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Antimicrobial Susceptibility Profiling of Staphylococcus aureus from Bovine Clinical Mastitis Milk Samples

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ABSTRACT

Mastitis is the most common disease of bovine, leads to reduced and poor quality of milk yield causing huge economic losses. The objective of the present study was to estimate the incidence of *Staphylococcus aureus* in bovine clinical mastitis and determine the antibiotic susceptibility. For this purpose, a total of 71 milk samples from bovine clinical mastitis were collected from different regions of the Capital of Bihar from January 2021 to March 2022. By conventional enrichment and plating, *S. aureus* was isolated from 52.11% of samples. Further 39.44% incidence of *S. aureus* was confirmed using biochemical and molecular tools. On antibiotic susceptibility testing, the isolates were 100% resistant to penicillin and amoxicillin, while higher resistance were shown to ampicillin, oxacillin, amoxicillin/clavulanic acid, cefoxitin, erythromycin, and tetracycline.

Keywords: Bovine, Clinical mastitis, *Staphylococcus aureus*, 16SrRNA, ABST

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INTRODUCTION

Mastitis is an altered condition of the mammary gland parenchyma caused by a wide range of pathogens, primarily bacteria. Mastitis can be clinical or subclinical, depending on the intensity and progression of the inflammation. Visible abnormalities, such as red and swollen udders, fever, and watery with flakes or clots in milk, make clinical mastitis obvious and simple to identify (Kaliwal and Kulkarni, 2013). Clinical mastitis is an economically significant illness that affects the dairy industry globally. Severe milk losses, decreased milk quality, higher veterinary and treatment costs, additional labour costs, and a higher risk of the culling. The affected dairy cows are just a few of the losses brought on by clinical mastitis. In addition to these financial implications, clinical mastitis is a recurrent condition that increases the risk of subsequent cases of mastitis during the current lactation or the subsequent lactation. The annual economic losses in India have been estimated as Rs.7,165.5 crores of which Rs.4,151.1 crores due to subclinical and Rs.3,014.4 crores from the clinical mastitis (Bansal and Gupta, 2009).

Escherichia coli, *Klebsiella* sp., *Staphylococcus aureus*, Coagulase Negative Staphylococci (CONS), *Streptococcus agalactiae*, *Streptococcus uberis*, and other streptococci species are the main pathogens responsible for clinical mastitis (He *et al.*, 2020). Among these, *S. aureus* is considered as one of the most significant and prevalent pathogenic microorganisms in bovine mastitis. Udder infection by bacteria such as *Staphylococcus aureus* results in its excretion through milk in variable numbers fluctuating from zero to 10^8 CFU/ml (Asperger and Zangerl, 2003). Staphylococcal mastitis is a major problem for dairy cows, affecting animal well-being with economic losses due to decreased milk quality and milk yield. As *S. aureus* may spread among cows during milking, a concerted approach is required to prevent its spread to healthy animals (Gomes and Henriques, 2016). *S. aureus* isolated from dairy animals is also a major cause of food poisoning in humans. Leukocidin, hemolysins, toxic shock syndrome toxin-1, fibronectin-binding proteins, and clumping factors are just a few of the many virulence factors carried by *S. aureus* that help it adhere to the extracellular matrix components of the host, harming host cells and impairing the immune system (Campos *et al.*, 2022).

Antimicrobials are the most frequently used in the dairy industry for dry cow therapy or the treatment of clinical mastitis and it is well known fact that the overuse or misuse of antibiotics promotes the development and maintenance of antibiotic resistance. Due to selective pressures from the widespread use of antibiotics, *S. aureus* has evolved antimicrobial resistance, and MDR strains of the bacteria, such as methicillin-resistant *S. aureus*, have appeared (Gomes and Henriques, 2016). Therefore, the present study was designed to assess the status of *S. aureus* in bovine clinical mastitis with their antibiotic susceptibility profile.

MATERIALS AND METHODS

Collection of samples

A total of 71 bovine clinical mastitis milk samples from cows and buffalo were collected aseptically from various locations in Patna, Bihar, India including Kautilya Nagar, Danapur, Digha, Maner, Phulwari Sharif, Ramana Road, Khagaul, and Raja Bazar.

Isolation and identification of *S. aureus*

One milliliter (ml) of each milk samples was taken in sterile

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test tubes with 10 ml of sterilized tryptone soya broth (TSB) mixed with 10% sodium chloride salt, and then test tubes were incubated at 37^{0}C for 24 hours. The samples showing turbidity in the broth were streaked onto mannitol salt agar (MSA) plate, and incubated at 37^{0}C for 24 hours. The plates exhibiting characteristic mannitol fermenter colonies of *S. aureus* with round and golden, yellow, or pale color were selected and was examined under oil immersion followed by catalase and tube coagulase test with human plasma.

