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ISOLATION AND ANTIMICROBIAL RESISTANCE IN *STAPHYLOCOCCUS* SPP. ASSOCIATED WITH CLINICAL MASTITIS IN DOMESTIC RUMINANTS

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ABSTRACT

A study was undertaken to determine the prevalence and *in vitro* antimicrobial susceptibility of *Staphylococcus* spp. isolated from 32 mastitic milk samples received from lactating cows (12), buffaloes (9) and goats (11) of Tonk district (Rajasthan). *Staphylococcus* spp. was isolated (n=29) from milk samples by routine cultural technique and confirmed by 16S rRNA sequencing and NCBI blast analysis. Among the isolates, prevalence of *S. aureus* was maximum (41.38%) followed by *S. chromogenes* (20.70%), *S. haemolyticus*, *S. gallinarum* (13.79% each) and *S. xylosus* (10.34%). On antimicrobial sensitivity test, the *Staphyloccocus* spp. isolates were found resistant to ampicillin (89.6%), methicilin (79.3%), polymixin-B (82.7%), penicillin-G (79.3%), cefixime (72.4%) and enrofloxacin (55.1%). The resistance against amoxicillin-clavulanic acid, ofloxacin, norfloxacin, amoxicillin and ciprofloxacin was <50%. The study indicated that *Staphylococcus* spp. are the major etiologies of mastitis in domestic animals with isolates being resistant to majority of commonly used antibiotics in the treatment of mastitis. All the *Staphylococcus* isolates tested were susceptible to bacitracin, chloramphenicol, ceftriaxone, imipenem, doxycycline, gentamicin, chlortetracycline, tetracycline, amikacin and vancomycin and could be used in treatment of mastitis caused by *Staphylococcus* spp. in the region.

Key words: Antibiotics, Antimicrobial resistance, Mastitis, *Staphylococcus* spp

Mastitis is an inflammatory condition of mammary gland caused by environmental or contagious pathogenic microorganisms including bacteria, algae and mycoplasma. In India, the total annual economic loss due to bovine mastitis was calculated as Rs. 7165.51 crore (Bansal and Gupta, 2009). Besides financial implications, the importance of mastitis in public health could not be disregarded. Contaminations of common foods such as milk, milk products and meat due to infectious agents acquired by mastitis have been reported as potential health risks and unsuitable for human consumption. It includes, *Staphylococcus* spp., *Streptococcus agalactiae*, *Corynebacterium bovis*, *Mycoplasma* spp., *Streptococcus uberis*, Coliforms (*Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp. and *Serratia* spp.), *Pseudomonas*, *Proteus* spp. and environmental *Streptococci* (Erskine et al., 2002) etc. Apart from this, most of these bacterial species are responsible for streptococcal toxicity, colibacillosis, streptococcal sore throat and brucellosis in humans.

Staphylococcus spp. is well known for its tolerance to a wide range of adverse circumstances and has the potential to develop resistance to almost all the antimicrobial agents used for the management of the disease (Barkema et al., 2009). Extensive use of antibiotics in the treatment and control of mastitis (Erskine et al., 2002) has possible implications for

human health through an increased risk for emergence of antibiotic resistant strains that may enter the human food chain. In view of the economic importance of the disease and increasing antimicrobial resistance of the microorganisms a study was undertaken to determine the prevalence and *in vitro* antibiotic susceptibility profiles of *Staphylococcus* spp. in the milk samples of mastitis affected cows, buffaloes and goats.

MATERIALS AND METHODS

During 2014 to 2016, a total of 32 milk samples from clinical mastitis affected cows (n= 12), buffaloes (n=9) and goats (n=11) received from Malpura and surrounding villages of Tonk district (Rajasthan) were used for the study. The history of animals revealed painful swelling of udder with redness. The milk colour was red brown with thick consistency. In some animals, udder was hard to touch, less painful and presence of watery milk from the teat of affected quarter. All the samples were processed for initial bacterial isolation on nutrient agar plates. The golden/white, opaque, smooth, glistening colonies suspected for *Staphylococci* were smeared on glass slide and stained by Gram's method and observed under microscope. The gram positive colonies showing grapes like clusters were sub-cultured on nutrient agar slants to obtain pure culture. Coagulase test was used to differentiate coagulase positive and negative *Staphylococci* isolates using rabbit plasma. The slants of suspected *Staphylococcus* spp. were maintained at 4°C till further processing.

