanwhile, 56.6% of the streptococcal isolate contained the tetracycline-conferring tetM gene and none of the streptococci contained the ermB gene. The 92.3% *E. coli* isolates contained the tetA gene and the tetB gene for tetracycline resistance.

Keywords: Antimicrobial, Multidrug, PCR, Prevalence, Resistance, Sirohi.

Abbreviations: CLSI: Clinical and Laboratory Standards Institute, SCM: Sub-Clinical Mastitis.

INTRODUCTION

The livestock sector has become an important element in the development and diversification of the agricultural sector in India's economy. The share of agriculture and allied sectors in gross value added (GVA) of the country at current prices is 17.8 per cent for the year 2019-20. In which Share of is Livestock 5.1 per cent. The livestock sector operates efficiently in terms of the production of milk, milk products, meat, eggs, value-added and per capita availability of different livestock products Suthar *et al.,* (2019).

Sub-clinical mastitis remains to be an obscure and latent form of this disease that poses a more serious economic concern to the dairy and livestock sector, as the incidence, is much higher in a dairy herd than in the clinical one Shaheen *et al.,* (2016). Early detection of mastitis with low cost and rapid screenings at the field level, hygienic farm management, biosecurity and awareness building among farmers will be helpful to control the clinical and SCM of dairy cows Kabir *et al*., (2017). Due to the multifactorial etiology and the risk of antibiotic resistance, the best method of mastitis treatment is to accurately identify the causative agent, which typically has been carried out by microbiological culture, a standard diagnostic tool till now. However, because the cultures of mastitis milk samples may not always result in bacterial growth, an increasing number of studies have shown the potential of molecular techniques to improve the diagnosis of mastitis, with high sensitivity and specificity Lima *et al.,* (2018). Keeping in view the above facts the present study was designed with the following objective:

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- Phenotypic characterization of the three most important bacterial pathogens (*Staphylococcus aureus,* streptococci species and *Escherichia coli*) isolated from bovine milk with subclinical mastitis and molecular characterization of some genes responsible for their antibiotic resistance.

MATERIALS AND METHODS

Location

The entire research was performed in the laboratory of the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Navania, Vallabhnagar, Udaipur. This study was conducted with all animal welfare and ethical

considerations in mind and was approved by the Establishment's Animal Ethics Board.

Sampling and general microbiologic analysis

About two hundred milk samples were collected under aseptic conditions from domesticated dairy Cattle and buffaloes (5-8-year age group) from organized and unorganized dairy farms. Out of these 200 milk samples, 100 milk samples were collected from cows and 100 milk samples were collected from buffaloes. The animal was examined Clinically and the samples of milk were taken following standardized aseptic procedures. These samples were kept on ice and transferred immediately to the laboratory.

Screening for scm by modified california mastitis test

Screening of the SCM was conducted by modifying the California mastitis test. The CMT was performed and interpreted as described by Kandeel *et al.,* (2018).

Estimation of somatic cell count

The udders were tested for SCM using the Modified California Mastitis Test and only those milk samples which were found positive for mastitis were used in the study. Somatic cell counts were determined by a Lactoscan milk analyzer (Belgium) according to the technique described by the manufacturer. The SCC value>5,00,000 cells/mL Hegde *et al.,* (2013) milk was taken as a criterion to declare milk/animal as sub-clinical mastitis/infected and these milk samples were subjected to cultural isolation.

Isolation and biochemical characterization

A total of 74 milk samples based on CMT and SCC were subjected to bacteriological examination for the isolation and identification of bacterial species in the milk samples, the techniques as per standard procedures by Markey *et al.,* (2013) were implemented.

Identification and biochemical analysis of *Staphylococcus aureus*, *Streptococcus* spp. and *E. coli.*

Pure cultures of isolates were submitted for gram staining and further by catalase test. The catalase-positive cultures were streaked on nutrient agar obliques and preserved at 4°C. From these slants, the pure cultures were subjected to various biochemical tests as per standard procedures Markey *et al.,* (2013). The isolated bacteria were identified up to specie level based on colony characteristics of individual primary isolate (Plate 1, 2 and 3).

Phenotypic detection of antibiotic resistance

All the *S. aureus*, Streptococcus and *E. coli* isolates obtained were subjected to in vitro antibiotic sensitivity test as per the Kirby-Bauer disc diffusion method on Muller Hinton agar according to Clinical and Laboratory Standards Institute guidelines Clinical and Laboratory Standards Institute guidelines (2018) and Bauer *et al.,* (1966). Results were interpreted according to CLSI recommendations.

