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Original Research

Phenotypic Antibiotic Resistance Pattern and Presence of *mecA* in *Staphylococcus aureus* Isolated from Bovine Mastitis

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Abstract

Emergence of antibiotic resistance in Staphylococcus aureus and in particular community associated methicillin-resistant S. aureus (MRSA) is one of the most serious problem. In the present study, 97 isolates of S. aureus from cases of clinical (n=49) and subclinical (n=48) mastitis were evaluated for phenotypic antibiotic resistance patterns. A total of 28 antibiotics belonging to 10 groups of antibiotics were tested. Isolates from clinical cases of mastitis had higher resistance than those from subclinical mastitis and resistance was observed in many newly developed antibiotics as well. Multidrug resistance (MDR) was observed in 83 of the studied isolates, and of these 18 exhibited extreme drug resistance (XDR) and one isolate demonstrated resistance to all the antibiotics (pan drug resistance, PDR). Though phenotypic methicillin resistance was observed in 25 isolates, mecA was present in only 3 isolates. The antibiotic resistance is mainly attributed to acquisition of resistance genes by genetic exchange. However, the present study revealed that there may be some other mechanisms associated with methicillin resistance in S. aureus.

Key words: Mastitis, *mecA*, Methicillin Resistance, *Staphylococcus aureus*

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Introduction

Mastitis, the inflammation of mammary gland, is one of the costliest diseases of dairy cattle resulting in the reduction of milk yield and quality. Annual economic losses due to subclinical and clinical mastitis in India have been estimated to be Rs. 4151.16 and Rs. 3014.35 crores, respectively with a total of Rs. 7165.51 crores (Bansal and Gupta, 2009). Mastitis is the single most common cause of antibiotic use in dairy farms. Mastitis therapy has a potential to develop antibiotic resistance, as antibiotics employed to treat mastitis often have a short duration of therapy. β-lactams, commonly used in therapy, have little or no activity against gram negative bacteria; infusion of dry cow therapy increases exposure time of intra-mammary

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bacteria; and efficacy of many therapeutics in lactating cows is quite low especially in chronic infections. Moreover, the use of antibiotics for treatment and control of mastitis often results in antibiotic residues in milk (Bansal *et al.*, 2011) above the maximum residue limits (MRLs) resulting in build-up of antibiotic-resistant organisms in human food chain (Jones and Seymour 1988; Seymour *et al.*, 1988).

Staphylococcus aureus is a major pathogen responsible for bovine mastitis (Lowy, 2003). *S. aureus* is a cause of skin infections and serious hospital infections including bacteremia and pneumonia in human beings. The emergence of antibacterial resistance in this pathogen is of growing concern as *S. aureus* associated bacteremia results in 20-40% mortality despite treatment (Mylotte *et al.*, 1987). In addition, community acquired-methicillin resistant *S. aureus* strains (CA-MRSA) that have evolved independently of hospitals are becoming widespread. Cattle, pigs and poultry are colonized with MRSA and the zoonotic transmission of such MRSA to humans via direct animal contact or environmental contaminations are a matter of concern (Kock *et al.*, 2013) indicating the need for surveillance and biosecurity measures in the animal health sector.

Phentoypic antibiotic susceptibility patterns have been studied previously in detail from clinician point of view in India (Mir *et al.*, 2014; Bansal *et al.*, 2015) but the development of antibiotic resistance in *S. aureus* has not been focused from community health aspects. Therefore, the present study has been undertaken to determine *in vitro* antibiotic resistance pattern of *S. aureus* in clinical and subclinical cases of mastitis and correlate the presence of *mecA* with the phenotypic methicillin resistance.

Materials and Methods

Confirmation of *S. aureus* Isolates

The study was carried out on 97 isolates of *S. aureus* isolated from cases of clinical (n=49) and subclinical (n=48) mastitis in dairy cows and buffaloes. All the isolates (n=20) from buffaloes belonged to subclinical mastitis. These strains were isolated from bovine milk samples in mastitis and milk quality laboratory of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana from July 2012 to June 2013. *S. aureus* organisms were identified on the basis of colony characteristics on blood agar, gram staining, clumping factor, growth characteristics on manitol salt agar, DNase agar, Baird parker agar and tube coagulase test (Quinn *et al.*, 2000). Individual isolates were stored in trypticase soy broth containing 30% glycerol in deep freeze (−80°C).

