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Research Article

PREVALENCE OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND ANTIMICROBIAL RESISTANCE PATTERN IN MASTITIC BUFFALOES OF JABALPUR, MADHYA PRADESH, INDIA

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ABSTRACT: The World Health Organization (WHO) identifies Methicillin resistant *Staphylococcus aureus* (MRSA) as a pathogen of higher risk to the world community. Livestock and the products it produces, which are frequently raised in subpar conditions, are one of the sources of MRSA emergence. Studies on the antimicrobial resistance (AMR) profile and prevalence of MRSA in dairy buffaloes were scarce in Jabalpur. Thus, using their antimicrobial pattern as well as phenotypic and genotypic characterization, the current study was aimed to ascertain the prevalence of methicillin-resistant *Staphylococcus aureus* in mastitic buffaloes in the Jabalpur region of Madhya Pradesh. The California Mastitis Test was used to screen 408 buffaloes' milk samples for mastitis (CMT). The mecA gene of methicillin-resistant *S. aureus* was molecularly characterized using polymerase chain reaction. Overall 25 percent (102/408) of the milk samples tested positive for CMT. 30.39 % (31/102) of the milk samples that tested positive for CMT had *S. aureus*, according to the results of the biochemical tests. mecA gene was identified in 16.1% (05/31) of the total 31 *S. aureus* isolates detected as MRSA. Only one *S. aureus* isolate was found to be multidrug resistant based on the antibiotic sensitivity test results.

Keywords: Buffalo, Mastitis, *S. aureus*, MRSA, CMT.

INTRODUCTION

Mastitis is defined as inflammation of the parenchyma of the udder or mammary glands. It leads to pathological malformations in the mammary glands, as well as physical, chemical and bacterial changes in milk [1]. Mastitis is thought to be the most frequent cause of overuse of antibiotics in dairy animals. Inappropriate dosage or duration of medication can also contribute to the development of antibiotic resistance without enhancing the effectiveness of treatment [2].

There are two main categories of mastitis: clinical and subclinical. Subclinical mastitis is characterized by symptoms associated with irregularities in the milk, such as milk clots, flakes, watery discharges, and blood, while clinical mastitis is characterized by local (such as swelling of the udder, heat, and pain) or systemic (such as fever, anorexia, and depression) symptoms [3]. Radostitis *et al.* [1] have classified udder pathogens, comprising bacterial, fungal, yeast, algal and leptospiral agents. Based on their principal reservoir and route of transmission, mastitis-causing bacteria can be divided into two categories. Environmental bacteria include *Streptococci* sp., *Klebsiella* sp., and coliforms like *E. coli*. These result from the buffalo's environment, which include mud, manure, and unhygienic bedding materials that come into contact with the teats. Contact with contaminated

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milk can spread pathogenic germs such as *Streptococcus agalactiae* and *Staphylococcus aureus* among cows [4].

About 30-40% of cattle in India suffer from mastitis due to *Staphylococcus aureus* [5,6]. Because MRSA provides resistance to all β-lactam anti-microbial drugs, treatment of *S. aureus* mastitis is extremely difficult. *S. aureus* organisms colonize abnormal teat ends or teat lesions. It can result in gangrenous, subclinical, chronic, acute, subacute, and per acute mastitis. The acute form of the disease typically manifests soon after parturition and tends to cause gangrene in the affected areas, which has a high fatality rate [7].

One of the key requirements for successfully preventing infections and providing therapy is early detection of the prevalence and spread of causing microorganisms. The epidemiological features of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in humans and animals have not been extensively studied in India. According to an ICAR-NIVEDI study, methicillin-resistant staphylococci in dairy cattle had a high overall frequency of MRSA [8]. The prevalence of methicillin-resistant *S. aureus* in mastitic buffaloes in the Madhya Pradesh region of Jabalpur is currently unidentified. In order to ascertain the prevalence of resistant *S. aureus* in mastitic buffaloes in the Jabalpur region of Madhya Pradesh, the current investigation was carried out using their antimicrobial pattern as well as phenotypic and genotypic characterization.

