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Antibiogram of methicillin resistant *Staphylococcus aureus* (MRSA) of animal origin from Chhattisgarh

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Abstract
Present investigation was made to study antibiogram profile of Methicillin Resistant *Staphylococcus aureus* (MRSA) of animal origin. MRSA isolates were obtained from bovine mastitis and wound infection of animals using cultural isolation and were confirmed by *S. aureus* specific thermonuclease (*nuc*) and MRSA specific *mecA* gene in PCR assay. A total of 13 MRSA isolates were obtained and subjected to antibiotic sensitivity test using 10 numbers of selected antibiotics by disc diffusion test. MRSA isolates exhibited variable degree of sensitivity towards various antibiotics. MRSA isolates were detected 100% senisitive to linezolid and imipenem followed by clindamycin (92.31%), tetracycline (76.92%), vancomycin and gentamycin (53.85%). Isolates showed higher rate of resistance towards amoxicillin (84.62%) and penicillin (76.92%). All the isolates were found resistant to cefoxitin and methicillin. It can be concluded from present study that isolates harbouring *mecA*/ MRSA gene have tendency to exhibit resistant towards multidrug and treatment option can be explored by studying antibiogram of MRSA of particular region.

Keywords: MRSA, *mecA* gene, antibiogram, disc diffusion test

Introduction
Staphylococcus aureus is an important pathogen of bovine mastitis and wound infection in animals. Emergence of deadly methicillin resistant *Staphylococcus aureus* (MRSA) in animals and its possible onward zoonotic transmission to human poses threat to animals as well public health [1]. The prevalence of MRSA has been dramatically rising in recent years and they show innate or acquired resistance to several antibiotics [2].
Routinely, conventional cultural method is being used for identification of *Staphylococcus aureus* but in most of the cases it failed to characterize MRSA. Methicillin resistance is mainly mediated by *mecA*, which encodes for penicillin binding protein, PBP2a, which has a low affinity for all the β-lactams compounds such as methicillin, cefoxitin, oxacillin, cloxacillin, dicloxacillin, and nafcillin [3]. Therefore, detection of conserved nucleotide sequences of *Staphylococcus aureus* (*nuc* gene) and MRSA (*mecA* gene) by PCR turn out to be essential for confirmatory diagnosis. However, harboring *mecA* gene is not sufficient for methicillin resistance because some *S. aureus* isolates that contain the gene are still shown to be susceptible to methicillin or some other antibiotics [4].
Despite of intensified antibiotic therapy, MRSA infections continue to be a threat as antibiotics fail to resolve the infections either due to development of resistance to several antibiotics or due to persistent chronic infections. This proposes the need to perform routine antimicrobial susceptibility test for MRSA. Findings of antibiogram could explore the appropriate treatment option and would therefore be helpful to clinicians as they rely only on antibiotics for treatment of the bacterial infections.

Materials and Methods
Location of Study
Present investigation was conducted at Department of Veterinary Microbiology, Veterinary College, Anjora, Durg (Chhattisgarh).

Samples

Samples included twelve MRSA isolates of animal origin available at repository of Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anjora, Durg. Isolates were revived using nutrient broth containing 6.5% NaCl followed by plating on tryptone soya agar.

Besides, attempt was made to isolate new MRSA from bovine mastitis during course of current study. For isolation of *Staphylococcus aureus* milk samples were collected from mastitic cow unresponsive to conventional antimicrobial therapy [5]. Cultural isolation of *Staphylococcus aureus* was done using nutrient broth containing 6.5% NaCl and plating on tryptone soya agar and mannitol salt agar. Isolates showing Gram positive cocci in clusters were identified presumptively as *Staphylococcus aureus*.