Antibiotic Sensitivity Testing

Susceptibility testing of bacterial isolates to antibiotics was performed on Mueller-Hinton agar (HiMedia) using the disc diffusion method (Quinn *et al.*, 2000). Briefly, a fresh colony of an individual isolate was transferred to a tube containing 5 ml nutrient broth. The mixture was incubated at 37ºC until light visible turbidity appeared; this was compared with the McFarland 0.5 turbidity standard. The suspension of test

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organism was streaked over the surface of Muller Hinton agar plates using a sterile disposable cotton swab. Commercially available discs (Hi-Media) of 28 antibiotics belonging to 10 groups were evaluated in the study. The discs were firmly placed on agar by means of sterile forceps and plates were incubated for 24 h at 37°C. The diameters of growth-inhibition were measured in millimeters and reported as susceptible, intermediate or resistant as per Clinical and Laboratory Standards Institute (CLSI 2008) guidelines. The antibiotic discs along with their concentration and interpretation criteria have been given in the Table 1. Antibiotics, for which interpretive criteria was not available as per CSLI guidelines, breakpoints of antibiotic in similar group were used. Thus, the breakpoint of ampicillin has been used for amoxicillin and that of oxacillin was used for cloxacillin. For streptomycin and neomycin, the breakpoints suggested by Petrovski (2011) were used.

Table 1: Antibiotic sensitivity test of *S. aureus* against different antibiotics

Group	Antibiotic (concentration in mcg)	Interpretation	Break Point (mm)	Cow				Buffalo		Total	
				CM		SCM					
				N= 46	%	N= 31	%	N= 20	%	N	%
β-Lactams	Oxacillin	S	≥13	28	60.9	23	74.2	19	95	70	72.2
	(OX:1)	I	11-12	2	4.3	0	0	0	0	2	2.1
		R	≤10	16	34.8	8	25.8	1	5	25	25.8
	Penicillin-G	S	≥29	5	10.9	14	45.2	11	55	30	30.9
	(P:2 U)	I	NA	NA	NA	NA	NA	NA	NA	NA	NA
		R	≤28	41	89.1	17	54.8	9	45	67	69.1
	Amoxycillin +	S	≥20	27	58.7	23	74.2	17	85	67	69.1
	Clavulanic acid	I	NA	NA	NA	NA	NA	NA	NA	NA	NA
	(AC:30)	R	≤19	19	41.3	8	25.8	3	15	30	30.9
	Ampicillin	S	≥29	8	17.4	17	54.8	14	70	39	40.2
	(A:10)	I	NA	NA	NA	NA	NA	NA	NA	NA	NA
		R	≤28	38	82.6	14	45.2	6	30	58	59.8
	Amoxycillin +	S	≥15	24	52.2	23	74.2	17	85	64	66
	Sulbactum	I	12-14	1	2.2	1	3.2	1	5	3	3.1
	(AS:30/15)	R	≤11	21	45.7	7	22.6	2	10	30	30.9
	Amoxycillin	S	≥29	5	10.9	14	45.2	11	55	30	30.9
	(AMX:10)	I	NA	NA	NA	NA	NA	NA	NA	NA	NA
		R	≤28	41	89.1	17	54.8	9	45	67	69.1
	Cloxacillin	S	≥13	37	80.4	29	93.5	19	95	85	87.6
	(COX:10)	I	11-12	2	4.3	1	3.2	0	0	3	3.1
		R	≤10	7	15.2	1	3.2	1	5	9	9.3
	Ceftrioxone +	S	≥21	17	37	22	71	16	80	55	56.7
	Sulbactum	I	14-20	23	50	4	12.9	2	10	29	29.9
	(CIS:30/15)	R	≤13	6	13	5	16.1	2	10	13	13.4
	Ceftrioxone +	S	≥21	29	63	21	67.7	16	80	66	68
	Tazobactum	I	14-20	13	28.3	5	16.1	3	15	21	21.[illegible]