The *S. aureus* isolates were confirmed by using primers of *S. aureus* specific 16S rRNA genes (Forward, 5'-AACTCTGTTATTAGGGAAGAAC-3' and reverse, 5'-CCACCTTCCTCCGGTTTGTCACC-3') (Moussa and Shibl, 2009) and thermonuclease (*nuc*) genes by using primers (Forward, 5'-GCGATTGATGGTGATACGGTT-3' and reverse, 5'- AGCCAAGCCTTGACGAACTAAAGC -3') (Brakstad et al., 1992). For amplification of *S. aureus* specific16S rRNA and thermonuclease (*nuc*) genes the PCR conditions were an initial denaturation for 5 min at 95°C, each PCR cycle (total 35 cycles) consisted of denaturation at 94°C for 30 sec, annealing at 58°C for 45 sec, elongation at 72°C for 45 sec and final extension at 72°C for 10 min. The PCR products were resolved on 1.5 % agarose gel electrophoresis and visualized with ethidium bromide staining. The *S. aureus* isolates were confirmed by amplification of specific product size of 756 and 270 bp for 16S rRNA and thermonuclease (*nuc*) genes, respectively (Plate 1).

For the molecular identification of suspected *Staphylococcus* isolates, sequencing was done by using bacterial 16S rRNA universal genes and amplified on PCR with primers of 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Lane, 1991). For all the *Staphylococcus* isolates specific sized band (1466 bp) (Plate 2) amplified by using 16S universal genes primers were excised from gel under UV light and subsequently purified using MinElute gel extraction kit (Qiagen). The eluted DNA was quantified using Nanodrop UV spectrophotometer and DNA concentration equalized at ~100 ng/µl and stored at -20°C until use. The purified products were sequenced by Xcelris genomics, India and compared with sequence available in the NCBI database.

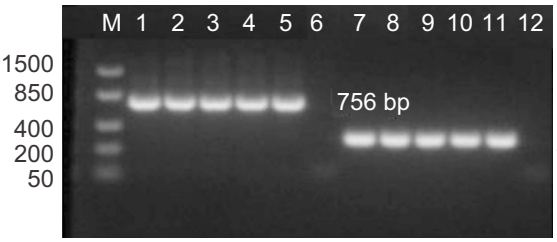

Plate 1. PCR profile of *S. aureus* (lane M: DNA ladder, lane 1-4: *S. aureus* isolates positive for 16S rRNA specific gene, lane 5 and 11: Known positive *S. aureus* (VTCCBAA999), lane 6 and 12: Non template control, lane 7-10: *S. aureus* isolates positive for thermonuclease gene

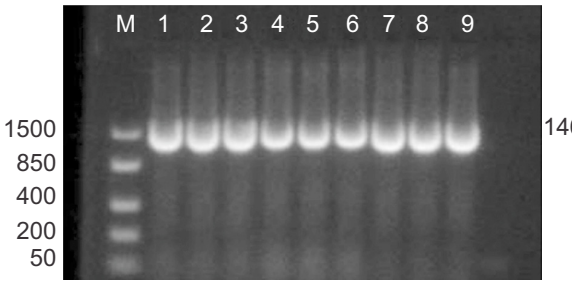

Plate 2. Representative bacterial 16S rRNA gene amplification of the *Staphylococcus* isolates (lane 1-9), lane M: DNA ladder

All the confirmed *Staphylococcus* isolates were tested for their susceptibility to 29 antibiotic discs (Hi-Media, Mumbai). The *in vitro* antimicrobial susceptibility test (AST) was performed following the disc diffusion method (Bauer et al., 1966). The results were interpreted for each antibiotic disk as per the CLSI and EUCAST standard as indicated by manufacturer.

RESULTS AND DISCUSSION

A total of 29 *Staphylococcus* spp. were isolated from the milk of clinically mastitis affected cows, buffaloes and goats (Table 1). Among the *Staphylococci*, *S. aureus* (41.38%) was more prevalent than other species. In cows and goats, 80.0 and 44.4% of isolates were *S. aureus*, respectively. The present prevalence was higher than previous reports of 5 to 18% in dairy cows and 5 to 10% in goats (Contreras and Rodriguez, 2011; Mahlangu et al., 2018). Similarly, Sharma et al. (2015) reported 50% incidence of *S. aureus* from clinical cases of mastitis; however, the difference in the prevalence of pathogens may be due to parity, type of sample, season and place of collection.

