

Research Article

ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF METHICILLIN RESISTANT AND METHICILLIN SENSITIVE *STAPHYLOCOCCUS AUREUS* FROM BOVINE MILK IN THE STATE OF HARYANA, INDIA

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Received 22 July 2024, revised 09 September 2024

ABSTRACT: Bovine mastitis is the principal leading cause of monetary losses to dairy farmers. This disease impacts the udder health as well as the quantitative and qualitative parameters of milk. The disease is multi-etiological, but *Staphylococcus aureus* (*S. aureus*) contributing to intramammary infections is the principal cause. Our work intended to go through the sensitivity pattern of *S. aureus* obtained from milk samples of bovines. The samples used in the study were received at College Central Laboratory, LUVAS, Hisar. The bovine milk samples were inoculated on blood agar to obtain bacterial isolates, followed by morphological and biochemical characterization. *S. aureus* was confirmed by phenotypic as well as molecular assays. Ninety-five staphylococci were preliminarily isolated from 381 quarter milk samples based on morphological features of a bacterial colony, Gram stain, catalase reaction, oxidase test, and HiStaph latex™ kit from bovines. Out of these, 86 *S. aureus* isolates were confirmed based on phenotypic (mannitol salt agar) as well as a molecular test (23S rRNA PCR). All these isolates were used for sensitivity profiling by Kirby Bauer disc diffusion method. Maximum sensitivity was observed for chloramphenicol and doxycycline, least against cloxacillin and methicillin. From 86 isolates, 62.79% were found to be multidrug-resistant (MDR). From MDR isolates, 11.11% were extensively drug-resistant (XDR) and none were pan-drug-resistant (PDR). The presence of a high percentage of MDR phenotypes among *S. aureus* isolates in this study draws our attention that treatment of animals must be carried out after the identification of pathogens, followed by patterns of sensitivity to antimicrobials.

Keywords: : Mastitis, MDR, *S. aureus*, Bovine milk.

INTRODUCTION

Bovine mastitis being a ubiquitous disease affects dairy animals globally [1, 2]. The disease is multi-etiological, but among intramammary infections on a farm like mastitis bacteria are the prime common cause. Both gram-positive (Staphylococci, Streptococci, *Listeria* sp.) and gram-negative (*E. coli*, *Klebsiella* sp., *Pseudomonas* sp., *Proteus* sp.) bacteria can cause the disease [3]. But *S. aureus* is the principal cause of mastitis in bovine. The mammary glands act as reservoirs of the bacteria, so it is highly prevalent in clinical, subclinical, and even chronic mastitis [4].

S. aureus being a potential zoonotic agent has been constantly associated with the rapid emergence of

MDR strains panworld in dairy mastitis [5, 6]. According to the 20th Livestock Census 2019, the total cattle and buffalo in India are 193.46 million and 109.85 million, respectively [7]. The state of Haryana has a total livestock population of 71,26,497; out of which 19,32,039 and 43,76,644 are cattle and buffalo, respectively [7]. The rate of occurrence of subclinical and clinical mastitis in India has been reported to be about 45% and 18%, respectively leading to tremendous losses to the dairy industry [8]. Since the beginning, antibiotics have been used as the primary treatment of choice against bacterial agents resulting in the occurrence of disease [5, 8]. However, due to unchecked employment of antibiotics for treatment, there has

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been constant emergence of antibiotic-resistant strains *viz.* MRSA has led to serious animal and public health issues [1, 9]. Methicillin resistance in *S. aureus* is linked to the presence of the *mecA* gene at the staphylococcal chromosomal cassette *mec* (SCC*mec*) and is linked with resistance to virtually all β -lactams [9, 10]. The increase in MDR isolates, with escalating number of MRSA infections has limited the therapeutic success as well as affected production levels [11, 12]. The existence of antibiotic residues in the consumption of milk along with MDR organisms in dairy foods has led to alarming implications for public health [11]. This has resulted in a negative impact on one-health, thus making antimicrobial resistance (AMR) a priority for global agencies [11, 12].

The unchecked utilization of antibiotics without sensitivity testing is one of the reasons for treatment failure in dairy mastitis [1, 2]. Therefore, it has become necessary to monitor the sensitivity patterns of organisms to assist veterinarians in choosing the right therapy [2]. *S. aureus* is an opportunistic pathogen which is a known pathogen of diseases in both humans and livestock. The occurrence of MRSA in bovine milk presents a probable risk to veterinarians, farm workers, and animals [12]. So, our study intends to isolate and evaluate antibiotic sensitivity data of MSSA as well as MRSA isolates obtained from clinical milk samples.