The biochemically confirmed *S. aureus* isolates were used to prepare the template DNA using the boiling and snap chilling technique as described by Kaushik *et al.* (2018) and confirmed by PCR amplification of 16S rRNA gene as per the primer pair and procedure described by Karmakar *et al.* (2016). The amplified products of 228 bp were examined using agarose gel electrophoresis (1.5%) with ethidium bromide staining (0.5 g/ml) and visualized using the gel documentation system (Vilber, France).

Antibiotic susceptibility profile of *Staphylococcus aureus*

The disc diffusion method (Wayne, 2002) was used to test for antibiotic susceptibility of isolates. The nutrient broth was inoculated with the test colony and kept overnight at 37^{0}C. By using a clean L-shaped spreader, approximately 100 µl of the growth culture was applied to Mueller-Hilton agar plates and thirteen different antibiotics, including ampicillin, oxacillin, vancomycin, cefoxitin, penicillin, amoxicillin/clavulanic acid, tetracycline, linezolid, erythromycin, chloramphenicol, gentamicin enrofloxacin, and amoxicillin were applied. All plates containing antibiotic discs were incubated for 18–24 hours at 37^{0}C. According to the recommendations of CLSI (2013), the zones of inhibition were measured using a calibrated zone scale (Hi-media, India), and the organisms were interpreted as sensitive, resistant, and intermediate against each antibiotic.

RESULTS AND DISCUSSION

In this study, a total of 71 bovines' clinical mastitis milk samples were processed for isolation of *Staphylococcus aureus*. By enrichment in TSB followed by plating on MSA, *S. aureus* characteristic colony was produced from 52.11% (37) samples. Further 28 (75.68%) isolates from these 37 samples were confirmed as *S. aureus* by microscopic examination, catalase test, coagulase test, and species-specific *16S rRNA* gene amplification (Fig. 1). The present study showed the incidence of *S. aureus* among bovine clinical mastitis as 39.44% (28/71). The samples of the present study were collected from the 09 different places of the capital of Bihar and its surrounding areas. The area-wise incidence representation showed an incidence of *S. aureus* among 66.67% of samples from Kautliya Nagar, 25.00% of Dhigha, 60.00% of Maner, 25.00% of Phulwari Sharif, 33.33% of Ramna Road, 50.00% of Khagaul, 100.00% of Rajabajar and 34.88% of samples from advance diagnostic laboratory, Bihar Veterinary College, Patna. However, *S. aureus* was not detected in the samples from Danapur. In the present work, the incidence of *S. aureus* in bovine clinical mastitis (39.44%) was in concordance with the findings of Chandrasekaran *et al.* (2014) who reported a similar prevalence of 40.4% of *S. aureus* in clinical bovine milk samples from Madras, India. However, in contrast to this study, a higher incidence of 50.00-56.00% was reported from Andhra Pradesh and Uttar Pradesh (Sharma *et al.*, 2015; Kutar *et al.*, 2015; Manasa *et al.*, 2019), while a lower incidence of 29.94- 33.33% from Ranchi and North Gujarat (Kumari *et al.*, 2020; Bhagat *et al.*, 2017) was also reported in India. Similarly, an incidence of bovine clinical mastitis of 10.78% to 32.0% was also reported from the southwest region of Western Australia, Iran, Southeast Europe, Estonia, and Tunisia (Aasmae *et al.*, 2003; Jamali *et al.*, 2014; Mehmeti *et al.*, 2016; Klibi *et al.*, 2018; Chung *et al.*, 2021).

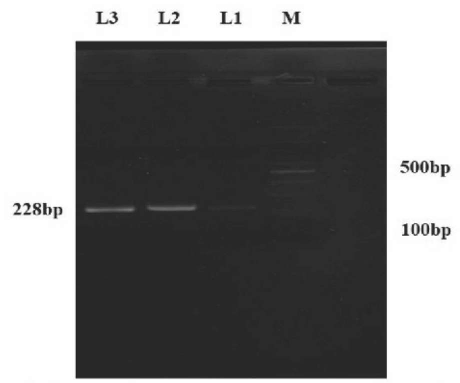

Fig.1 : *Staphylococcus aureus* species specific 16S rRNA gene amplification.
M : 100 bp DNA marker
L1: Positive Control amplification of 228 bp 16S rRNA gene
L2- L3: PCR amplification of *S. aureus* isolates from samples.