MATERIALS AND METHODS

A total of 408 buffaloes milk samples were screened from private dairy farms, and adopted villages in and around Jabalpur city for the presence of subclinical mastitis by California mastitis test (CMT) [9,10]. Aseptically collected milk from diseased quarters or udders with a CMT score of ≥1 was transferred in ice-filled tubes and stored at - 20°C until needed. Methicillin-resistant *S. aureus* was isolated using the EFSA-published protocol's guidelines [11] and Markey *et al.* [12] respectively.

For phenotypic characterization of Methicillin Resistant *Staphylococcus aureus*, all the isolates were assessed by Disk diffusion method, for cefoxitin (30µg disc) resistance. The samples were then pre enriched in Mueller Hinton (MH) (HiMedia, India) broth supplemented with 6.5% NaCl at 35ºC for a time period of 16 to 24 hours. Finally, the isolates were enriched with Tryptone Soya Broth (Hi Media) consisting of 3.5mg/L cefoxitin (HiMedia, India) and 75 mg/L Aztreonam (HiMedia, India) at 35ºC for a time period of 16 to 24 hours.

HiChrome MeReSa agar plates (supplemented with cefoxitin and methicillin, HiMedia, India) were used as selective agar. The plates were inoculated with the isolates and were incubated at 35ºC for 16-20 Hours. Later, five colonies of MRSA (blue green colonies) were inoculated on tryptone soy agar, further they were incubated at 35ºC for 16-20 hours. Biochemical tests were used to characterize the phenotype of the presumed isolates of *S. aureus* [12].

The isolates were also positive for mannitol fermentation, haemolysis on sheep blood agar (SBA), susceptibility to novobiocin 5 µg disc, and resistance to polymyxin B 300 µg disc.

The mecA gene was detected by PCR and the genotypic characterization of methicillin-resistant *S. aureus* (MRSA) was done [11]. Instagene (BioRad) was used for DNA isolation. ATCC 43300 *S. aureus* culture was used as a positive control. The following Primer sequence was used to amplify mecA gene: F - TCC AGA TTA CAA CTT CAC CAG CAG G, R- CCA CTT CAT ATC TTG TAA CG (20µM) [13]. DreamTaq DNA polymerase is supplied in 2X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each and 4 mM $MgCl_2$. It contains a density reagent and two dyes which migrate with 3-5 kb DNA fragments and the yellow dye migrates faster than 10bp DNA fragments in 1% agarose gel. Cyclic conditions for amplification of mecA gene used were, initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 59°C for 1 min, extension at 72°C for 1min (30 cycles) and final extension at 72°C for 10 min.

Agarose gel electrophoresis was used to analyze the PCR-amplified products. 1.5% (w/v) agarose gel (horizontal) in 1X Tris Borate EDTA (TBE) buffer (pH 8.3) was used, and the reaction was run for two hours at 80 Volts. The Gel documentation system (Alpha Innotech) was used to observe and capture photographs of the PCR results. The molecular weight marker, a 100-base pair (bp) ladder from Promega, USA, was used to compare the samples. Positive confirmation was obtained by amplification of the 168 bp *S. aureus* mecA gene.

The multidrug resistance profile of methicillin resistant *S. aureus* isolates against 10 antimicrobial agents (HiMedia) (Table 1) was performed by disk diffusion assay as per CLSI procedure [14]. Resistance to at least 3 classes of antibiotics was considered as MDR strain.

RESULTS AND DISCUSSION

Prevalence of *S. aureus* from mastitic milk sample

The conventional methods were used for isolation of *S. aureus* isolates from 102 mastitic milk screened by California mastitis test. All the 31(30.39%) isolates of *S. aureus* identified tentatively based on colony characteristics of individual isolate in MeReSa agar and Gram's staining. Twenty-two isolates showed both alpha and beta haemolysis in sheep blood agar (SBA). Nine isolates showed no haemolysis on SBA.