Table 1. Isolation of *Staphylococcus* spp. from mastitis affected animals

Species	Cow (n=10)	Buffalo (n=10)	Goat (n=9)	Overall (n=29)
S. aureus	8 (80.00)	-	4 (44.44)	12 (41.38)
S. chromogenes	-	4 (40.00)	2 (22.22)	6 (20.70)
S. xylosus	2 (20.00)	-	1 (11.11)	3 (10.34)
S. haemolyticus	-	2 (20.00)	2 (22.22)	4 (13.79)
S. gallinarum	-	4 (40.00)	-	4 (13.79)

Figures in parentheses are percentages

Although *S. aureus* is one of the most important pathogens for mastitis in animals(41.38%), coagulase negative *Staphylococci* (CNS) are recognised as agents associated with intramammary infections in most of the countries and described as emerging pathogens for mastitis (El-Jakee et al., 2013). In our study, CNS such as *S. chromogenes* (20.70%), *S. haemolyticus* (13.79%), *S. gallinarum* (13.79%) and *S. xylosus* (10.34%) were isolated from the milk of mastitis affected cows, buffaloes and goats. *S. chromogenes* was reported as the most commonly isolated CNS from both clinical and sub-clinical bovine mastitis in Finland (Taponen et al., 2006) and in intramammary infection in Washington State (Quirk et al., 2012). However, in the present study, *S. chromogenes* from cows was not isolated, but in buffaloes and goats, 40.00 and 22.22% of the *Staphylococci* were S. *chromogenes,* respectively. *S. chromogenes* can colonise the skin and act as udder pathogens as they are well-adapted to the udder (Thorberg et al., 2009). The CNS species along with *S. hemolyticus* and *S. xylosus* may cause long-lasting udder health problems in animals. *S. xylosus* from cows (20.00%) and goats (11.11%) and *S. haemolyticus* from buffaloes (20.00%) and goats (22.22%) were isolated from mastitic milk, which falls in line with the previous reports of intra mammary infections caused by these organisms (Taponen et al., 2006; Thorberg et al., 2009). *S. gallinarum* strains (CNS) were first isolated from chickens and a pheasant (Devriese et al., 1983) and were considered as lowly pathogenic though they have been implicated in various human infections. *S. gallinarum* strains have also been isolated from sub-clinical and clinical cases of mastitis in sheep and goat flocks from the states of Pernambuco and Bahia in Northeastern Brazil (Franca et al., 2012). In our study *S. gallinarum* has been isolated in 40.0% of mastitic milk of buffaloes. The association of this *Staphylococcus* species for mastitis needs to be investigated.

Based on the AST results, susceptibility and resistance of 29 *Staphyloccocus* spp. were calculated against different antibiotics (Table 2). The emergence of multidrug resistance (MDR) in *Staphylococci* (*S. aureus* and CNS) is a problem for concern in animal production and issues related to public health (Virdis et

al., 2010). The total resistance calculated against different antibiotics indicated that about 70 to 90% of the *Staphylococci* isolated were resistant to ampicillin, methicillin, penicillin-G, polymyxin-B and cefixime. About 27 to 55% *staphylococci* showed resistance to enrofloxacin, amoxyclav, ofloxacin, norfloxacin, amoxycillin and ciprofloxacin. The isolates of *S. aureus* were 100% resistant to ampicillin, enrofloxacin, methicillin, polymixin-B and penicillin-G and 66 to 84% showed resistance to amoxyclav and quinolones (ofloxacin, norfloxacin and ciprofloxacin). *S. aureus* isolates also showed resistance to cefaxime (33.3%) and amoxicillin (50%). It was reported that the methicillin resistant *Staphylococcus* strains have been observed to be multidrug resistant, such as aminoglycosides, macrolides, lincosamides, streptogramins, tetracycline *etc*. which were commonly used in the treatment of mastitis (Kumar et al., 2010). The *Staphyloccocus* spp. were 100% susceptible to bacitracin, cloramphenicol, ceftriaxone, imipenem, doxycycline, gentamycin, chlortetracycline, tetracycline, amikacin and vancomycin.

Table 2. Antibiotic resistance profiles (%) of *Staphylococcus* spp. isolated from cows, buffaloes and goats