MATERIALS AND METHODS

Isolation and presumptive identification of *S. aureus* from milk samples

Clinical milk samples obtained in College Central Laboratory, LUVAS, Hisar, Haryana directly from cattle owners around Haryana were used in this study. The received samples were immediately inoculated on 5% sheep blood agar and placed for incubation at 37°C for 24 hours. The colonies were presumptively recognized as *S. aureus* based on gram staining, catalase, and oxidase tests along with a HiStaph latex™ kit.

Phenotypic and molecular characterization of *S. aureus*

The bacterial colonies identified presumptively were additionally ensured phenotypically by inoculation on mannitol salt agar (MSA), *i.e.* specific media for identifying *S. aureus*. The MSA plates were allowed to incubate at 37°C for 24 h. The molecular confirmation was achieved by polymerase chain assay (PCR) assay targeting the 23S rRNA gene. For this, DNA isolation was done using a commercially available QIAamp

Blood Kit (Qiagen, Germany) as per instructions detailed by the manufacturer. The PCR was done using published primers (Forward: 5' AGC GAG TCT GAA TAG GGC GTT T 3' and Reverse: 5' CCC ATC ACA GCT CAG CCT TAA C 3') by Shome *et al* [13], using similar thermocycler conditions. The DNA template of *S. aureus* ATCC 700699 was added to carry out a reaction in positive control. The PCR products of size approximately 894 bp were seen by the UV illuminator after running 1% agarose gel electrophoresis (AGE).

Phenotypic and molecular identification of MRSA

S. aureus isolates were characterized for harboring methicillin resistance based on culture sensitivity test (CST) by Kirby Bauer's disc diffusion method as per CLSI guidelines [14]. Methicillin discs of concentration 5 μ g were used for identifying methicillin-resistant isolates. The isolates were molecularly directed for the presence of a methicillin-resistant gene, *mecA*. For this published genes (F: 5' TCC AGA TTA CAA CTT CAC CAG G 3'; R: 5' CCA CTT CAT ATC TTG TAA CG 3') by Oliveira and deLencastre [15], were used following similar conditions. The genomic DNA extracted from *S. aureus* ATCC 700699 and *S. aureus* ATCC 25923 were added to carry the reaction in positive and negative controls, respectively. The PCR products of size approximately 162 bp were seen by UV illuminator after running 1% agarose gel electrophoresis (AGE).

Culture sensitivity assay of *S. aureus* isolates

The sensitivity results of the *S. aureus* isolates were obtained by Kirby Bauer's disc diffusion method ensuing guidelines given by CLSI [14]. The sensitivity profiling was performed using 16 antibiotics belonging to 10 antibiotic classes, as mentioned in Table 1. Comparative analysis of the antibiogram of MRSA and methicillin-sensitive *S. aureus* was also done to obtain an overview of drug susceptibility.

RESULTS AND DISCUSSION

Mastitis in bovines has been considered one of the most devastating infectious diseases affecting dairy [16, 17, 18]. Although there have been extensive studies related to the identification and antibiogram assessment of *S. aureus* [19, 20], the organism still stands as the leading etiology [20, 21, 22]. Haryana state has 1932039 and 4376644 cattle and buffalo, respectively [7], and the occurrence of mastitis is also on the higher end. Yadav *et al.* [23] reported about

Table 1. List of antibiotic discs used in the study along with their concentration.

| Sl. No. | Group of Antibiotics | Antibiotics | Concentration |
|---------|----------------------|-----------------|---------------|
| 1 | Cephalosporins | Cefoxitin | 30 µg |
| | | Cefoperazone | 75 µg |
| | | Ceftriaxone | 30 µg |
| 2 | Glycopeptides | Vancomycin | 30 µg |
| 3 | Quinolones | Enrofloxacin | 10 µg |
| | | Ciprofloxacin | 10 µg |
| 4 | Oxazolidinones | Linezolid | 30 µg |
| 5 | Suphonamides | Sulfisoxazole | 300 µg |
| 6 | Tetracyclines | Doxycycline | 30 µg |
| | | Oxytetracycline | 30 µg |
| 7 | Lincosamide | Clindamycin | 02 µg |
| 8 | Chloramphenicols | Chloramphenicol | 30 µg |
| 9 | Aminoglycosides | Neomycin | 30 µg |
| | | Gentamicin | 30 µg |
| 10 | β- lactams | Cloxacillin | 30 µg |
| | | Methicillin | 05 µg |

38% prevalence of mastitis in Southern Haryana, whereas Bhanot *et al.* [24] reported > 75% of bovines to be affected in the eastern part of Haryana. Kumar and Sharma [25] reported a 60% prevalence of mastitis disease in bovines in the Hisar district of Haryana, whereas Manoj *et al.* [26] reported around 86% prevalence across Haryana. Since, a large portion of the bovine population is affected by the disease and *S. aureus* is the principal cause, the current study intended to analyze the occurrence and profiling of antibiogram of *S. aureus* isolated from clinical milk samples from across the state of Haryana.