Screening of MRSA

MRSA isolates were rescreened for MRSA specific *mecA* gene (Forward- 5' AAAATCGATGGTAAAGGTTGGC 3'and Reverse- 5' AGTTCTGCAGTACCGGATTTGC 3') by conventional PCR [6]. Confirmation of newer isolates as *Staphylococcus aureus* was done using *Staphylococcus aureus* specific *nuc* gene [7] primer (Forward- 5' GCGATTGATGGTGATACGGTT 3'and Reverse- 5' AGCCAAGCCTTGACGAACTAAAGC 3') and further confirmation as MRSA by MRSA specific *mecA* based primer in PCR. Briefly, genomic DNA was extracted from MRSA isolates by boiling and snap chilling method [8]. Extracted genomic DNA is subjected to PCR protocol as per PCR cyclic conditions given in Table 1.

Antibiotic sensitivity test

MRSA isolates were subjected to antibiotic sensitivity test by disc diffusion method as described by Bauer *et al*. (1966) [9] and as per the recommendation of CLSI (2019) [10]. Antibiotics which are used commonly as marker and treatment of MRSA were selected for AST during present study (Table 2). To perform this test, a loopful of the culture was inoculated in 5.0 ml of nutrient broth and incubated at 37^0C for 2-4 hr till the opacity of broth culture get matched with Mc-Farland's turbidity standard 0.5. Each broth culture was then evenly spread on to Mueller Hinton Agar Petri-plates (90 mm diameter with uniform bottom surface) with the help of cotton swabs. The inoculated Petri-plates were kept at room temperature for 15-20 min to allow the inoculum to be adsorbed on the surface. Each of the antimicrobial discs was placed with the help of flame sterilized forceps on the plates at an equal distance of 25 mm. The plates were further incubated at 37 ^{0}C for 18-24 hrs. The results as sensitive, intermediate sensitive and resistant were interpreted as per CLSI (2019) [10] guidelines.

Results and Discussion

MRSA isolates

During present study, a total of five isolates from bovine mastitis were confirmed as *Staphylococcus aureus* on the basis of cultural properties shown on tryptone soya agar (Fig. 1) and mannitol salt agar (Fig. 2) and revelation of 270 bp product of thermonuclease (*nuc*) gene in PCR as shown in earlier study [7]. Only one isolate out of five *S. aureus* was found positive for *mecA* gene (Fig 3) and confirmed as MRSA. All twelve MRSA isolates of repository were reported positive for *mecA* gene and yielded 533 bp PCR products which is similar to previous findings [6].

Antibiogram of MRSA

Each MRSA isolate was resistant to at least two or more antibiotics under study (Table 3 and Fig. 4). All 13 (100%) MRSA isolates was reported resistant to methicillin and cefoxitin. Isolates showing resistance towards methicillin and cefoxitin are considered to be marker for MRSA [1] as cefoxitin induces *mecA* gene of MRSA and it was therefore observed in concordance to PCR [11]. Although cefoxitin disc is known for its good sensitivity and specificity but a single false negative or positive result may mislead MRSA cases. Therefore, cefoxitin disc diffusion method can be employed for initial screening of MRSA but it must be followed by confirmation with gold standard PCR.

Present study reported re-emergence of penicillin susceptibility in MRSA isolates (23.80%). Present observation can be correlated with findings of Cheng *et al.* (2016) [12] who also reported 28% penicillin susceptible *S. aureus* among humans. Likewise, Chabot *et al.* (2015) [13] and Crane (2014) [14] also observed resurgence of penicillin sensitivity in *S. aureus*. Due to increased rate of staphylococcal resistance and emergence of MRSA, penicillin was not being used in clinical practice over a long period of time. Back to penicillin susceptibility could be attributed to narrow use of penicillin in the MRSA chemotherapy currently.

Only 2 (15.38%) out of 13 MRSA isolates were sensitive to amoxicillin whereas 84.62% isolates were found resistant. Similarly, higher resistance to amoxicillin was reported by Al-Zaidi and Al-Sulami (2013) [15], Imran *et al.* (2015) [16], Nasution *et al.* (2018) [17] and Shah *et al.* (2019) [18]. Resistance of MRSA to beta-lactam antibiotics including penicillin, amoxicillin, methhicillin and cefoxitin is attributed to the presence of the *mecA* gene.