Genome-based detection of antibiotic resistance

For Staphylococcal isolates, *mecA* that codes for an altered penicillin-binding protein (PBP2a) and *blaz* genes coding for beta-lactam gene were targeted. For Streptococcal isolates, *ermB* and *tetM* genes which confer resistance to macrolides and tetracycline respectively were targeted in PCR. For *E. coli*., the genes *tetA* and *tetB* confer resistance to tetracycline were targeted in PCR. The oligonucleotide primer sequences and the corresponding amplicon sizes for detecting antibiotic resistance genes in different PCR tests are listed in Table 1.



Plate 1: Representative image of Mannitol fermentation by *S. aureus.*

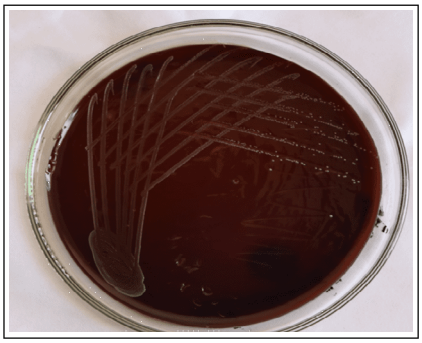


Plate 2: Representative image of *Streptococcus* on Edward's media.

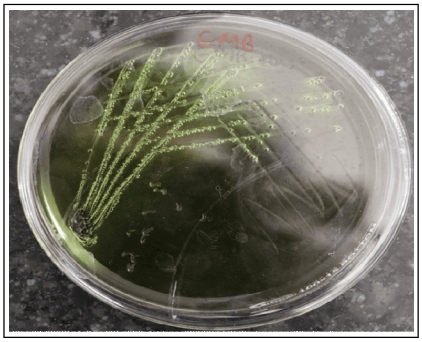


Plate 3: Representative image of *Escherichia coli* on Eosin Methylene blue agar.

RESULTS AND DISCUSSION

Among the 200 milk samples, CMT was found positive in 45% (n=90) samples. Our results were comparable with other workers Busanello *et al.,* (2017); Olivares-Pérez *et al.,* (2017); Ahmed *et al.,* (2018); Algammal *et al.,* (2020); Abed *et al.,* (2021). A similar, result was also obtained by Birhanu *et al.,* (2017).

Further, the collected milk samples were subjected to measurement of SCC. The result of the SCC of 200 milk samples indicated the prevalence of SCM as 37% (n=74). These findings are in complete agreement with Hegde *et al.,* (2013), Nithinprabhu, (2010) and Javia *et al.,* (2018).

These 74 milk samples were cultured for primary isolation of predominant pathogens like *S. aureus, Streptococci* and *E. coli* found positive for the presence of these bacteria. Out of these 74 positive samples for SCC, 72 samples had bacterial growth and while in the 02 samples bacterial growth was absent. A total of 97 isolates were recovered from these milk samples either as single or mixed infections. The etiological prevalence of SCM caused by *S. aureus,* (54/200, 27%), *Streptococcus* spp. (30/200, 15%) and *E. coli* (13/200, 6.5%) respectively either as single and or as mixed infections. Similar findings were also reported by Lakshmi and Jayavardhanan (2016) and Sztachanska *et al.,* (2016).

Antibiogram study of individual isolates of *S. aureus, Streptococcus* and *E. coli*

All the 54 isolates of *S. aureus* showed varying degrees of resistance to different antibiotics. The highest resistance was observed for Penicillin-G (88.9%), Tetracycline (83.3%), Erythromycin (81.5%) and Ampicillin (75.9%) respectively. Total (51.9%) isolates of Staphylococci were resistant to Methicillin. The lowest levels of resistance were observed in Ceftriaxone and Co-trimoxazole (20.4%) and (25.9%) respectively. The least resistance (16.7%) was observed against Gentamicin. The antimicrobial resistance of *S. aureus* isolates in our current study are comparable with the finding's other researchers Hoque *et al.,* (2018), Pal *et al.,* (2017), Preethirani *et al.,* (2015).