The major risk factors for developing mastitis include herd size, bedding material, and milking of mastitic cows (Abebe *et al.* 2016). These variations in the occurrence of bovine mastitis are possible, due to the difference in adopted management and hygienic practices at different dairy farms. Besides farm practices and sanitation, the mastitis incidence among bovines was reported to be significantly associated with several animal factors such as the breed of animals, milk yield, stage of lactation, and the morphology of udder (Sharma *et al.*, 2007). The climatic conditions also affect the prevalence of mastitis (Schultze 1985). Many bacterial pathogens have been isolated and known as etiological agents of mastitis in dairy animals, but *S. aureus* has been reported as the major pathogen that produces both sub-clinical and clinical intra-mammary infections (Sharma and Sindhu, 2007). Thus, the prevalence rate of ~17% as observed in the present investigation, might be attributed to intensive management and lack of proper hygiene and adoption of suitable control measures. Besides financial losses, mastitis has public health implications through injudicious use of antibiotics and other drugs having adverse effects on humans and concerns of antibiotic resistance. Improper and non-judicious use of antibiotics can form antibiotic residues in milk and meat of treated animals and thus can lead to development of antibiotic resistance (White and McDermott, 2009).

Antibiotic sensitivity profile of *S. aureus*

The antibiotic susceptibility test was performed by disc diffusion method using 13 antibiotics namely-ampicillin, oxacillin, vancomycin, cefoxitin, penicillin, amoxicillin/ clavulanic acid, tetracycline, linezolid, erythromycin, chloramphenicol, gentamicin, enrofloxacin and amoxicillin (Fig. 2). The antibiotic susceptibility study of *S. aureus* isolates from bovine clinical mastitis milk samples revealed that all isolates were resistant to ampicillin and penicillin while

96.43% were resistant to amoxicillin followed by 92.86% to oxacillin, 75.00% to cefoxitin, 57.14% to erythromycin, 53.57% to amoxicillin/clavulanic acid, 46.43% to tetracycline, 28.57% gentamicin, 25.00% to chloramphenicol and 3.57% to vancomycin and enrofloxacin. The isolates showed a susceptibility of 100.00% to linezolid followed by 67.86 % to enrofloxacin, 64.29% to chloramphenicol, 60.71% to gentamicin, 46.43% to amoxicillin/clavulanic acid and vancomycin, 35.71% to tetracycline, 25.00% to cefoxitin, 17.86% to erythromycin, 7.14% to oxacillin and 3.57% to amoxicillin. The isolates also showed an intermediate susceptibility of 50.00% to vancomycin, 28.57% to enrofloxacin, 25.00% to erythromycin, 17.86% to tetracycline, and 10.71% to chloramphenicol and gentamicin.

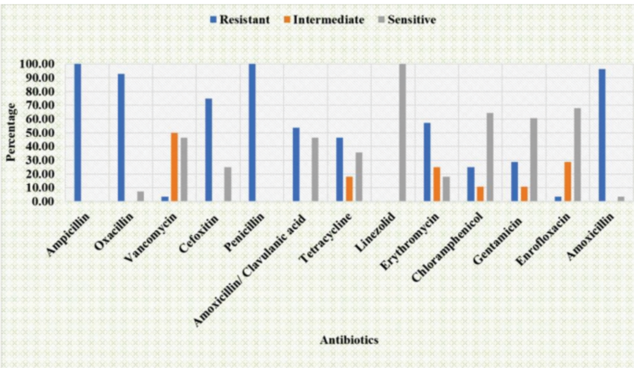

Fig. 2: Antibiotic susceptibility profile of Staphylococcus aureus isolates of bovine clinical mastitis milk

The area wise representation of antibiotic susceptibility test data showed that all *S. aureus* isolates of Kautilya Nagar were resistant to ampicillin, oxacillin, penicillin, and amoxicillin while 50% resistance with vancomycin and amoxicillin/ clavulanic acid. Further, all isolates were found susceptible to cefoxitin, linezolid, chloramphenicol and enrofloxacin while 50% to vancomycin, amoxicillin/clavulanic acid, erythromycin, and gentamicin. The isolates showed 100% intermediate resistance with tetracycline and 50% with erythromycin and gentamicin.

All isolates obtained from Digha were found resistant to penicillin and amoxicillin followed by 87.5% resistance with ampicillin and oxacillin, 75.0% to cefoxitin, 62.5% to amoxicillin/clavulanic acid, 37.5% to erythromycin and gentamicin, and 12.5% to vancomycin, and chloramphenicol. The isolates showed intermediate resistance of 75.0% with vancomycin, 37.5% to erythromycin, 12.5% to chloramphenicol and gentamicin. All isolates were found susceptible to linezolid and enrofloxacin followed by 50.0% susceptibility with gentamicin, 37.5% with amoxicillin/ clavulanic acid, 25.0% cefoxitin and erythromycin, and 12.5% with ampicillin, oxacillin, and vancomycin.