53.85% isolates of MRSA were reported sensitive gentamicin during present study. More or less similar pattern of gentamicin sensitivity was observed by Al-Zaidi and Al-Sulami (2013) [15], Ahmad *et al.* (2015) [19], Selvabai *et al.* (2017) [20], Krishnan *et al.* (2019) [21] and Arjyal *et al.* (2020) [22]. However Shah *et al.* (2019) [18] reported increased sensitivity to gentamicin. In contrast, reduced susceptibility of MRSA towards gentamicin was reported by Mahmood *et al.* (2010) [23] and Goyal *et al.* (2013) [24]. Enzymatic alteration of aminoglycosises by aminoglycoside modifying enzyme was reported to be the major mechanism of resistance to gentamycin [25].

53.85% isolates of MRSA were reported sensitive to vancomycin during present study. Existance of vancomycin resistant MRSA during present study was well supported by Shah *et al.* (2019) [18], Nasution *et al.* (2018) [17] and Al-Zaidi and Al-Sulami (2013) [15]. In contrast, Mahmood *et al.* (2010) [23], Selvabai *et al.* (2017) [20], Sonth *et al.* (2015) [26], Li *et al.* (2017) [27], Sharma *et al.* (2017) [28] and Krishnan *et al.* (2019) [21] reported 100% sensitivity of MRSA to vancomycin. Vancomycin, a glycopeptide antibiotic usually remains a drug of choice for treatment of MRSA infections. However emergence of *S. aureus* with vancomycin resistance during last few decades [29, 30] warrants immediate attention of clinician and researcher. Further studies need to be done in different regions of country to better define vancomycin resistance in MRSA.

Despite of frequent use of tetracycline in veterinary medicine, MRSA isolates sensitivity (76.92%) to tetracycline was

comparatively higher as compared with beta-lactam, aminoglycoside and glycopeptides group of antibiotics. In agreement with present findings, Mausam *et al.* (2017) [31], Al-Zaidi and Al-Sulami (2013) [15], Shah *et al.* (2019) [18] and Arjyal *et al*. (2020) [22] also observed increased and stable rate of sensitivity of MRSA isolates towards tetracycline. In contrast, Mohammed *et al.* (2018) [32] found 41.3% MRSA isolates resistant to tetracycline. The extended spectrum tetracyclines still appear to be a reasonable treatment option in areas where MRSA strains are susceptible to the tetracyclines.

92.31% of MRSA isolates were found sensitive to clindamycin which corresponds to the findings of Arjyal *et al.* (2020) [22] who reported 100% sensitivity of MRSA isolates towards clindamycin. In line with present observation Selvabai *et al.* (2017) [20], Sharma *et al.* (2017) [28], Li *et al.* (2017) [27], Ahmad *et al*. (2015) [19], Al-Zaidi and Al-Sulami (2013) [15] and Kumar *et al*., (2011) [33] reported fairly similar rate of sesnistivity towards clindamycin. Whereas higher rate of resistance towards clindamycin was reported by Krishnan *et al.* (2019) [21], Sohail and Latif (2018) [34] and Goyal *et al.* (2013) [24]. Clindamycin, a lincosamide drug could be used to treat MRSA infection because of its good efficacy and pharmacokinetic properties.

All the isolates were found sensitive to linezolid and imipenem. Likewise present study, Mahmood *et al.* (2010) [23], Goyal *et al*. (2013) [24], Li *et al.* (2017) [27], Sharma *et al.* (2017) [28], Selvabai *et al.* (2017) [20], Krishnan *et al.* (2019) [21] and Arjyal *et al.* (2020) [22] also reported 100% sesnisitivity of MRSA towards linezolid whereas 3.8% resistance was observed by Sonth *et al.* (2015) [26]. Variable degree of resistance to imipenem was reported by Al-Zaidi and Al-Sulami (2013) [15] and Samra and Gadba (1993) [35]. Imipenem was reported to have stronger bactericidal activity than other beta-lactams. Increased susceptibility of MRSA towards linezolid and imipenem corresponds to its narrow use in MRSA chemotherapy.