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	(CIT:30/10)	R	≤13	4	8.7	5	16.1	1	5	10	10.3
	Ceftrioxone	S	≥21	17	37	22	71	15	75	54	55.7
	(CI:10)	I	14-20	21	45.7	4	12.9	3	15	28	28.9
		R	≤13	8	17.4	5	16.1	2	10	15	15.5
	Ceftazidime	S	≥18	11	23.9	19	61.3	11	55	41	42.3
	(CA:30)	I	15-17	11	23.9	5	16.1	5	25	21	21.7
		R	≤14	24	52.2	7	22.6	4	20	35	36.1
	Cefoperazone	S	≥24	7	15.2	16	51.6	13	65	36	37.1
	(CS:75)	I	NA	NA	NA	NA	NA	NA	NA	NA	NA
		R	≤23	39	84.8	15	48.4	7	35	61	62.9
	Ceftixozime	S	≥20	26	56.5	22	71	17	85	65	67
	(CK:30)	I	15-19	3	6.5	2	6.5	2	10	7	7.2
		R	≤14	17	37	7	22.6	1	5	25	25.8
	Cefuroxime	S	≥18	28	60.9	24	77.4	17	85	69	71.1
	(CU:30)	I	15-17	2	4.3	1	3.2	1	5	4	4.1
		R	≤14	16	34.8	6	19.4	2	10	24	24.7
Aminogylcosides	Gentamicin	S	≥15	34	73.9	21	67.7	9	45	64	66
	(G:10)	I	12-14	7	15.2	9	29	10	50	26	26.8
		R	≤11	5	10.9	1	3.2	1	5	7	7.2
	Neomycin	S	≥15	27	58.7	19	61.3	11	55	57	58.8
	(N:30)	I	13-14	9	19.6	7	22.6	5	25	21	21.7
		R	≤12	10	21.7	5	16.1	4	20	19	19.6
	Streptomycin	S	≥14	37	80.4	27	87.1	18	90	82	84.5
	(S:25)	I	NA	NA	NA	NA	NA	NA	NA	NA	NA
		R	≤13	9	19.6	4	12.9	2	10	15	15.5
	Amikacin	S	≥17	26	56.5	24	77.4	12	60	62	63.9
	(AK:30)	I	15-16	11	23.9	2	6.5	6	30	19	19.6
		R	≤14	9	19.6	5	16.1	2	10	16	16.5
Floroquinolones	Enrofloxacin	S	≥22	23	50	27	87.1	17	85	67	69.1
	(EX:10)	I	18-21	11	23.9	3	9.7	2	10	16	16.5
		R	≤17	12	26.1	1	3.2	1	5	14	14.4
	Ciprofloxacin	S	≥21	13	28.3	22	71	15	75	50	51.6
	(CF:5)	I	16-20	17	37	6	19.4	4	20	27	27.8
		R	≤15	16	34.8	3	9.7	1	5	20	20.6
	Moxifloxacin	S	≥24	5	10.9	19	61.3	12	60	36	37.1
	(MO:5)	I	21-23	7	15.2	5	16.1	3	15	15	15.5
		R	≤20	34	73.9	7	22.6	5	25	46	47.4
Sulphonamide	Co-Trimoxazole	S	≥16	20	43.5	23	74.2	18	90	61	62.9
	(CO:25)	I	11-15	7	15.2	4	12.9	0	0	11	11.3
		R	≤10	19	41.3	4	12.9	2	10	25	25.8
Macrolide	Erythromycin	S	≥23	6	13	9	29	7	35	22	22.7
	(E:10)	I	14-22	38	82.6	21	67.7	13	65	72	74.2

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		R	≤13	2	4.3	1	3.2	0	0	3	3.1
Tetracycline	Tetracycline	S	≥19	29	63	26	83.9	17	85	72	74.2
	(T:10)	I	15-18	4	8.7	1	3.2	1	5	6	6.2
		R	≤14	13	28.3	4	12.9	2	10	19	19.6
Lincosamide	Clindamycin	S	≥21	15	32.6	14	45.2	8	40	37	38.1
	(CD:2)	I	15-20	25	54.3	13	41.9	11	55	49	50.5
		R	≤14	6	13	4	12.9	1	5	11	11.3
Oxazolidinone	Linezolid	S	≥21	31	67.4	25	80.6	11	55	67	69.1
	(LZ:30)	I	NA	0	0	NA	NA	NA	NA	NA	NA
		R	≤20	15	32.6	6	19.4	9	45	30	30.9
Chloramphenicol	Chloramphenicol	S	≥18	42	91.3	28	90.3	18	90	88	90.7
	(C:30)	I	13-17	4	8.7	3	9.7	2	10	9	9.3
		R	≤12	0	0	0	0	0	0	0	0
Glycopeptide	Teicoplanin	S	≥14	17	37	21	67.7	10	50	48	49.5
	(TE:30)	I	Nov-13	22	47.8	8	25.8	9	45	39	40.2
		R	≤10	7	15.2	2	6.5	1	5	10	10.3

S: Sensitive, I: Intermediate, R: Resistant, NA: Not applicable, N: Number of isolates, CM: Clinical mastitis, SCM: Subclinical mastitis, Break Point: Zone of inhibition in mm

Criteria for Phenotypic Antibiotic Resistance

Phenotypic antibiotic resistance pattern was interpreted as per guidelines of Magiorakos *et al.* (2012). Isolates resistant to three or more antibiotics belonging to different groups were classified as multidrug resistant (MDR). Among MDR isolates, isolates susceptible to only two antibiotics belonging to two different groups were considered extreme drug resistant (XDR), while resistance to all the antibiotics was considered as pan-drug resistant (PDR).

DNA Extraction

After overnight inoculation of an individual bacterial colony in brain heart infusion (BHI, HiMedia) broth, 1 ml culture was pelleted at 7500 rpm for 5 min in refrigerated centrifuge (Thermo Scientific). The pellet was suspended in 180 µI lysozyme enzyme solution and incubated at 37ºC for 30 min. Bacterial DNA was extracted using QIAamp DNA mini kit (Qiagen) as per manufacturer guidelines. Eluted genomic DNA was stored at −20°C until use.

Amplification of *mecA* and Agarose Gel Electrophoresis

The PCR assays used 100 pg of DNA template in a 25 µl reaction mixture with 13 µl of Taq DNA Master Mix (Qiagen) and 250 nM of each oligonucleotide primer (Genaxy) for amplification of *mecA* (Murakami *et al.*, 1991). PCR products were analyzed using conventional agarose gel electrophoresis in 1% w/v agarose (Genaxy). The amplified products were run in agarose gel in 1x TBE buffer (Genaxy) containing ethidium bromide at 0.1 µg/ml. Quantitative DNA Markers (Genaxy) were used as molecular size markers.