All the 31 isolates obtained in this study revealed typical cultural and biochemical characteristics *viz.* positive for coagulase and catalase reaction, mannitol fermentation on mannitol salt agar, novobiocin susceptibility, polymyxin B resistance and haemolysis on sheep blood agar are considered for preliminary identification of *S. aureus* strains [15,12]. Similar findings were observed in present study. No isolates obtained any atypical biochemical properties.

Characterization of MRSA from mastitic milk sample

After screening of 102 CMT positive milk samples for methicillin resistance in *S. aureus*, 5 isolates were found MRSA positive after phenotypic and genotypic characterization for both *S. aureus* and methicillin resistance.

In the present study, 5 (4.9%) samples of *S. aureus* were found positive for mecA gene (Fig.1), hence confirmed them as MRSA. Song *et al.* [16] confirmed MRSA by detection of the mecA gene from 165 (25.4%) *S. aureus* from mastitis milk samples in Korea and 13.9% (23/165) detected as MRSA. In a similar study, conducted by Chandrasekaran *et al.* [17] in the southern state of Tamil Nadu, India, 3.0% (12/401) MRSA was detected in the mastitic milk samples. Similarly, Shrestha *et al.* [18] found 6.9% (2/29) mecA gene of *S. aureus* in mastitis milk samples of Nepal. Likewise, Li *et al.* [19] in 2014 conducted a study in the area of Northwestern China and determined high prevalence (56.5%, 121/214) of *S. aureus*, but only one isolate (0.46%) was MRSA positive. Shrivastava *et al.* also studied the characteristics and prevalence of MRSA in mastitis dairy cattle [20]. They concluded 16.47% positivity rate of mecA gene as a gold standard for assessing resistance against methicillin. If precautions are not taken, the public's health could be at risk due to MRSA in dairy buffalo milk. The circulation of various MRSA amongst the cattle and buffalo population poses a risk of transmission to human population through direct and indirect contact, through food chain and animal handlers. To minimize the risk, fast and early diagnosis of MRSA strains can be of help. It may help in establishing effective treatment strategies against disease caused by MRSA infections.

Antibiogram profile of *S. aureus* isolates

Phenotypically characterized 31 *S. aureus* isolates revealed different susceptibility patterns by disc diffusion method (Fig. 2). Only one *S. aureus* isolate (493) was multidrug resistant.

Susceptibility pattern of antibiotics in descending order were vancomycin, chloramphenicol and tetracycline 100% (31/31), gentamicin (30/31), linezolid 93.54% (29/31), clindamycin 83.87% (26/31), erythromycin 77.41% (24/31), ciprofloxacin and cefoxitin 74.19% (23/31) and 29.03% mupirocin (09/31).

The frequency of MRSA in dairy animals was recently investigated by Shrestha *et al.* [18]. *S. aureus* isolates were found to be 100% resistant to ampicillin and, to a lesser extent, to ciprofloxacin, gentamicin, and cefepime.

In the present study, resistance pattern to erythromycin was found to be similar to Luini *et al.* (2015) [20]. Salauddin *et al.* [22] examined multidrug resistance in *S. aureus* isolated from bovine mastitic milk. The isolates depicted resistance to vancomycin and tetracycline, and intermediate resistance to chloramphenicol and erythromycin . Prabhu *et al.* [23] investigated antimicrobial susceptibility pattern of *S. aureus* and stated that it was highly susceptible to chloramphenicol (100%) followed by enrofloxacin (97.14%), kanamycin (85.75%), streptomycin (82.85%), cefalexin (74.28%) and gentamicin (65.71%) [22]. In contrast, isolates were highly resistant to tetracyclines (74.28%), penicillin (71.42%) and ampicillin (45.71%). Similarly, Singh *et al.* [24] identified antibiotic resistance determinants mecA and blaZ in 46 and 27 isolates in 300 bovine mastitis. In a study conducted by Abdalhamed *et al.* [25], MRSA isolates from 150 mastitic milk samples exhibited complete resistance to both penicillin and methicillin.

