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DETECTION OF METHICILLIN RESISTANCE IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE MASTITIS#

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ABSTRACT

Mastitis is one of the most prevalent and costly disease of dairy industry. Among the multitude of pathogens, S. *aureus* is a major contagious pathogen responsible for both the clinical and subclinical mastitis in dairy cattle all over the world. The emergence of methicillin resistant strains of *S. aureus* has gained global attention due to its ability to resist therapy and the potential risk of carriage to humans, resulting in numerous life threatening and therapy refractory infections. In this study, a total of twenty two *S. aureus* isolates from bovine clinical mastitis were evaluated for their susceptibility towards methicillin by both phenotypic and genotypic methods. It was found that although 81.82 per cent of the isolates possessed the *mecA* gene, only 45.45 per cent of the isolates were resistant to methicillin by the *in vitro* disc diffusion assay. This study indicates a high prevalence of the methicillin resistance among *S. aureus* isolates from bovine mastitis.

Keywords: Antimicrobial resistance, Mastitis, *mecA*, *Staphylococcus aureus*

INTRODUCTION

Dairying forms an integral part of the total farming system in the country. One of the persistent challenges faced by the dairy farmers all over the world is the increasing occurrence of intramammary infections often compounded by the mounting tide of antimicrobial resistance. It provokes a negative economic impact on the farmers and ultimately endangers the national economy. Besides, the milk from such animals poses significant zoonotic

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risk if consumed raw or improperly pasteurised, by vulnerable groups such as children, pregnant women, elderly people or people suffering from immunosuppressive disorders, due to the shedding of bacteria and their toxins in milk.

Among the multitude of mastitis pathogens, *Staphylococcus aureus* is frequently recognized worldwide as a highly adaptable and versatile contagious mastitis pathogen that lives and multiply in the mammary gland and spreads from cow to cow during milking. The success of this pathogen and its ability to cause a wide range of infections are the result of its extensive virulence factors and the relentless onslaught by antibiotic-resistant strains. In recent times, the outbreaks of community associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) and livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) infections has been considered as a rapidly emerging cause of numerous life threatening and therapy refractory infections of humans, and dairy cows are recognized as one of the potential reservoirs. Therefore, surveillance of antimicrobial resistance among mastitis causing bacteria especially *Staphylococcus* spp. is of vital importance for prevention of mastitis and for securing human health and global food safety. Taking in to account the above mentioned factors, the present study was undertaken to find the prevalence of methicillin resistance among *S. aureus* isolated from bovine mastitis.

MATERIALS AND METHODS

Sample collection

The midstream lacteal secretions of 83 dairy cows suffering from clinical mastitis in and around Thrissur district, Kerala were collected over a period from March 2019 to July 2020.

Isolation and identification of *Staphylococcus aureus*

Isolation of bacteria was attempted by direct streaking of a loopful of milk sample on to brain heart infusion agar (BHIA- M211, Himedia), followed by incubation of the plates at 37 ^{o}C for 12 to 18 h. The isolates were identified based on morphological characteristics on Gram's staining. The presumptively identified *S. aureus* isolates were further verified by direct streaking and subsequent incubation at 37 °C for 24 h on mannitol salt agar (MSA-M118, Himedia) which was prepared according to the manufacturer's instruction. Biochemical characterisation of the isolates were done as per Barrow and Feltham (1993) and Quinn *et al*. (2013). All the staphylococcal isolates obtained were further characterised up to species level with the help of a biochemical test kit

procured from Himedia (HiStaph test kit, KB004) as per manufacturer's guidelines.