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The DNA bands were visualized and imaged using the Molecular Imager® ChemiDoc™ XRS+ imaging system (Bio-Rad).

Results

Clinical Interpretation of Antibiotic Sensitivity Testing

A total of 97 *S. aureus* isolates consisting of 77 isolates from cows (subclinical=31, clinical=46) and 20 isolates from buffaloes (subclinical=17, clinical=3) were evaluated for antibiotic sensitivity testing. Susceptibility of individual *S. aureus* isolates to antibiotics is presented in Table 1. It is clear from the table that isolates from clinical cases of cows had higher resistance than isolates from subclinical mastitis and resistance was also observed against linezolid (30.9%), an antibiotic that has been developed recently. To simplify the results of antibiotic sensitivity test from clinician point of view (shown in Table 1), susceptibility profile of *S. aureus* isolates to different antibiotics has been presented in Table 2. Clinical isolates demonstrated resistance to most of the antibiotics used (ampicillin, amoxycillin, ceftazidime, clindamycin, ciprofloxacin, ceftrioxone-sulbactum, co-trimoxazole, cefoperazone, ceftrioxone, erythromycin, moxifloxacin, penicillin, teicoplanin), while in comparison, subclinical isolates demonstrated resistance to a fewer antibiotics (amoxycillin, clindamycin, erythromycin, penicillin, gentamicin). Overall, chloramphenicol, co-trimoxazole, cloxacillin, oxacillin and streptomycin showed substantial *in vitro* susceptibility to *S. aureus*.

Table 2: Susceptibility profile of *S. aureus* isolates to different antibiotics

Species	**Mastitis Status**	**Susceptibility of *S. aureus* isolates**			
		High (≥90%)	**Moderate (75-89.9%)**	**Low (50-74.9%)**	**Resistant (<50%)**
Cow	Clinical (n=46)	C	COX, S	AK, AC, AS, CIT, CU, CK, EX, G, LZ, N, OX, T	A, AMX, CA, CD, CF, CIS, CO, CS, CI, E, MO, P, TE
	Subclinical (n=31)	C, COX	AK, CU, EX, LZ, S, T	A, AC, AS, CA, CF, CIS, CIT, CO, CS, CI, CK, G, N, MO, OX, TE	AMX, CD, E, P
Buffalo (n=20)	17 subclinical + 3 clinical	C, CO, COX, OX, S	AC, AS, CF, CIS, CIT, CI, CU, CK, EX, T	AK, A, AMX, CA, CS, LZ, MO, N, P, TE	CD, G, E

OX: oxacillin P: penicillin AC: amoxycillin + clavulanic acid A: Ampicillin AS: amoxycillin + sulbactum AMX: amoxycillin COX: cloxacilliin CIS: ceftrioxone + sulbactum CIT: ceftrioxone + tazobactum CI: ceftrioxone CA: ceftazidime CS: cefoperazone CK: ceftixozime CU: cefuroxime G: gentamicin N: neomycin S: streptomycin AK: amikacin EX: enrofloxacin CF: ciprofloxacin MO: moxifloxacin CO: co-trimoxazole E: erythromycin T: tetracycline CD: clindamycin LZ: linezolid C: chloramphenicol TE: teicoplanin

Phenotypic Antibiotic Resistance Patterns

Eighty three out of 97 isolates were found to be MDR. Of these, 18 isolates were XDR, and one isolate was resistant to all the antibiotics tested (PDR). The antibiotic resistance status of an individual isolate against all the antibiotics within 10 groups has been presented in Fig. 1 and their relationship in terms of MDR,

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XDR and PDR is shown in the diagram (Fig. 2). Interestingly, only 14 isolates (14.4%) were identified as non-MDR indicating high level of resistance in *S. aureus* strains isolated from cases of bovine mastitis.

Isolate	**Antibiotics (1-28) within Antibiotic groups (A-J)**																												**Resistance Status**
No*	**A**														**B**				**C**			**D**	**E**	**F**	**G**	**H**	**I**	**J**	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	
1.																■									■				Non-MDR
2.						■						■				■									■				MDR
3.																■							■		■				MDR
4.		■	■	■		■						■				■				■	■				■				MDR
5.	■	■		■		■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■			XDR
6.																													Non-MDR
7.																■		■					■		■				MDR
8.																■							■		■				MDR
9.																■							■						Non-MDR
10.															■														Non-MDR
11.															■	■							■						Non-MDR
12.	■	■				■		■	■	■	■	■	■	■											■				Non-MDR
13.		■				■										■							■						MDR
14.	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■					■	■	■				■		MDR
15.		■		■		■						■													■				Non-MDR
16.		■				■									■	■							■						MDR
17.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■					■	■	■		■			■	MDR
18.																■						■			■				MDR
19.		■		■		■						■				■							■						MDR
20.		■				■						■			■	■	■	■	■	■	■		■	■	■	■	■	■	XDR
21.		■				■						■			■	■	■	■		■	■		■		■	■		■	MDR
22.		■	■	■	■	■				■	■					■												■	MDR
23.															■	■		■					■		■	■		■	MDR
24.						■									■	■		■		■	■		■		■	■		■	MDR
25.																■							■					■	MDR