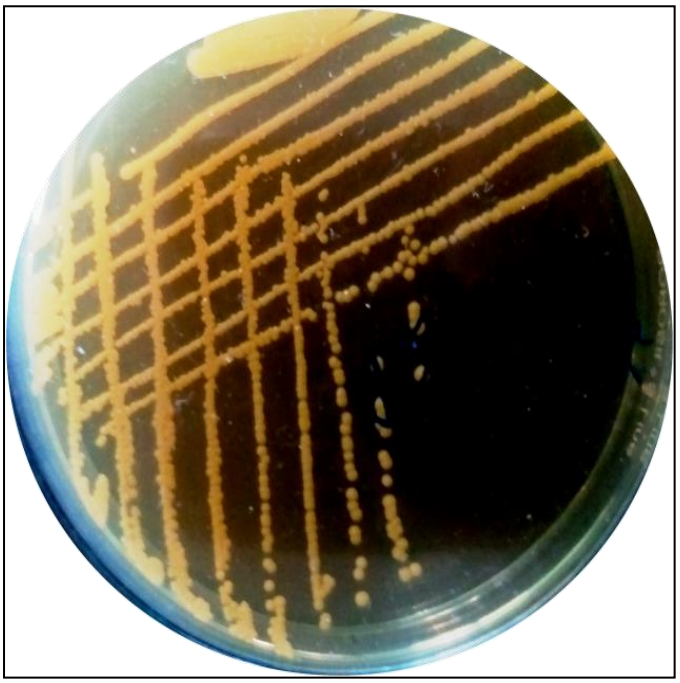

Fig 1: Golden yellow pigmented colony of *S. aureus* on tryptone soya agar

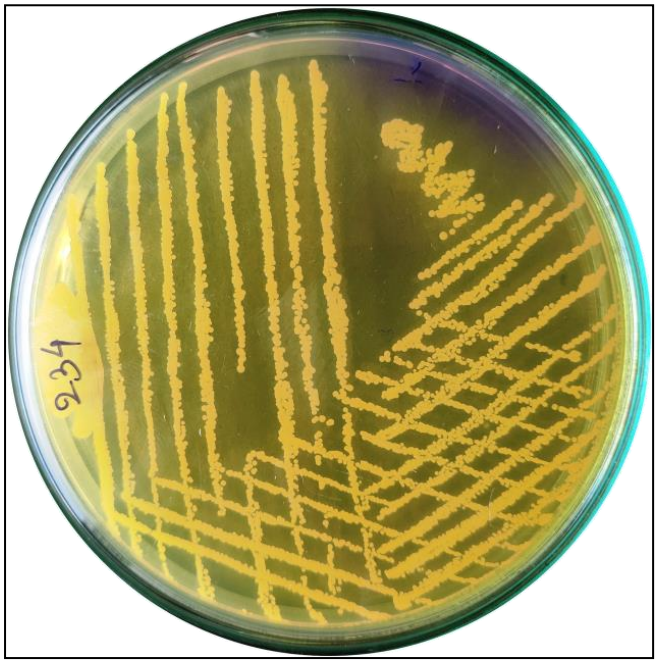

Fig 2: Yellow colony of *S.aureus* on mannitol salt agar

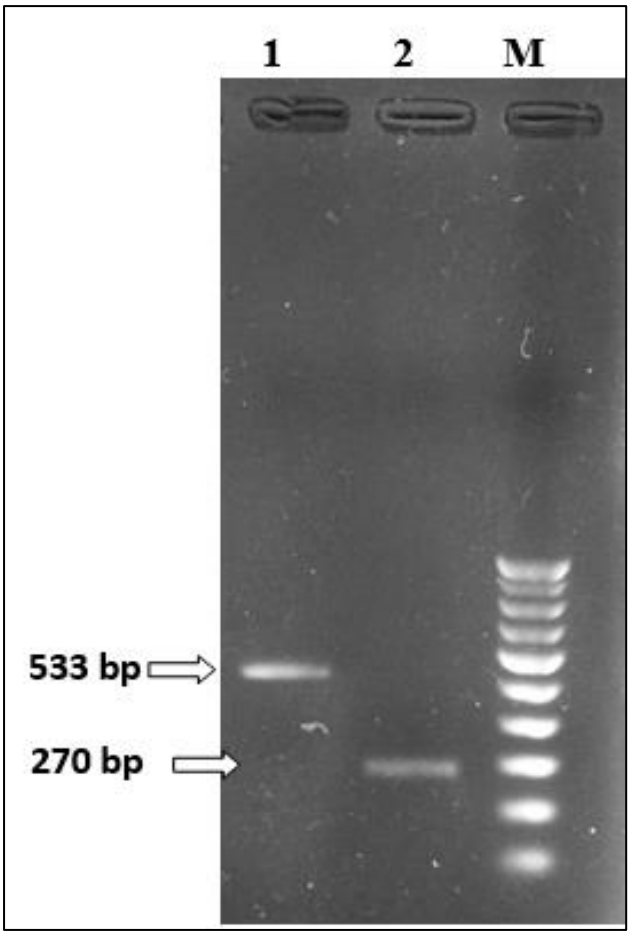

Fig 3: Amplification of *nuc* and *mecA* gene by PCR Lane M: 100 bp DNA Ladder Lane 1: *S. aureus* isolate positive for *nuc gene* Lane 2: *S. aureus* isolate positive for *mecA gene*

