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Antimicrobial drug resistance of *Staphylococcus aureus* from clinical bovine mastitis in Chhattisgarh state

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Abstract
The study was carried out to investigate the incidence and antimicrobial susceptibility pattern of *Staphylococcus aureus*. Further isolates were screened for presence of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from bovine mastitis in Chhattisgarh. A total of 300 milk samples were collected from clinical cases of mastitis. Milk samples were processed for isolation and identification of *Staphylococcus aureus* followed by Antibiogram using standard bacteriological procedures. Overall, incidence of *Staphylococcus aureus* was 54.66%. Drug sensitivity revealed 83.5% resistance against penicillins followed by methicillin 31.09%, gentamicin 17.6%, tetracycline 12.8%, cefepime 26.82%, erythromycin 11.58%, clindamycin 4.8%, amoxyclav 60.9%, amoxicillin 68.29%, linezolid 3.04% and vancomycin (0.006%). Results indicated that these isolates exhibit the multidrug resistant (MDR) pattern which is an alarming situation to both human and animal health. However, present findings are useful in selection of antibiotics in the treatment of bovine mastitis.

Keywords: Bovine, *Staphylococcus aureus*, mastitis, antibiotic resistance

Introduction
During the past six decades anti-microbial agents have played a critical role in reducing the burden of infectious diseases in livestock and have played pivotal role in industrialization and globalization of livestock industry. Inappropriate and indiscriminate use of anti-microbial agents in livestock for therapeutic, prophylactic, metaphylaxis and growth promoting reasons results in strong selection pressure that allows the resistant strain to grow and rapidly replace susceptible isolates. Mastitis has become a greatest challenge in India. Mastitis is very complex disease, with indiscriminate use of antibiotics, leading more difficult to treat the disease. In a dairy herd, 70% problem is due to mastitis, because of low quantity and quality of milk (Sumathi *et al*, 2008) [30].

In India, prevalence of bovine mastitis due to *Staphylococcus aureus* was around 30-40% (Patel *et al*., 2012) [20]. The economic losses due to mastitis in India have increased about 115 folds in the last five decades and presently the loss due to mastitis is to the tune of Rs 7165.51 crores per annum (Bansal and Gupta, 2009) [3]. Multiple cause of mastitis is a great problem for clinical veterinerian (Jian *et al*, 2010). Main pathogens that caused clinical and subclinical mastitis are *Streptococcus uberis* and *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and the number of *Staphylococcus aureus* and *Streptococcus agalactiae* isolates depended on herd size (Kalmus *et al*, 2011) [13].

As a remedy, many antibiotics are used, but therapeutic outcome of *Staphylococcus aureus* is poor. The *Staphylococcus aureus* is a Gram positive cocci, one of the most important bacteria in *Micrococcaceae* family. Treatment becomes more challenging when bacteria acquires resistance against various antibiotics leading to no cure but an antibiotic load in an animal body. In-vitro antimicrobial sensitivity test are done to look ahead for herd level treatment. It is necessary to identify the particular bacteria causing mastitis and its sensitivity test, so that animal could be saved from unnecessary antimicrobial load and sooner to increase the quality and quantity of milk (Gentilini *et al*, 2000) [9]. Looking to the importance of *Staphylococcus aureus* in mastitis, this study was carried out to investigate the incidence and its antimicrobial sensitivity profile in Chhattisgarh state of India.

Materials and Methods
Milk samples were collected from the lactating cows and buffaloes with clinical mastitis at the small holder farms as well as large farms. Prior to samples collection, the udder, teats and

adjacent flank areas were thoroughly washed and dried with sanitary paper towel and the teats were disinfected with 70% alcohol. Ten milliliters of milk from each animal was collected. The milk samples were transported in an ice box to the laboratory within 3 hours. A total of 300 milk samples were collected. Milk samples were inoculated in Mueller Hinton Broth with 6.5% salt, incubated at 37^0C for 18-24 hrs. After growth in broth, streaked on Bai red Parker agar (BPA) plate. Colonies from BP agar plate is transferred to Tryptone soy agar plate for appearance of characteristic colony. Then the *Staphylococci* were identified on the basis of Gram's staining, pigment production and biochemical reactions: catalase activity, coagulase test (rabbit plasma), mannitol fermentation, polymixin B and novobiocin susceptibility. (Zmantar *et al*., 2008; Kumar *et al*., 2010) [31, 14].