Isolation of genomic DNA

The DNA was extracted from the presumptively identified *S. aureus* isolates using snap chill method (heat lysis method) as described by Junior *et al.* (2016) with slight modifications. For this, approximately three millilitres of overnight grown *S. aureus* culture in mannitol salt broth (MSB-M383, Himedia) was centrifuged at 10,000 rpm for 10 min at 4 °C (Hispeed Centrifuge, KEMI). The pellet formed was resuspended in one milliliter of sterile nuclease (DNAase & RNAase) free milli-Q-water (ML064, Himedia), followed by centrifugation at 10,000 rpm for 10 min at 4 °C and the supernatant was discarded. The pellet obtained was further resuspended in 100µl tris EDTA buffer with pH 8, mixed in a vortex and kept in boiling water bath for 15 min. Then snap chilled on crushed ice for 30 min and centrifuged at 10,000 rpm for 10 min, at 4 °C. After that the supernatant containing DNA was collected in sterile nuclease free centrifuge tubes and further checked for concentration and purity using a Nanodrop Spectrophotometer (Nanodrop™ 1000 Spectrophotometer). The DNA samples with 260/280 ratio greater than 1.8 were selected and used as template for the polymerase chain reaction (PCR).

Genotypic characterisation of methicillin resistance

The genotypic characterisation of methicillin resistance was done by polymerase chain reaction targeting the *mecA* gene using the primers specific for them. The PCR was performed in a total volume of 25 µl reaction mixture by combining the reagents as shown in Table 1 using the programmable S1000 Thermal cycler, (BioRad, USA). The details of the primer used and the PCR protocol are shown in Table 2 and 3 respectively. PCR products were analysed on 1.2 per cent agarose gel stained with ethidium bromide, visualised under the ultraviolet transilluminator ((Genei™, Bengaluru) and photographed using GelDoc apparatus (Doc™ Gel EZ imager, BIO-RAD, USA).

Phenotypic characterisation of methicillin resistance

In-vitro antibiotic sensitivity of the isolates was studied using Kirby Bauer disc diffusion method (Bauer *et al.,* 1966) as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017). Antibiotic discs with known concentration in microgram (mcg) or international unit (IU) per disc were used in the study. The details of the antibiotic discs are shown in the table below (Table 4).

Fig.1: Biochemical tests for dentification of *Staphylococcus aureus*
A: Catalase test, B: Oxidase test, C: Coagulase test

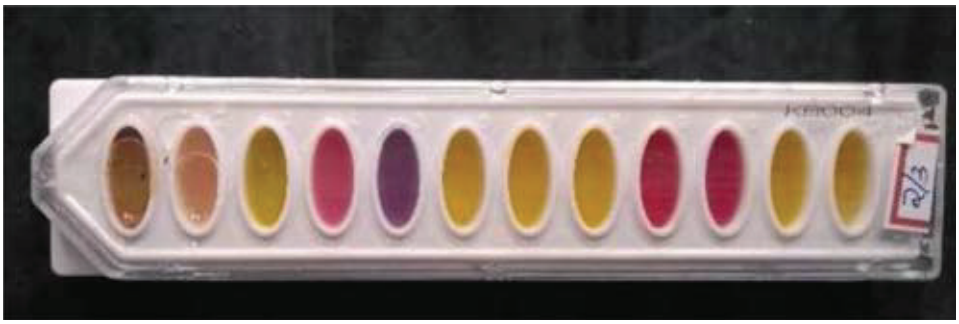
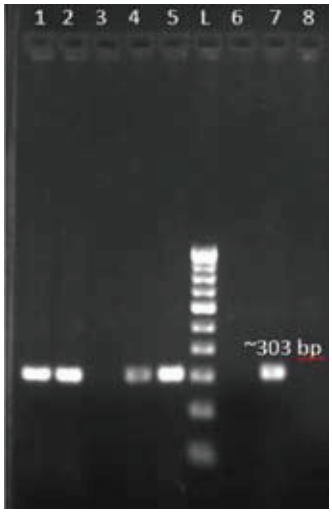


Fig.2: Biochemical tests used for identification of *Staphylococcus aureus* using commercial biochemical test kit (VP test, Alkaline phosphatase, ONPG, Urease, Arginine, Mannitol, Sucrose, Lactose, Arabinose, Raffinose, Trahalose, Maltose)