For the Streptococci isolate, a high level of antibiotic resistance was observed for Methicillin (93.3%) and Tetracycline (53.3%). These isolates were less resistant to Erythromycin (30%), Penicillin-G (16.7%), Ampicillin (16.7%), Gentamicin (13.3%) and Ceftriaxone (10%). Co-trimoxazole is the most sensitive antibiotic. Similar results were also observed by Javia *et al.,* (2018) and Preethirani *et al.,* (2015).

All the 13 *E. coli.* isolate, showing a high resistance towards the Penicillin-G (100%) Methicillin (92.3%), Tetracycline (92.3%), Erythromycin (76.9%) and Ampicillin (76.9%) while Ceftriaxone (38.5%) was the least resistant. Similar higher resistance to oxytetracycline was reported by Alekish *et al.,* (2013) and Das *et al.,* (2017). The percentage sensitivity and resistance of three isolates- *S. aureus, Streptococcus* and *E. coli.* to the individual antibiotics is given in Table 2.

Genotypic detection of antibiotic resistance gene

Antimicrobial resistance is conferred by the presence of resistance genes that can be linked to genetic elements. Table 3 signifies the genotypic pattern of antibiotic resistance in three isolates- *S. aureus, Streptococcus* and *E. coli.* Among all the *S. aureus* isolates examined in this study, the overall detection rate of the *mecA* gene was (51.9%), 28 out of 54 indicating the high prevalence of methicillin-resistant (MRSA) strains as they yielded an amplification product of 583 bp and *blaZ* gene (81.4%) 44 isolates could be identified as methicillin-resistant as they yielded an amplification product of 816 bp. Of all the 30 isolates of Streptococci, none of the isolates were found to be positive for the *erm gene* as they did not yield the amplified product of 639 bp and hence were recorded as *ermB* negative. Among 30 isolates 17 (56.6%) isolates were found to carry *tetM* genes as they showed an amplification product of 397 bp size. Out of 13 isolates of *E. coli.* 12 isolates were found to carry the *tetA* gene and *tetB* gene, as they showed amplification products of 887 bp size and 773 bp size, respectively. Fig 1 to 5 showed amplification products of the respective gene and the size of the antibiotic resistance gene.

Table 1: Oligonucleotide primer sequences and amplicon sizes for antibiotic resistance gene.

Bacterial species and their gene	Oligonucleotide primer sequence (5′-3′)	Amplicon size (bp)	Reference
Staphylococcus aureus (*mec A*)	AGAAGATGGTATGTGAAGTTGATG TATGTGCGATTGTATTG	583	Al-makhzoomy *et al.,* (2018)
Staphylococcus aureus (*blaZ*)	ACTTCAACACCTGCTGCTTTCTGA CCACTTTTATCAGCAACC	172	Martineau *et al.,* (2000)
Streptococcus genus (*ErmB*)	GAAAAGGTACTCAA CCAAATAAGT AACGGTACTTAAATTGTTC	442	Dogan *et al.,* (2005)
Streptococcus genus (*tetM*)	TTATCAACGGTTTATCAGGCGTATAT ATGCAAGACG	397	Lopardo *et al.,* (2003)
E. coli (*tetA*)	GTGAAACCCAACATACCCGAAGGC AAGCAGGATGTG	887	Maynard *et al.,* (2003)
E. coli (*TetB*)	CCTTATCATGCCAGTCTTGCACTGC CGTTTTTTCGCC	773	Maynard *et al.,* (2003)

Table 2: The percentage sensitivity and resistance of three isolates- *S. aureus, Streptococcus* and *E. coli.* to the individual antibiotics.

Antibiotic disc	*S. aureus*			Streptococcus			*E. coli*		
	R	I	S	R	I	S	R	I	S
Ampicillin	41	09	4	5	6	19	10	2	1
Gentamicin	9	10	35	4	5	21	2	1	10
Methicillin	28	**7**	19	28	1	1	12	1	0
Penicillin-G	48	1	5	5	7	18	13	0	0
Ceftriaxone	11	3	40	3	4	23	5	0	8
Tetracycline	45	5	4	16	6	8	12	1	0
Erythromycin	44	8	2	9	13	8	10	2	1
Co-trimoxazole	14	13	27	0	2	28	1	0	12

S-Sensitive, I-Intermediate, R-Resistant.

Table 3: Genotypic pattern of antibiotic resistance of *Staphylococcus*, *Streptococcal* and *E. coli* isolated from bovine milk samples.