Antibiotic	*S. aureus*	*S. chromogenes*	*S. haemolyticus*	*S. Xylosus*	*S. gallinarum*	Overall
Ampicillin	100.0	50.0	100.0	100.0	100.0	89.6
Amoxyclav	83.3	0.0	50.0	0.0	0.0	41.3
Bacitracin	0.0	0.0	0.0	0.0	0.0	0.0
Chloramphenicol	0.0	0.0	0.0	0.0	0.0	0.0
Cefepime	0.0	0.0	50.0	0.0	0.0	6.8
Ceftriaxone	0.0	0.0	0.0	0.0	0.0	0.0
Ceftazidime-T	0.0	0.0	50.0	0.0	0.0	6.8
Imipenem	0.0	0.0	0.0	0.0	0.0	0.0
Doxycycline	0.0	0.0	0.0	0.0	0.0	0.0
Enrofloxacin	100.0	0.0	100.0	0.0	0.0	55.1
Erythromycin	16.6	0.0	0.0	0.0	0.0	6.8
Kanamycin	0.0	0.0	50.0	0.0	0.0	6.8
Methicillin	100.0	50.0	100.0	0.0	100.0	79.3
Ofloxacin	83.3	0.0	50.0	0.0	0.0	41.3
Nitrofurantoin	0.0	0.0	50.0	0.0	0.0	6.8
Norfloxacin	83.3	0.0	0.0	0.0	0.0	34.4
Novobiocin	0.0	0.0	0.0	100.0	0.0	10.3
Polymyxin-B	100.0	100.0	50.0	0.0	100.0	82.7
Penicillin-G	100.0	50.0	100.0	0.0	100.0	79.3
Streptomycin	0.0	0.0	50.0	0.0	0.0	6.8
Gentamicin	0.0	0.0	0.0	0.0	0.0	0.0
Cefixime	33.3	100.0	100.0	100.0	100.0	72.4
Chlortetracycline	0.0	0.0	0.0	0.0	0.0	0.0
Amoxicillin	50.0	0.0	100.0	0.0	0.0	34.4
Cloxacillin	0.0	0.0	50.0	0.0	0.0	6.8
Tetracycline	0.0	0.0	0.0	0.0	0.0	0.0
Amikacin	0.0	0.0	0.0	0.0	0.0	0.0
Vancomycin	0.0	0.0	0.0	0.0	0.0	0.0
Ciprofloxacin	66.6	0.0	0.0	0.0	0.0	27.5

The multidrug resistance of *Staphylococci* observed in the present study could be due to the indiscriminate use of beta–lactam antibiotics and related intramammary infusions for the treatment of mastitis which leads to the development of resistance due to the production of beta-lactamases and low-

affinity penicillin-binding protein, PBP2A (Bush, 2018). Methicillin-resistant *S. aureus* strains are known to have the potential to complicate treatment of mastitis in animals. These strains have been considered as a potential risk to other exposed animals in the farm, farm workers and associated veterinary officials (Juhasz-Kaszanyitzky et al., 2007). Indiscriminate use of antibiotics and intramammary preparations used by the owners without veterinary control could be one of the reasons for increasing incidence of these strains.

Apart from *S. aureus* isolates MDR was also seen in CNS isolates of the present study. Most of the CNS isolates (*S. chromogenes, S. hemolyticus, S. xylosus and S. gallinarum*) showed resistance to ampicillin, enrofloxacin, methicillin, novobiocin, polymixin-B, penicillin-G, cefixime and amoxicillin. These isolates were also resistant to amoxyclav, cefapime, ceftazidine-T, kanamycin, ofloxacin, nitrofurantoin, streptomycin and cloxacillin. A study conducted in South Africa on non-pasteurized milk reported MDR as a common phenomenon observed in almost all *S aureus* isolates as well as in around 10% of the CNS (Pekana et al., 2017). In contrast, the present study reports higher resistance in CNS to commonly used antibiotics such as methicillin, ampicillin, enrofloxacin, penicillin-G, amoxicillin, novobiocin, amoxyclav, cefapime, ceftazidine-T, kanamycin, ofloxacin, nitrofurantoin, streptomycin and cloxacillin. It also indicated alarming drug resistance frequencies of the tested antibiotics. It was also observed that incidence of antibiotic resistance was higher in *S. aureus* and CNS in comparison to other reports. It was also hypothesized previously that CNS may serve as a reservoir for the transfer of antimicrobial resistance genes to *S. aureus*. Therefore, isolation of CNS from mastitis cases should not be ignored as super antigens (a family of potent immunostimulatory exotoxins) have been demonstrated not only in *S. aureus* but in CNS also isolated from mastitis (Park et al., 2011).

In conclusion, increasing multidrug resistance in *Staphylococcus* species against commonly used antibiotics in the treatment of mastitis is a major concern to the public health, as the milk may act as carrier of resistant *Staphylococci* strains and can transmit infection to humans *via* contaminated milk. The antibiotics such as bacitracin, chloramphenicol, ceftriaxone, imipenem, doxycycline, gentamicin, chlortetracycline, tetracycline, amikacin, and vancomycin found sensitive against *Staphylococcus* isolates which can be used in the therapeutic management of mastitis in domestic ruminants. Further, to counteract antibiotic resistance, there is need to explore the options of alternative therapies for the treatment of mastitis such as Ayurveda drugs and phage therapy.

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