Antibiotic susceptibility

Antibiotic sensitivity (by Kirby - Bauer disk diffusion method) screening of the bacterial isolates were analyzed against 14 different antimicrobial agents namely methicillin (10 ug, 30ug), penicillin (10units), erythromycin (15ug), amoxycillin (30ug), amoxyclav (30ug), cefepime (30 ug), clindamycin (2ug), linezolid (30ug), gentamicin (10ug), cefoxitin (30ug), vancomycin (30ug) and tetracycline (30ug). Isolates were categorized as either susceptible or resistant based upon interpretive criteria according to guide lines of CLSI (2014) [7].

Preparation of Inoculum for Disc diffusion test

The modified Kirbi-Bauer disc diffusion method was followed. The diffusion test was done for each isolate on Mueller-Hinton agar plates. 20 ml of medium was poured into 90 mm diameter sterile Petri dishes to a depth of 4mm on a level surface to make the depth of the medium uniform and left at 37^0C temperature overnight to check sterility. Isolates were inoculated in nutrient broth and incubated at 37^0C for 2-4hrs. Standardized bacterial suspension was saturated with a sterile Dacron tip swab and excess culture was removed by turning the swab against the side of the tube. Inoculum was spread evenly over the entire surface of the Mueller-Hinton agar plates by swabbing back and forth across the agar in three directions to give a uniform inoculum to the entire surface. These plates were allowed to dry before applying discs and after 15 minutes, discs of given potency were applied on the inoculated plates with the help of sterile forcep. These plates were then placed in an incubator at 35 ± 2°C for 24 hours in inverted position. After 24 hours of incubation, plates were examined and the zone of inhibition was measured. The results were interpreted as per CLSI (2014) [7] guidelines.

Detection of minimum inhibitory concentration of *Staphylococcus aureus* against Oxacillin-Vancomycin.

The MIC was tested for random ten isolates which showed resistance against methicillin and cefoxitin antibiotic disc. The isolated viable colonies from overnight agar plate were suspended into broth tubes. An inoculum turbidity of at least 0.5 McFarland was adjusted by the addition of sterilized plain broth (NCCLS, 2004) [19]. A sterile swab was dipped into the suspension to press out excess fluid and then the entire surface of the agar plate was swabbed evenly in all directions by the same sterile swab. After that, the agar surface was left for 15 minutes on the bench or in an incubator to dry and an oxacillin-vancomycin strip was applied onto the agar surface using sterile forcep. Once applied, the strips was attached firmly on the Mueller-Hinton agar and not allowed to move at all. Finally, the plates were incubated at 37˚C for a full 24 hours. The MIC result was read where the ellipse intersects the MIC scale on the strip. Also, the point of complete inhibition of all growth including hazes and isolated colonies were read.

Results and Discussion

Out of 300, 164 isolates were positive for *Staphylococcus aureus* on the basis of biochemical tests and Gram staining. All of the *Staphylococcus aureus* isolates were found catalase positive, oxidase negative, fermentative by O-F test, urease positive, coagulase positive and failed to grow on Mac-conkey agar. Growth on Baired Parker agar shows that the isolates were lecithinase, lipase positive and showed tellurite reduction. Presence of coagulase enzyme is considered as a criteria for pathogenicity of *Staphylococcus aureus*. All 164 isolates of *Staphylococcus aureus*, were resistant to one or more antimicrobial agents tested by this method (table 1). Resistance was detected highest against penicillin (83.5%), which is in agreement with the reports of (Abera *et al*, 2010., Shiferaw *et al*, 2009., Ayano *et al*, 2017) [1, 25]. Also in India, high resistance against penicillin reported by Chandrashekaran *et al*. (2014) [6]. Resistance against other antibiotics were gentamicin (17.6%), tetracycline (12.8%), cefepime (26.82%), erythromycin (11.58%), clindamycin (4.8%), amoxyclav (60.9%), amoxicillin (68.29%) and linezolid (3.04%). After penicillin, isolates were more resistant to amoxicillin than amoxyclav, next high resistance is found against cefepime, which is 26.82%. Gentamicin resistance is quite high than the data reported by Feng *et al*. (2016), whereas, isolates were less resistant against erythromycin and tetracycline. Sori *et al*. (2011) [28] reported almost similar results to the present work including clindamycin (4.8%) showing very less resistance, and (only) single isolate was found resistant to vancomycin which is in concerence with work reported by (Pati and Mukherji, 2016., Soares *et al*, 2012) [21, 27] in which all the isolates were susceptible to vancomycin. There is no such report available regarding the significant amount of vancomycin resistant *Staphylococcus aureus* in dairy animals in India. The present study also revealed the presence of vancomycin intermediate *Staphylococcus aureus*, which is an indication of future vancomycin resistant strains of *Staphylococcus aureus.*