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26.		■				■		■	■	■	■	■	■	■	■	■	■	■			■		■		■	■			MDR
27.															■	■	■	■			■	■	■		■	■		■	MDR
28.															■	■		■		■	■		■		■	■		■	MDR
29.															■	■		■			■		■					■	MDR
30.		■		■		■										■							■		■				MDR
31.															■								■		■	■	■	■	MDR
32.											■												■						Non-MDR
33.											■					■													Non-MDR
34.											■	■																	Non-MDR
35.		■	■	■	■	■		■	■	■	■	■	■			■													MDR
36.																■							■						Non-MDR
37.		■	■	■	■	■	■	■	■	■	■	■	■				■			■	■		■	■	■			■	MDR
38.	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■			■	■	■	■						MDR
39.	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■		■		■	XDR
40.	■	■	■	■	■	■		■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■		■	XDR
41.	■	■		■		■		■	■	■	■	■				■		■		■	■		■		■	■		■	MDR
42.				■		■					■					■					■		■					■	MDR
43.		■		■		■					■	■	■		■	■	■	■		■	■	■	■		■	■	■	■	XDR
44.		■	■	■		■					■	■				■	■	■	■	■	■	■	■		■	■	■	■	XDR
45.		■		■		■									■	■							■	■					MDR
46.																									■				Non-MDR
47.		■													■	■	■						■		■				MDR
48.		■													■	■							■						MDR
49.		■				■			■		■	■													■				Non-MDR
50.		■		■		■		■		■					■	■			■				■	■	■				MDR
51.		■	■	■	■	■		■	■	■	■	■	■	■	■	■	■		■	■	■						■		MDR
52.						■						■				■	■				■		■					■	MDR
53.		■		■		■						■				■				■	■	■	■	■					MDR
54.		■	■	■	■	■					■	■				■	■	■	■	■	■				■	■			MDR
55.		■	■	■	■	■	■	■		■	■	■			■	■	■	■	■	■	■		■	■	■	■		■	XDR

56.	□	■	□	■	□	■	□	■	□	■	■	□	□	□	■	■	■	■	■	■	■	■	■	■	■	■	□	■	XDR
57.	■	■	□	■	□	■	■	■	□	■	■	■	■	□	□	■	■	■	□	■	■	■	■	■	■	■	■	■	PDR
58.	□	■	□	■	□	■	□	□	□	■	■	□	■	■	□	■	■	■	□	■	■	□	■	□	■	■	□	■	MDR
59.	■	■	■	■	■	■	■	□	□	□	□	■	□	■	□	■	■	□	■	■	■	■	■	□	■	□	□	■	MDR
60.	■	□	□	□	□	■	□	□	□	□	■	□	□	□	□	■	□	■	□	■	■	□	■	□	■	■	□	■	MDR
61.	□	■	□	■	□	■	■	■	■	■	□	■	□	□	□	■	□	□	■	■	■	■	□	■	□	□	□	■	MDR
62.	□	■	□	■	□	■	□	□	□	□	□	■	□	□	□	■	□	□	□	□	□	□	□	■	□	□	□	■	MDR
63.	□	■	■	■	■	■	□	□	□	■	■	■	□	□	■	■	■	■	■	■	■	■	■	■	■	□	□	■	XDR
64.	□	■	□	□	□	■	□	□	□	□	■	■	□	□	□	■	□	□	□	■	■	□	■	□	■	■	□	□	MDR
65.	□	■	■	■	■	■	□	■	□	□	■	■	□	□	□	□	■	□	□	□	□	□	□	■	■	□	□	□	MDR
66.	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	XDR
67.	■	■	□	■	□	■	□	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	□	□	■	MDR
68.	■	■	■	■	■	■	■	■	□	■	■	■	■	■	□	□	□	■	□	■	■	□	■	■	□	□	□	□	MDR
69.	□	■	□	■	□	■	■	■	■	■	■	■	□	□	□	□	□	□	□	□	■	□	■	□	□	□	□	□	MDR
70.	□	■	□	■	□	■	□	■	□	■	■	■	□	□	□	■	□	□	□	□	■	□	■	□	□	□	□	■	MDR
71.	□	■	□	■	□	■	■	■	■	■	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	Non-MDR
72.	□	■	■	■	■	■	□	□	□	□	□	■	□	□	□	■	□	□	■	■	■	□	■	□	■	□	□	□	MDR
73.	■	■	□	■	□	■	□	■	□	■	■	■	■	■	□	■	■	■	■	■	■	□	■	□	■	■	□	■	MDR
74.	□	■	■	■	■	■	■	■	■	■	□	■	□	□	□	■	□	■	■	■	■	■	■	□	■	■	□	■	XDR
75.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	□	□	□	■	■	■	■	□	■	□	□	□	MDR
76.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	□	■	□	□	□	MDR
77.	■	■	□	■	□	■	■	■	■	■	■	■	■	■	■	□	□	□	□	□	■	■	■	□	■	□	□	□	MDR
78.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	□	□	□	■	■	■	■	□	□	□	□	□	MDR
79.	■	■	■	■	■	■	■	■	□	□	■	■	■	■	□	■	□	□	■	■	■	■	■	■	■	□	□	■	XDR
80.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	□	□	□	□	MDR
81.	■	■	□	■	■	■	□	■	□	□	■	■	■	■	□	■	□	□	■	■	■	■	■	■	■	□	□	■	XDR
82.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	□	□	■	■	■	■	■	□	□	□	MDR
83.	■	■	□	■	□	■	■	■	■	■	■	■	■	■	□	■	■	■	□	□	■	■	■	■	□	□	□	□	MDR
84.	□	■	□	■	□	■	□	■	■	■	■	■	□	□	□	■	□	□	□	□	□	□	■	□	□	□	□	□	MDR
85.	□	■	□	■	□	■	□	■	□	■	□	□	□	□	□	■	□	□	■	□	□	□	□	■	□	□	□	□	MDR