Fig. 3: Agarose gel electrophoresis of *mecA* specific PCR of *Staphylococcus aureus* (Source : Original)



Lane L : DNA marker (100 bp)
Lane 6 : Negative control
Lane 7 : Positive control
Lane 1, 2, 4, 5 : Positive samples (756 bp)
Lane 3, 8 : Negative samples

Table 1. Components of PCR reaction mix

Components	**Volume** (µl)
Master Mix (2X PCR Smart mix, Takara, Japan)	12.5
Forward Primer (100nM/ml, Sigma Aldrich)	1
Reverse Primer (100nM/ml, Sigma Aldrich)	1
Nuclease Free Water	5.5
Template DNA	5
Total	**25**

Table 2. Primer used for genotypic characterisation of methicillin resistance *S. aureus*

Gene	Primer	Amplicon Size	Reference
Mec A	Forward: TGG CTA TCG TGT CAC AAT C Reverse: CTG GAA CTT GTT GAG CAG AG	303 bp	Archana, 2018

Table 3. PCR protocol for amplification of *mecA* gene by PCR

Sl. No.	PCR Programme		Temperature (ºC)	Time
1.	Initial Denaturation		95	3 min
2.	Denaturation	30 cycles	95	45 sec
3.	Annealing		60	1 min
4.	Extension		72	1 min
5.	Final Extension		72	7 min
6.	Hold		4	10 min

Table 4. Antibiotic concentration of discs used and zone interpretation strategies for antibiogram (CLSI, STANDARDS)

Sl. No.	Antibiotic disc used	Abbrevation and antibiotic concentration per disc	Zone Diameter (nearest whole mm)		
			Resistant	Intermediate	Susceptible
1.	Amoxicillin-Sulbactam	AMS(30 / 15mcg)	32- 43		
2.	Ceftriaxone	CTR (30 mcg)	13 or less	14 – 20	21 or more
3.	Ceftriaxone-Sulbactam	CIS (30 / 15 mcg)	24 – 30		
4.	Cotrimoxazole	COT(23.75/1.25mcg)	14 or less	14 – 16	17 or more
5.	Enrofloxacin	EX (10 mcg)	22- 30		
6.	Gentamicin	GEN (50 mcg)	25 – 33		
7.	Methicillin	MET (10 mcg)	17 – 22		
8.	Penicillin G	P (10 U)	28 or less	-	29 or more
9.	Tetracycline	TE(30 mcg)	19 or less	19 – 21	22 or more

RESULTS AND DISCUSSION

In the present study, isolation of bacteria from bovine mastitic milk was achieved by standard microbiological culture techniques. Upon initial inoculation of 83 mastitic milk samples on to BHIA, 26 samples (31.33 per cent) did not produce any colonies whereas, 57 samples yielded bacterial growth (68.67 per cent), among which *S. aureus* was isolated from 22 samples (26.5 per cent). The bacterial isolates were identified based on their morphology, colony characteristics on selective media (Barrow and Feltham, 1993; Quinn *et al*., 2013).

The isolates which gave positive catalase (Fig. 1A) and coagulase tests (Fig. 1C) and negative oxidase test (Fig. 1B) were presumptively identified as *Staphylococci* spp. as per Barrow and Feltham (1993) and Quinn *et al*. (2013). Furthermore, the species level characterisation of the isolates were done with the help of a biochemical test kit procured from Himedia (HiStaph test kit, KB004) as per manufacturer's guidelines and all the 22 isolates were identified as *S. aureus* (Fig.2).

PCR has been considered as the gold standard for the precise identification of MRSA. It helps in rapid, simple and accurate identification of the AMR profiles and could be used in clinical diagnosis as well as in epidemiological studies to track the spread of antibiotic resistance (Strommenger *et al*., 2003).