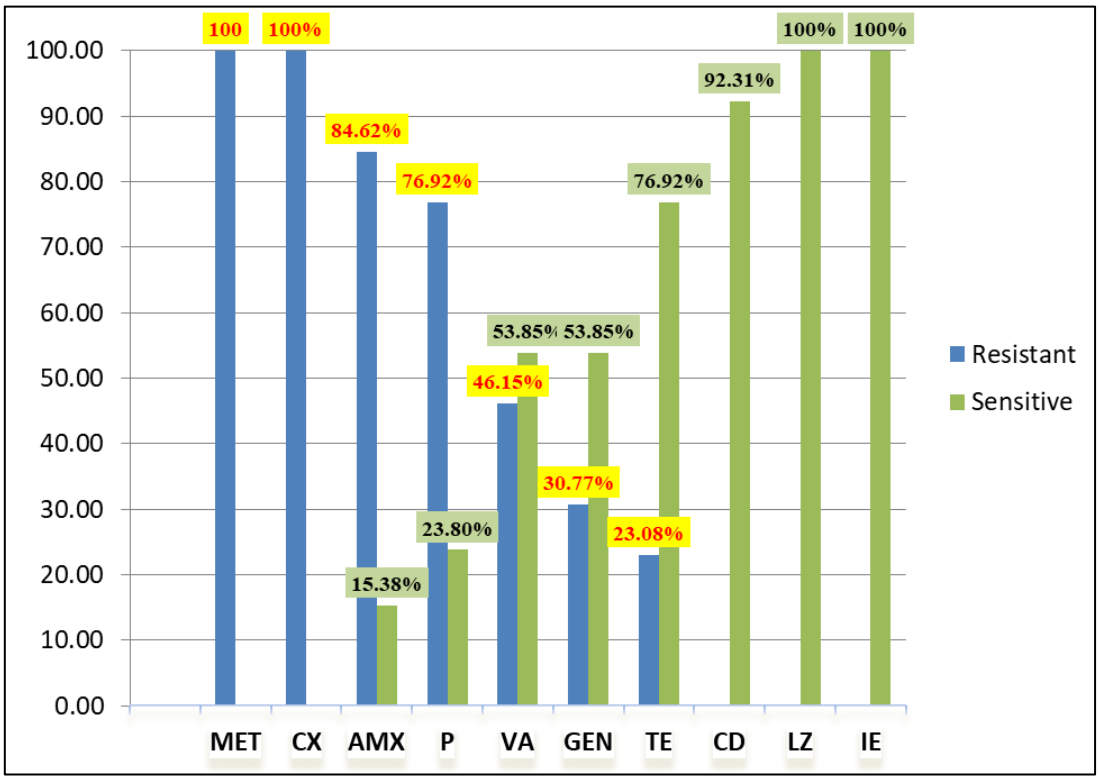

Fig 4: Antibiotic sensitivity pattern of MRSA

Table 1: PCR cyclic conditions

Particulars	*nuc* gene	*mecA* gene		
Denaturation	94 °C for 3 minutes	94 °C for 3 minutes		
Denaturation	94 °C for 30 seconds	94 °C for 30 seconds		
Annealing	60 °C for 30 seconds	60 °C for 30 seconds		35 cycles
Extension	72 °C for 30 seconds	72 °C for 40 seconds		
Final extension	72 °C for 5 minutes	72 °C for 7 minutes		
Hold	4 °C	4 °C		

Table 2: Details of antimicrobial discs and their concentrations

S. No.	Antibiotics	Antibiotic disc	Interpretation		
			Sensitive	Intermediate	Resistant
1.	Methicillin	MET10 µg	14	10-13	9
2.	Penicillin-G	P 2 units	≥ 26	-	≤ 26
3.	Amoxycillin	AMX 10µg	20	-	19
4.	Cefoxitin	CX30 µg	≥ 22	-	≤ 21
5.	Tetracycline	TE30 µg	≥ 19	15-18	≤ 14
6.	Gentamycin	GEN 10µg	≥ 15	13-14	≤ 12
7.	Vancomycin	VA 30µg	21	-	17
8.	Imipenem	IE 10µg	≥ 23	-	≤ 19
9.	Linezolid	LZ30 µg	≥ 21	-	≤ 20
10.	Clindamycin	CD2 µg	≥ 21	15-20	≤ 14

Table 3: Antibiogram of MRSA of animal origin in Chhattisgarh

SN	Antibiotics	MRSA isolates												
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	Methicillin (10 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R
2	Cefoxitin (30 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R