Isolation, identification, and characterization of *S. aureus*

Three hundred and eighty-one (n=381) quarter milk samples from 101 animals were screened for isolation of *S. aureus*. Based on Gram's stain, catalase test, oxidase test, and HiStaph latexTM test, 95 (24.93% *i.e.* 95/381) bacterial colonies were presumptively taken as *S. aureus*. Based on colony morphology on specific media and PCR method, 86 (22.57% *i.e.* 86/381) isolates were pointed as *S. aureus*. This was in accordance with Mistry *et al.* (23%) [27], and Sharma *et al.* (21.73%) [28]. This percentage is lower than as reported by Charaya *et al.* (38.04%) [19]; Sharma *et al.* (51.56%) [29]; Chhabra *et al.* (63.54%) [20]; Rana *et al.* (>39%) [30].

Phenotypic and genotypic identification of MRSA isolates

Out of 86 *S. aureus* isolates confirmed based on PCR, 45 (52.33%) and 41 (47.67%) isolates were found to be MRSA and MSSA by phenotypic method, respectively. On the other hand, 19 (22.09%) isolates harbored *mecA* gene in their genome. Haran *et al.* (4%) [31] and Rana *et al.* (7.61%) [30] reported lower prevalence, whereas Hoque *et al.* (26.6%) [32] and Mistry *et al.* (48.7%) [27] reported higher prevalence of MSSA compared to our study. The National Action Plan on AMR (NAP-AMR) [8], India reported that MRSA proportion had increased to 47% in bovine mastitis by 2014, which is much higher than our study. The prevalence of aetiological agents that cause mastitis and the disease itself is higher in the Northern part of India, including the state of Haryana. This may be due to rising cases and a higher population of bovines in this area [33].

Antibiotic sensitivity pattern of *S. aureus*

The 86 *S. aureus* isolates characterized by PCR were used for the analysis of the antibiogram. The bacteria were categorized as sensitive, intermediate, and resistant after a comparison of inhibition zones with breakeven points given by CLSI [14]. The isolates possessed the highest sensitivity to chloramphenicol (90.70%), proceeded by doxycycline (87.21%), linezolid (86.05%), oxytetracycline (83.72%), gentamicin (80.23%), vancomycin (77.91%) and sulfisoxazole (77.91%), cefoperazone (76.74%), enrofloxacin (75.58%), neomycin (74.42%), clindamycin (72.09%), cefoxitin (70.93%), ciprofloxacin (69.77%), ceftriaxone (62.79%), cloxacillin (52.33%) and least against methicillin (47.67%) (Fig. 1). Out of 86 isolates, 54 (62.79%) isolates were identified as multidrug-resistant (MDR). From MDR isolates, 6 (11.11%) isolates were identified as extensively resistant (XDR) and no isolate was found to be pan-drug resistant (PDR). The sensitivity percentages in our study were lower than Charaya *et al.* [19], Sharma *et al.* [29], and Neelam *et al.* [34]; whereas values were higher than Chhabra *et al.* [20] and Rana *et al.* [30]. In a study conducted by Pankaj *et al.* [35], they found 100% sensitivity toward cloxacillin, ceftriaxone, and cefoperazone. Mistry *et al.* [27] reported 100% sensitivity towards oxacillin, cefoxitin, vancomycin, and linezolid. In contrast to this, none of the antibiotics was found to be 100% sensitive in our study. Brahma *et al.* [36] reported 100% sensitivity toward vancomycin and linezolid. Mubarack *et al.* [37] reported 100% sensitivity