Methicillin resistance in staphylococcal isolates is conferred by the acquisition of a genetic element, the staphylococcal cassette chromosome (SCCmec) carrying the *mecA* gene which encodes an altered penicillin binding protein - PBP2a/PBP2' - which has reduced affinity for β lactam antibiotics (Paterson *et al.*, 2014). Therefore, in the current study, PCR targeting the *mecA* gene was also used for the identification of MRSA. The findings of the present study revealed the presence of *mecA* gene in 18 (81.82 per cent) out of the total 22 *S. aureus* isolates (Fig. 3). The presence of genotypic resistance towards *mecA* was analogous to that of the study conducted by Choi *et al*. (2003) who reported 89 per cent prevalence of *mecA* gene among staphylococcal isolates from blood, sputum, urine and pus samples from tertiary teaching hospitals in Korea. However, the previous study by Kulangara *et al*., (2017) on dry bovine udders from the same locality, recorded a comparatively higher prevalence of *mecA* gene in *S. aureus* (95 per cent). This could be attributed to the indiscriminate prophylactic use of intramammary preparations in dry cows that may have exacerbated the development of persistent therapy refractory infections. Varying trends regarding the presence of *mecA* gene was being reported by Amrithapriya (2019) and Ciftici *et al.*, (2009) who reported the presence of *mecA* gene in 18.18 per cent and 30.7 per cent of the staphylococcal isolates, respectively.

In the present study, the 22 *S. aureus* isolates were subjected to antibiogram employing methicillin, penicillin and other seven antibiotics that are frequently being used in mastitis. It was found that all the isolates were resistant to penicillin. Ceftriaxone was found to be the most effective antimicrobial with only four isolates (18.18 per cent) being resistant. Thirteen isolates (59.1 per cent) each were found to be resistant against each amoxicillin-sulbactam, sulphadiazine-

trimethoprim and gentamicin. Twelve isolates (54.54 per cent) were resistant to tetracycline followed by ten isolates (45.45 per cent) each being resistant to ceftriaxone sulbactam and methicillin.

A comparison of the phenotypic and genotypic methods for assessment of methicillin resistance revealed that eventhough 81.82 per cent of the isolates possessed the *mecA* gene, only 45.45 per cent of the isolates were resistant to methicillin by the *in vitro* disc diffusion assay. This disparity between the phenotypic and genotypic resistance could be attributed to the phenomenon of the heterogeneous expression of methicillin resistance *in vitro*, with levels of resistance ranging from phenotypically sensitive to borderline resistant and highly resistant subpopulations (Chambers, 1997). The variation in phenotypic expression of methicillin resistance often depends on the inoculation size, incubation temperature, p^H or salt concentration of the medium and exposure to β lactam antibiotics (Choi *et al*., 2003). The presence of methicillin sensitive *mecA* positive strains might be because the methicillin resistance is not consistently expressed and certain auxillary genes such as *femA*, *mecR* and the gene encoding β lactamase plasmid may participate in the control of its expression. Hence, it is recommended that diverse variants of the gene should be covered for genotypic profiling of AMR (Hamid *et al.,* 2017*)*. Similar findings were reported by Choi *et al*. (2003) and Ciftici *et al*. (2009) wherein the methicillin resistant, *mecA* negative strains was attributed to the non PBP-2a dependent mechanisms such as hyper production of β lactamase and alteration of PBP types or due to the involvement of *mecC*, a divergent homolog of *mecA* in the expression of resistance.

SUMMARY

In conclusion, the results of the present study indicated a higher prevalence of methicillin resistance among *S. aureus* isolated from bovine mastitis. This must be considered as an alarming situation as it poses a serious threat to animal welfare as well as human health due to the possibility of direct transmission of staphylococci or its AMR determinants to human strains. A comparison of the phenotypic and genotypic AMR revealed the existence of phenotypic sensitivity to the antimicrobial agent in the presence of associated AMR genes. This finding assumes paramount significance, since these genes can be easily transferred to form multidrug resistant isolates. It was therefore concluded that a combination of both phenotypic and genotypic methods is warranted to unravel the complexities of the AMR situations and to tackle AMR before it becomes a global catastrophe.

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