Bacterial isolates	Target genes	No. positive sample	Percentage	No. negative sample	Percentage
Staphylococcus aureus (n=54)	*mec A*	28	51.9	26	48.1
	blaZ	44	81.4	10	18.5
Streptococcus spp. (n=30)	*ermB*	NA	-	NA	-
	tetM	17	56.6	13	43.3
E. coli (n=13)	*tetA*	12	92.3	1	7.6
	tetB	12	92.3	1	7.6

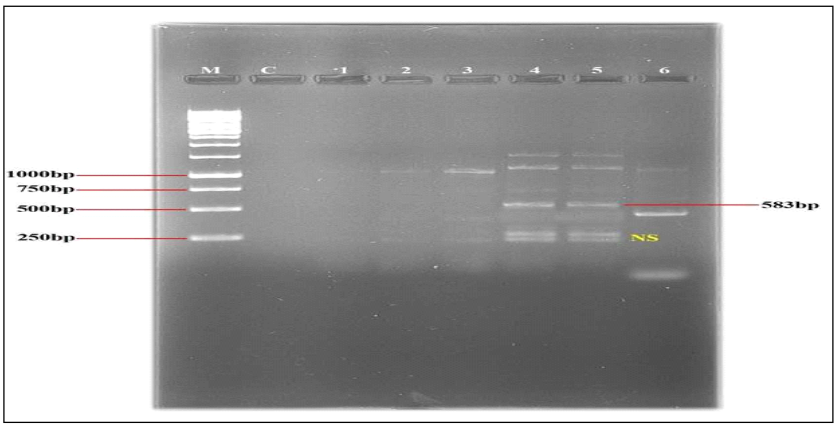


Fig 1: Detection of antibiotic resistance gene by PCR amplification of *mecA* gene (583 bp) of *S. aureus* isolated from subclinical bovine mastitis milk.

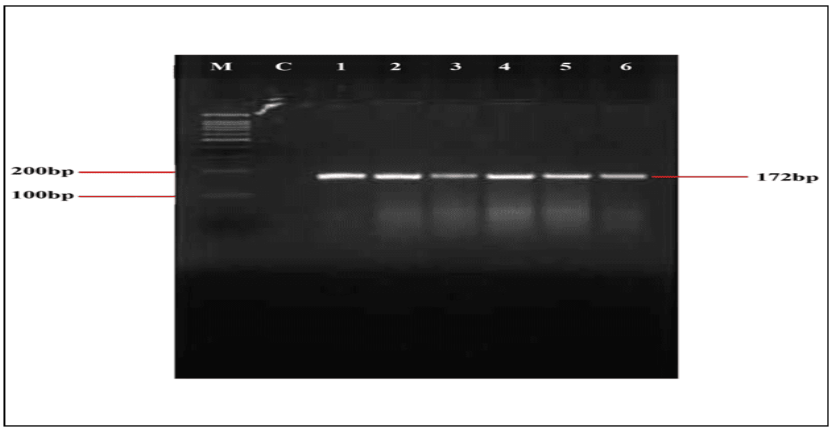


Fig 2: Detection of antibiotic resistance gene by PCR amplification of *blaZ* gene (172 bp) of *S. aureus* isolated from subclinical bovine mastitis milk.

These findings were supported by the results of previous studies describing the associations between resistance phenotypes and resistance genes of different bacteria. Haran *et al.,* (2012), Awad *et al.,* (2017), Shrivastava *et al.,* (2018) and Abed *et al.,* (2021). The inconsistency of the genotype-phenotype association of AMR could be explained by resistance phenotypes that can be expressed upon the stimulation of many different genetic factors that have not