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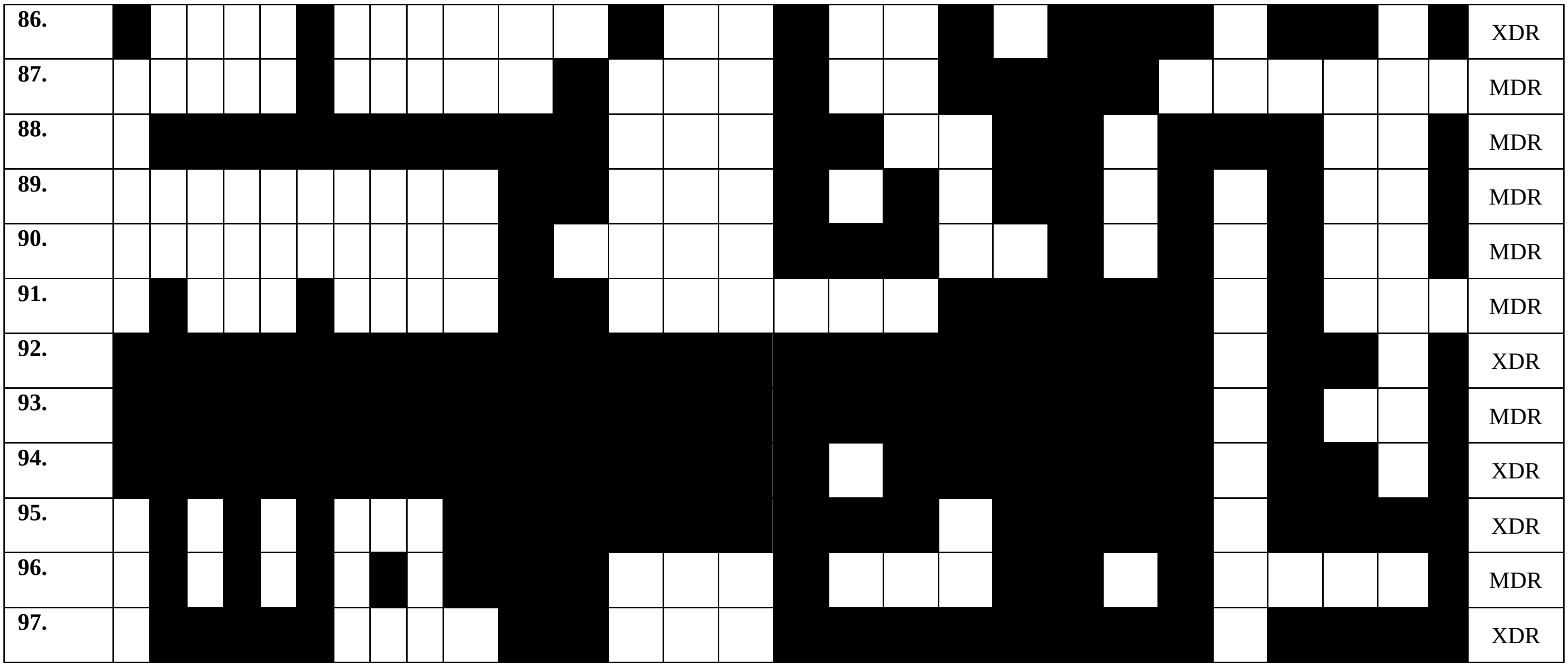