All isolates of Maner were resistant to ampicillin, oxacillin, cefoxitin, penicillin, amoxicillin/clavulanic acid, tetracycline, chloramphenicol, and amoxicillin followed by 66.67% resistant with erythromycin, and gentamicin, 33.33% resistance with enrofloxacin. All isolates were intermediate resistance with vancomycin followed by 33.33% with erythromycin, and gentamicin. All isolates were susceptible with linezolid followed by 66.67% with enrofloxacin.

All isolates of Phulwari Sharif were resistant to ampicillin, oxacillin, cefoxitin, penicillin, amoxicillin/clavulanic acid, erythromycin, chloramphenicol, and amoxicillin. The isolates also showed an intermediate resistance of 100% to vancomycin, tetracycline and enrofloxacin. All isolates showed susceptibility to linezolid and gentamycin.

All isolates of Ramana Road were resistant to ampicillin, oxacillin, cefoxitin, penicillin, amoxicillin/clavulanic acid, tetracycline, erythromycin, gentamycin, and amoxicillin. All isolates showed susceptibility to only linezolid. The isolates also showed an intermediate resistance of 100% to vancomycin, chloramphenicol, and enrofloxacin.

All isolates of Khagaul were resistant to ampicillin, oxacillin, cefoxitin, penicillin, amoxicillin/clavulanic acid, and amoxicillin. All isolates showed susceptibility to vancomycin, tetracycline, linezolid, gentamicin, and enrofloxacin. The isolates also showed an intermediate resistance of 100% to erythromycin and chloramphenicol.

All isolates of Raja Bazar were resistant to ampicillin, oxacillin, cefoxitin, penicillin, tetracycline, erythromycin, and amoxicillin. The isolates showed resistance of 50.00% to chloramphenicol and gentamicin, and 25.00% to amoxicillin/clavulanic acid. All isolates showed susceptibility to linezolid and enrofloxacin, while 75.00% to vancomycin and amoxicillin/clavulanic acid and 50.00% to chloramphenicol and gentamicin. The isolates also showed an intermediate resistance of 25.00% to vancomycin.

All isolates of advanced diagnostic laboratory, BASU, Patna were resistant to ampicillin and penicillin. The isolates showed resistance of 93.33% to oxacillin and amoxicillin followed by 73.33% to cefoxitin, 46.67% to amoxicillin/ clavulanic acid and erythromycin, 33.33% to tetracycline, 20.00% to gentamicin and 6.67% to chloramphenicol. All isolates showed susceptibility to linezolid, while 86.67% to chloramphenicol, 73.33% to gentamicin, 60.00% to tetracycline and enrofloxacin, 55.33% to vancomycin and amoxicillin/clavulanic acid, 26.67% to cefoxitin and erythromycin and 6.67% to oxacillin and amoxicillin. The isolates also showed an intermediate susceptibility of 46.67% to vancomycin, 40.00% to enrofloxacin, 26.67% to erythromycin, and 6.67% to tetracycline, chloramphenicol, and gentamicin.

The indiscriminate use of antibiotics without antimicrobial susceptibility testing of contributory organisms is presumed to be one of the most significant reasons for the failure of mastitis treatment and results in anti-microbial resistance of the organisms against commonly used antimicrobials (Owens *et al*., 1997). Antimicrobial susceptibility testing can add in selection of antibiotics for the bovine mastitis treatment caused by *S. aureus* without development of resistance, if used in proper doses. In this study, *S. aureus* isolates from bovine clinical mastitis were found to be highly resistant to antimicrobial agents. The results of this study showed that among the 13 antibiotics tested, 100% of *S. aureus* isolates were resistant to penicillin. The bovine mastitis causing *S. aureus* has developed resistant to many antibiotics like penicillin, ampicillin, streptomycin, tetracycline and oxytetracycline (Lange *et al*., 1999). Consistent with the results of this study, Varela-Ortiz *et al.* (2018) reported 100% resistance of *S. aureus* isolates from subclinical bovine mastitis to various antibiotics, including penicillin. Similar findings have been reported from other countries (Gentilini *et al*., 2000; Malinowski *et al*., 2002).

CONCLUSIONS

According to the findings of present study, it could be concluded that *S. aureus* is a significant pathogen contributing to bovine clinical mastitis in and around Patna, Bihar. The frequent use of antibiotics in intra-mammary infections without sensitivity testing, the prescription of the incorrect medications, or the administration of an incomplete course of treatment to animals could all be contributing factors to the widespread development of antibiotic resistance. The results of the study can be utilised in choosing the best medications to treat multidrug resistance *S. aureus* induced bovine mastitis in the study area.

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