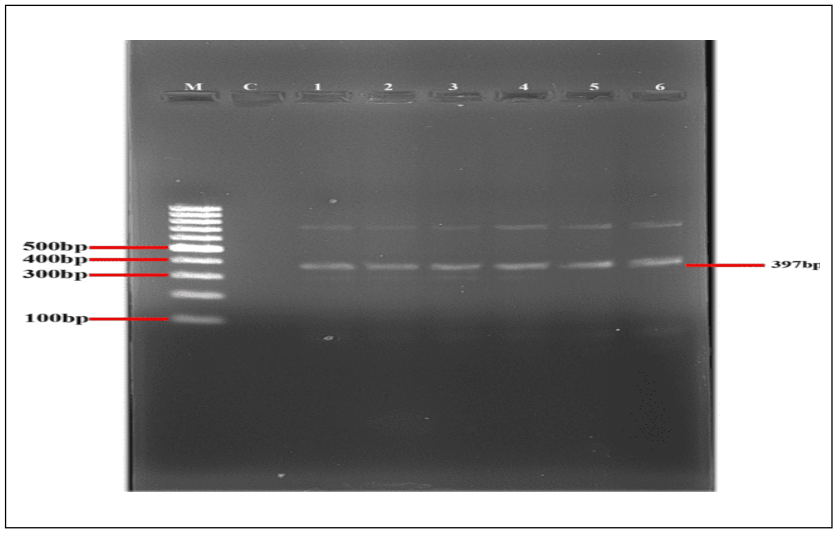


Fig 3: Detection of antibiotic resistance gene by PCR amplification of 397 bp *tetM* gene of *Streptococcus* isolated from subclinical bovine mastitis milk.

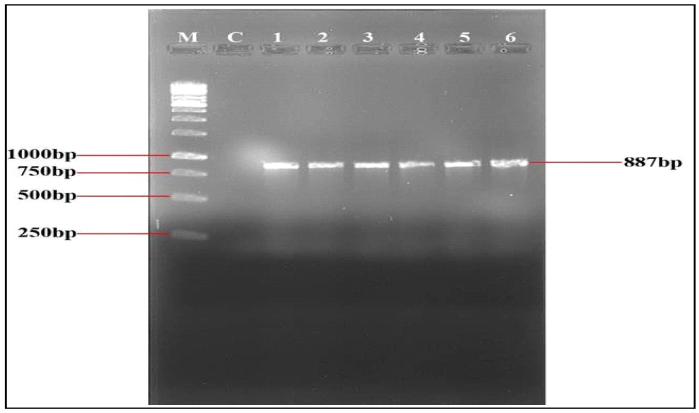


Fig 4: Detection of antibiotic resistance gene by PCR amplification of *tetA* gene (887 bp) of *E. coli* isolated from subclinical bovine mastitis milk.

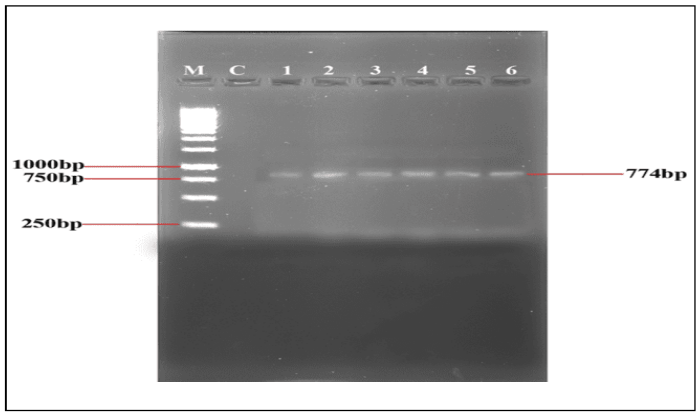


Fig 5: Detection of antibiotic resistance gene by PCR amplification of *tetB* gene (774 bp) of *E. coli* isolated from subclinical bovine mastitis milk.

been investigated in this study and each factor may present a unique epidemiological character as studied by Boerlin *et al.,* (2005) and Van *et al.,* (2020).

CONCLUSION

The results of the current study indicate high levels of multi-drug antibiotic resistance among bacteria that commonly cause mastitis, particularly ampicillin, penicillin, tetracycline, erythromycin and methicillin. However, the highest sensitivity was conferred to ceftriaxone, gentamicin and co-trimoxazole, which suggests judicious use of these antibiotics in the treatment of bovine subclinical mastitis. To limit antibiotic resistance exerted by the pathogen, an appropriate selection of antibiotics based on the identification of bacterial species responsible for mastitis is highly critical. Analysis of antimicrobial resistance patterns of the major bacterial species in a geographical region is of great value in the choice of an appropriate antibiotic drug for treatment and the prevention of intramammary infections. General public health, hygiene and hygiene measures must be observed. Development of cultural awareness campaigns targeting the general public explaining the importance of protecting antibiotics and using them only when necessary. Human health hazards are also associated with the consumption of raw or unpasteurized milk and dairy products. The findings of this study warrant the need for strategies that focuses on enhancing dairy farmers' awareness about regular screening for subclinical and mastitis in animals and judicious use of antibiotics.

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Conflict of interest: None.

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