Antibiotic resistance in bacteria is a matter of deep concern, worldwide. In dairy herds, mastitis is the most common cause to use antibiotics and in same track it is hazardous to both animals and human. *Staphylococcus aureus* is a Gram-positive cocci bacteria which causes an array of infections in economically important livestock including cattle, buffaloes, sheep and goats. It causes peracute gangrenous, acute and often chronic mastitis in cattle and buffaloes that responds poorly to antibiotic therapy. According to the previous studies, relatively high prevalence of *Staphylococcus aureus* is most likely be attributed to the wide distribution of organism inside the mammary glands (Jones *et al*, 1998) [12]. Presence of *Staphylococcus. aureus* is clear indication of most prevailing pathogen in case of mastitis (Kumar *et al*, 2010) [14]. In the present study, overall incidence of *Staphylococcus aureus* in clinical mastitis, was 54.66%, which is much higher than that of the findings of (Ayano *et al*, 2017., Lakew *et al*, Abera *et al*, 2010, Mekibib *et al*, 2010 and Sori *et al*, 2011) [1, 17, 28] in which the isolation rate of *Staphylococcus. aureus*

was 28.65%, 41.4%, 42.10%, 47.5% and 39.44% respectively. Higher than this, Hoque *et al*, 2018 reported the herd level mastitis, *Staphylococcus aureus* prevalence was 73.3%. In India, several workers have reported the prevalence of *Staphylococcus aureus* (Sharma *et al*, 2015, Mubarackka *et al*, 2012 and Suleiman *et al*, 2012) [24, 18, 29].

Now days, antimicrobial resistance is a serious matter and is mainly due to irrational use of antibiotics to treat the infections in which diagnosis is not proper or disease has multiple cause. Moreover, there is increased incidence of MRSA (livestock associated and human associated MRSA). MRSA is the strain of *Staphylococcus aureus* that has developed resistance against various antibiotics mainly to methicillin, penicillin, oxacillin and various other antibiotics. These strains called superbugs which are reservoir of many antmicrobial resistance genes (Batabyal *et al*, 2012) [4]. It has also been stated that MRSA isolates which are resistant to β-lactam antibiotics may induce cross resistance to vancomycin. In the present study, besides, prevalence of *Staphylococcus aureus*, resistance against methicillin (10µg) was 51 (31.09%), methicillin (30 µg) 32 (19.5%) which is quite high. Oxacillin-vancomycin and cefoxitin resistant *Staphylococcus aureus* isolates were used as indicator of MRSA (Fernandes *et al*, 2005, Rai and Tiwari, 2016) [8, 23]. In India, recently, authors reported about MRSA (Paul *et al*, 2015, Mausam *et al*, 2016, Rai and Tiwari, 2016) [22, 16, 23]. The present work showed the significant number of methicillin disc diffusion resistant *Staphylococcus aureus* isolates, which is an indication of mecA positive MRSA. The range of MIC of oxacillin-vancomycin resistant strips for the tested isolates are given in the (table: 2). for the oxacillin MIC strip, all of the isolates were sensitive at MIC of 1.5 mcg/ml. However, only one isolate was found to be resistant at concentration of 0.75 and 1.

Table 1: Antibiotic sensitivity of *Staph. aureus* strains from clinical bovine mastitis

Antibiotic	No of resistant isolated	Percentage of resistant isolates
Penicillin	137	83.5%
Amoxicillin	112	68.29%
Amoxyclav	100	60.9%
Methicillin(10µg)	51	31.09%
Cefepime	44	26.82%
Methicillin(30µg)	32	19.5%
Gentamicin	29	17.6%
Tetracycline	21	12.8%
Erythromycin	19	11.58%
Clindamycin	8	4.8%
Linezolid	5	3.04%

Table 2: Minimum inhibitory concentration of oxacillin and vancomycin of *Staphylococcus aureus* isolates.

S. No	Name of antibiotic	No of resistant *Staphylococcus aureus* isolates at given concentration of antibiotic (mcg/ml)				
		0	**0.75**	**1**	**1.5**	**2**
1	Oxacillin	8/10	1/10	1/10	-	-
2	Vancomycin	2/10	-	4/10	3/10	1/10

Conclusion

The incidence of *Staphylococcus aureus* in bovine clinical mastitis was 54.66%. Overall, antimicrobial resistance to penicillin, cefepime, methicillin, amoxyclav, tetracycline, amoxicillin, cefoxitin, clindamycin, gentamicin, erythromycin and linezolid was found in bovine mastitis cases. Incidence of *Staphylococcus aureus* causing mastitis and Antibiogram studies would provide useful information and alertness alarm to producers, farmers and veterinarians for the management of farms. But resistane to methicillin among bovine mastitic milk represents major threats for transmission of this multidrug resistant to human beings. It further highlighted the necessity of enforcement of hygienic implementations and practices within dairy facilities.

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