CONCLUSION

The present study highlighted the occurrence of MRSA in milk of buffaloes suffering with mastitis in and around Jabalpur. It provides the base line data on distribution of MRSA and molecular basis of its antibiotic resistance. The baseline data can help understand the distribution pattern and resistance trends

Table 1. Multi-drug resistance profile of *Staphylococcus aureus*.

Sl. No.	Antibiotics	Antibiotic disc	Conc. (mcg)	Group
1.	Ciprofloxacin	CIP30	30	Fluoroquinolone
2.	Erythromycin	E15	15	Macrolide
3.	Gentamicin	GEN10	10	Aminoglycoside
4.	Linezolid	LZ30	30	Oxazolidinones
5.	Vancomycin	VA30	30	Glycopeptide
6.	Cefoxitin	CX30	30	Cephalosporin
7.	Chloramphenicol	C30	30	Chloramphenicol
8.	Clindamycin	CD2	2	Lincosamide
9.	Tetracycline	TE10	10	Tetracycline
10.	Mupirocin	MUP200	200	Carboxylic acid

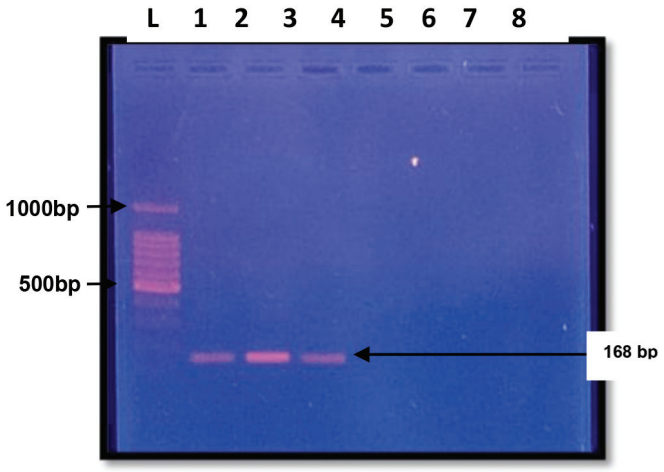

Fig. 1. Agarose gel electrophoresis showing PCR amplified product (168 bp) of mecA gene. [(From left to right L, 1, 2 and 3): L depicts 100 bp DNA ladder; Lane 1 and 2 depict product of (mecA) gene; Lane 3 depicts positive control]

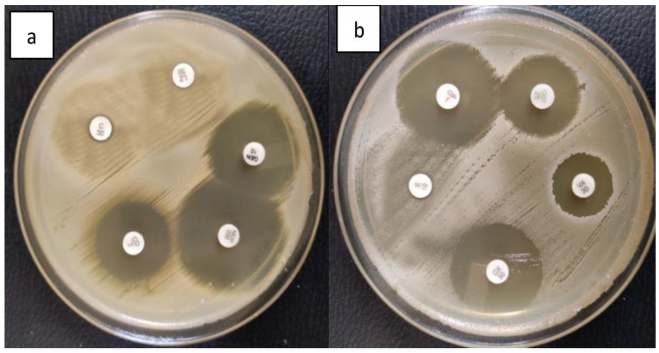

Fig. 2. Antibiotic susceptibility pattern of *S. aureus* isolate 493. [(From left to right a-b): a-b depicts antibiotic susceptibility pattern of isolate no. 493 sensitive to ciprofloxacin, chloramphenicol, cefoxitin, gentamicin, vancomycin and tetracycline whereas resistance to erythromycin, linezolid and clindamycin]

of MRSA, which can further help clinicians for formulations of treatments. It can also help in information of the antibiotics are being used to treat MRSA infections. Judicial use of antibiotics and strict monitoring on its sale is the need of the hour which can prevent the spread of resistant bacteria amongst animals and man.

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