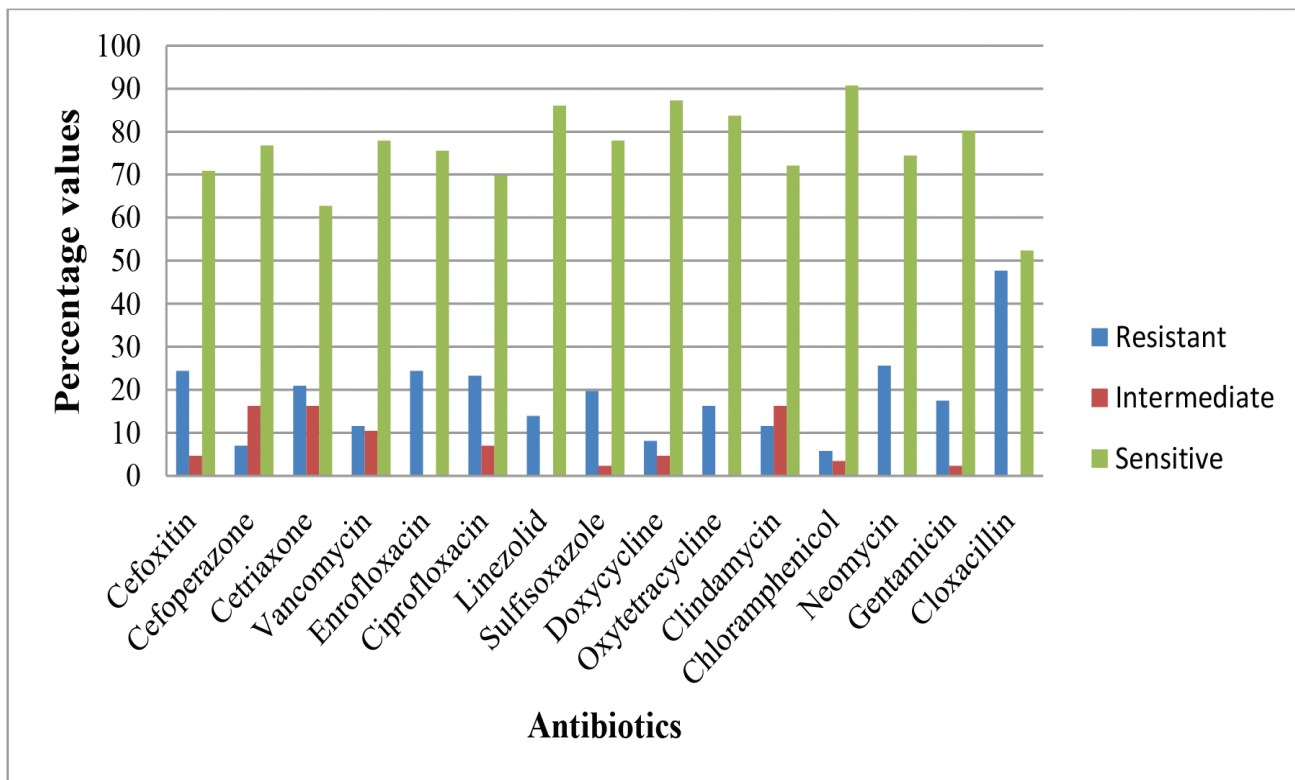


Fig. 1. showing antibiogram pattern of *S. aureus* isolates from milk samples against various antibiotics used for treatment in bovine mastitis.