86.	■					■							■			■			■		■	■	■		■	■		■	XDR
87.						■						■				■			■	■	■	■							MDR
88.		■	■	■	■	■	■	■	■	■	■	■				■	■			■	■		■	■	■			■	MDR
89.											■	■				■		■		■	■		■		■			■	MDR
90.											■					■	■	■			■		■		■			■	MDR
91.		■				■					■	■							■	■	■	■	■		■				MDR
92.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■		■	XDR
93.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■			■	MDR
94.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■		■	■		■	XDR
95.		■		■		■				■	■	■	■	■	■	■	■	■		■	■	■	■		■	■	■	■	XDR
96.		■		■		■		■		■	■	■				■				■	■		■					■	MDR
97.		■	■	■	■	■					■	■				■	■	■	■	■	■	■	■		■	■	■	■	XDR

*A: β-Lactams B: Aminoglycosides C: Floroquinolones D: Sulphonamide E: Macrolide F: Tetracycline G: Lincosamide H: Oxazolidinone I: Chloramphenicol J: Glycopeptide; 1: oxacillin 2: penicillin 3: amoxicillin + clavulanate 4: ampicillin 5: amoxicillin + sulbactum 6: amoxycillin 7: cloxacilliin 8: ceftrioxone + sulbactum 9: ceftrioxone + tazobactum 10: ceftrioxone 11: ceftazidine 12: cefaperazone 13: ceftixozime 14: ceforoxime 15: gentamicin 16: neomycin 17: streptomycin 18: amikacin 19: enrofloxacin 20: ciprofloxacin 21: moxifloxacin 22: cotrimazole 23: erythromycin 24: tetracycline 25: clindamycin 26: linezolid 27: chloramphenicol 28: teicoplanin; *1-48 isolates are from cases of subclinical mastitis, and 49-97 are from clinical mastitis*

Fig. 1: Antibiotic resistance status of individual *S. aureus* isolates

Phenotypic Antibiotic Resistance Patterns

Eighty three out of 97 isolates were found to be MDR. Of these, 18 isolates were XDR, and one isolate was resistant to all the antibiotics tested (PDR). The antibiotic resistance status of an individual isolate against all the antibiotics within 10 groups has been presented in Fig. 1 and their relationship in terms of MDR, XDR and PDR is shown in the diagram (Fig. 2). Interestingly, only 14 isolates (14.4%) were identified as non-MDR indicating high level of resistance in *S. aureus* strains isolated from cases of bovine mastitis.

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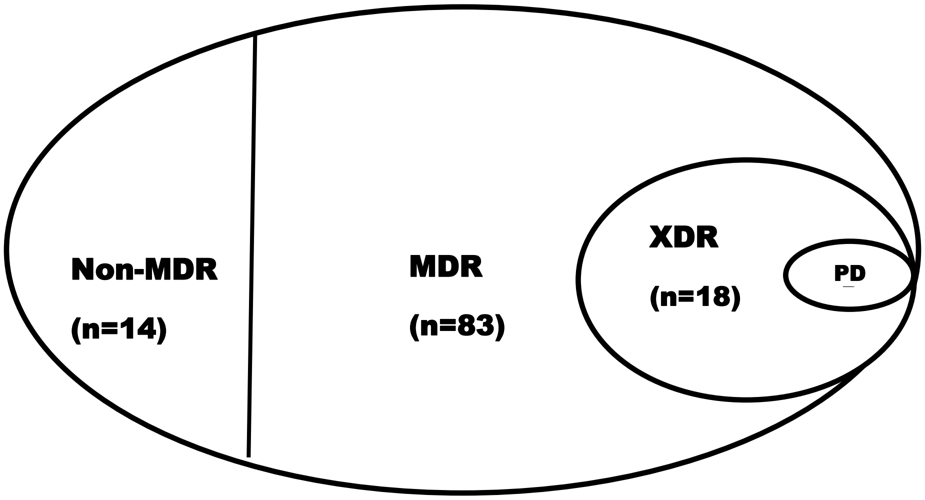

Fig. 2: Diagram showing the extent of phenotypic antibiotic resistance in *S. aureus*

Phenotypic Methicillin Resistance and Presence of *mecA*

A high level of phenotypic methicillin resistance (25.8%) was observed in *S. aureus* (Table 1). To determine, whether this resistance is actually associated with the *mecA* presence, the genomic DNA was extracted and PCR amplification targeting *mecA* was performed on all *S. aureus* isolates. Surprisingly, *mecA* was present (Fig. 3) in only 3 isolates of cows with clinical mastitis.