3	Amoxicillin (10 µg)	R	R	R	S	R	S	R	R	R	R	R	R	R
4	Penicillin-G (2 units)	R	R	R	S	R	S	R	R	R	R	R	S	R
5	Vancomycin (30 µg)	S	R	R	S	R	S	R	R	R	S	S	S	S
6	Gentamycin (10 µg)	R	S	S	S	IS	S	S	S	R	R	IS	S	R
7	Tetracycline (30 µg)	S	S	S	S	R	S	R	S	S	S	S	S	R
8	Clindamycin (2 µg)	S	S	S	S	S	S	S	S	S	IS	S	S	S
9	Linezolid (30 µg)	S	S	S	S	S	S	S	S	S	S	S	S	S
10	Imipenem (10µg)	S	S	S	S	S	S	S	S	S	S	S	S	S

Note: S- Sensitive, IS- Intermediate sensitive, R-Resistant

Conclusion

Methicillin Resistant *Staphylococcus aureus* (MRSA) obtained from animal origin were reported resistant to atleast two or more antibiotics tested. MRSA isolates were found completely resistant to methicillin and cefoxitin followed by amoxicillin. Methicillin and cefoxitin could be therefore considered as good phenotypic marker for MRSA. Linezolid and imipenem were reported most effective antibiotics against MRSA followed by clindamycin, tetracycline, vancomycin and gentamycin. Present study reports emergence of penicillin susceptibility in 23.08% MRSA isolates which demands detailed investigation in different region so that reuse of penicillin therapy against MRSA can be explored.

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