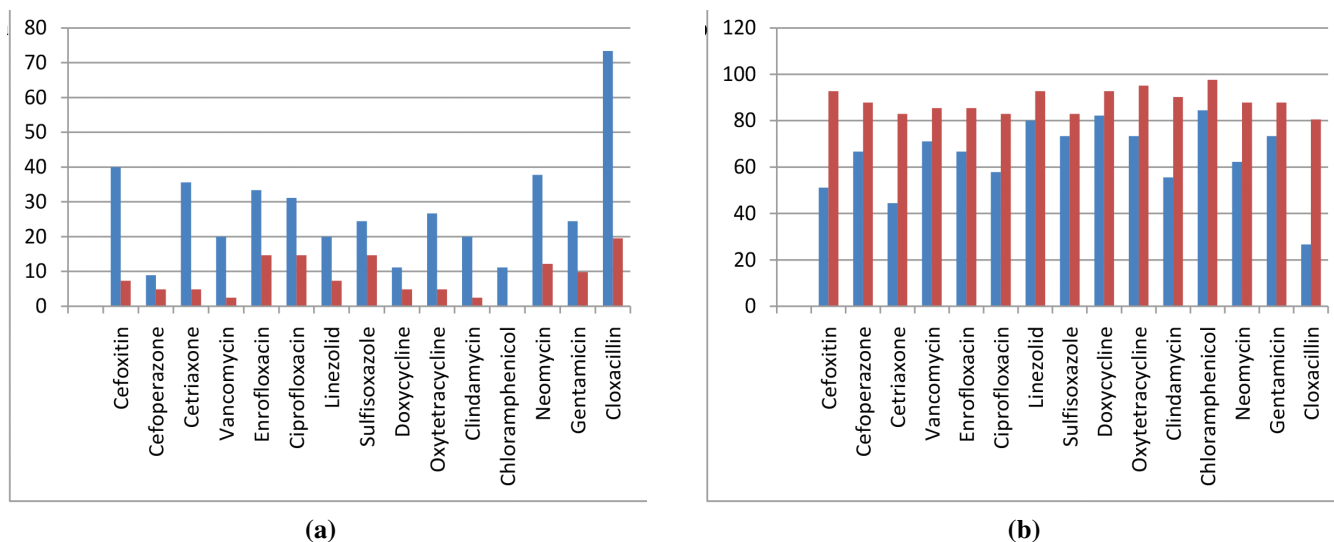


Fig. 2. Comparative resistance (a) and susceptibility (b) pattern of MRSA (blue bars) and MSSA (red bars) isolates from bovine milk samples.

towards gentamicin. Manoj *et al.* [26] carried out a temporal study on bovine mastitis in Haryana from 2019-2024. They reported *S. aureus* to be most sensitive to gentamicin, chloramphenicol, and ceftriaxone over the years. They also found that there was an increase in the

sensitivity of the antibiotics in the later years of the study period [26].

Comparative analysis of antibiogram of MRSA and MSSA isolates

In vitro, analysis of drug sensitivity of MRSA and MSSA isolates elucidated that these isolates show

variability in their susceptibility patterns. All the isolates resistant to methicillin phenotypically were used as MRSA and others as MSSA for comparative antibiogram. MSSA isolates showed highest susceptibility towards chloramphenicol, followed by linezolid>gentamicin, oxytetracycline, sulfisoxazole>vancomycin>cefoperazone, enrofloxacin>neomycin>ciprofloxacin>clindamycin>cefoxitin>ceftriaxone>cloxacillin (Fig. 2b). On the other hand, MRSA isolates depicted the highest susceptibility towards chloramphenicol, followed by oxytetracycline>doxycycline, linezolid, cefoxitin>clindamycin>cefoperazone, neomycin, gentamicin>vancomycin, enrofloxacin>ceftriaxone, ciprofloxacin, sulfisoxazole>cloxacillin (Fig. 2b). We can also interpret that MRSA isolates exhibit higher resistance percentage values towards different antibiotics as compared to MSSA (Fig. 2a). On comparison of antibiogram between MRSA and MSSA isolates, we found the difference in sensitivity patterns and also the percentage values of resistance towards various antimicrobials were higher in MRSA isolates. This highlights the havoc being created by MRSA isolates in the failure to control the disease. We found MDR occurrence to be 62.79% of the total *S. aureus*, i.e. the isolates showing resistance to ≥ 3 antibiotic classes and isolates resistant to methicillin were also counted in this [38]. The comparable results were reported by Mistry *et al.* [27]. Rana *et al.* [30] reported 100% isolates as MDR in their study, whereas a lower percentage (41.5%) was reported by Ren *et al.* [39].

The ineffectiveness of the antibiotics against *S. aureus* mastitis can be linked to the indiscriminate use of antibiotics [30]. Antibiogram patterns show variations among different farms, and geographical regions depending upon the ease of availability of antibiotics and their use in a particular area. So, sensitivity tests of bacterial cultures using various antibiotics should be mandatory before the commencement of therapeutics [30]. The rise in the number of MDR organisms is a substantial threat in tackling mastitis-affected animals as it fails therapeutic and preventive interventions. The higher number of MDR isolates in the study points out the unchecked use of antibiotics by the dairy sector [30, 40, 41]. This threatens not only the animals but also public health due to the entry of antibiotic residues and MDR organisms into the food chain through dairy animals and their byproducts [35, 40, 41].

CONCLUSION

Raw milk is used for consumption as well as for processing various dairy products. This has led to the entrance of MDR bacteria and antibiotic residues in humans through the food chain. Results of our study highlight that there is increasing resistance amongst MRSA and MSSA isolates against various antibiotics used in veterinary treatment. Therefore, we should strictly monitor antibiotic use in dairy animals to prevent public health concerns arising from the indiscriminate use of antibiotics and ensure treatment following sensitivity testing. On comparison of data, we concluded that despite variation in antibiogram, chloramphenicol and doxycycline can be used in treatment irrespective of being MRSA or MSSA, as both of these showed >80% sensitivity in all the isolates.

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Cite this article as: Kaur J, Lather A, Kamboj S, Singh M, Manoj J, Chhabra R. Antimicrobial susceptibility profile of methicillin resistant and methicillin sensitive *Staphylococcus aureus* from bovine milk in the state of Haryana, India. *Explor Anim Med Res.* 2024; 14(Superbug Spl.), DOI:10.52635/eamr/14(S2)74-80.