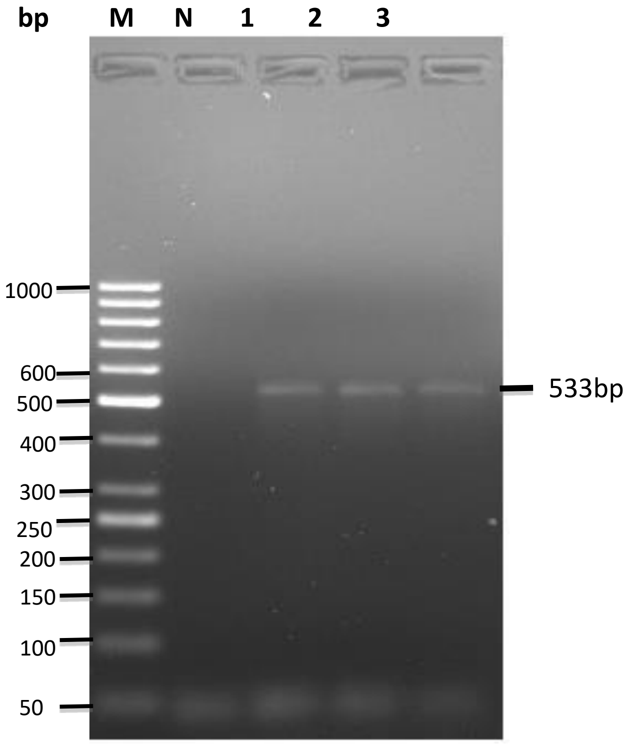

Fig. 3: Agarose gel electrophoresis of amplified DNA targeting *mecA* in *S. aureus*. Lanes 1-3: positive isolates revealing 533 bp product, M: Fermentas GeneRuler 50 bp DNA ladder, N: *mecA* negative isolate.

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Discussion

S. aureus, a major pathogen of bovine mastitis reported worldwide (Karimuribo *et al.*, 2006; Haftu *et al.*, 2012; Mir *et al.*, 2014), is of special public health concern and has been found to cause many serious and life-threatening infections in humans. Antibiotic susceptibility of pathogens varies in different parts of world and is a widely used clinical tool in bovine mastitis to select the most appropriate antibiotic. In the present study, a wide range of 28 antibiotics belonging to 10 groups were evaluated to understand antibiotic resistance patterns. Most of the isolates from clinical and subclinical mastitis (Table 2) were susceptible to chloramphenicol (90.7%), cloxacillin (87.6%) and streptomycin (84.5%). The highest sensitivity shown by chloramphenicol in the present study may be attributed to its restricted use in veterinary practice. Most of the previous studies on mastitis have evaluated the antibiotic sensitivity and resistance patterns comprising only a few antibiotics. Therefore, resistance patterns in those studies could not describe the presence of MDR, XDR and PDR strains in livestock sector. This elaborative study has clearly demonstrated that most of the currently used antibiotics may not be efficacious in mastitis owing to presence of MDR strains.

MRSA strains were first reported from cases of bovine mastitis in 1972 (Devriese *et al.*, 1972). Since then, MRSA strains have become a cause of major public health concerns due to their possible transmission between livestock species and human beings. Studies conducted on human *S. aureus* isolates in India have reported a high prevalence of MRSA ranging between 12 and 40% (Verma *et al.*, 2000; Kali *et al.*, 2013). In present study as well, 25 out of 97 (25.8%) *S. aureus* isolates from bovine milk exhibited phenotypic methicillin resistance. MRSA have been found to be resistant to most of the commonly used antibiotics including the aminoglycosides, chloramphenicol, flouroquinolones, lincosamides, macrolides, sulphonamides, tetracycline and trimethoprim-sulfamethaxazole (Mandell *et al.*, 1995; Feng *et al.*, 2008). Only a few antibiotics including linezolid and vancomycin are effective against MRSA and recent studies have indicated the emergence of resistance against these antibiotics as well (Thati *et al.*, 2011; Gu *et al.*, 2013).

To determine the reasons behind high prevalence of MRSA in Indian strains of *S. aureus* isolated from bovine mastitis, it was planned to compare the presence of methicillin resistance (n=25) with the presence of *mecA*. DNA was extracted from 97 individual *S. aureus* isolates and PCR amplification targeting *mecA* was carried out. The gene was present only in 3 of the 97 *S. aureus* isolates. Similar to our findings, *mecA* could be found in 3 of the 18 phenotypic methicillin resistant *S. aureus* isolates from bovine mastitis cases in Turkey (Turutoglu *et al.*, 2009). Most of the previous studies have demonstrated lower (7 to 11.6%) *mecA* presence (McKay 2008; Jamali *et al.*, 2014 and Reshma *et al*, 2017), however Pu *et al* (2014) has reported prevalence to be quite high (47.6%). The antibiotic resistance is mainly attributed to acquisition of resistance genes by genetic exchange. However, the present study confirmed the results of earlier workers

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who indicated that there might be some other mechanisms associated with phenotypic methicillin resistance (Vesterholm-Nielsen 1999; Olsen, 2006).

Conclusion

In conclusion, this study has shown the emergence of MDR, XDR and PDR in *S. aureus* of bovine mastitis origin. The presence of MRSA strains in bovine milk has public health implications, and needs elaborative research work.

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