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Phenotypic and Genotypic Characterization of Antibiotic Resistant *Staphylococcus aureus* in Bovine Milk Samples in Ludhiana, Punjab, India

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A B S T R A C T

A brief study was carried on forty *Staphylococcus aureus* isolates from Bovine milk from Ludhiana, Punjab, India for detecting the antibiotic resistance profile using E-strips and genotypically targeting various antibiotic resistance genes. The antibiotics used for phenotypic resistance detection were Penicillin, Gentamicin, Erythromycin, Clindamycin, Tetracycline, Chloramphenicol, Ciprofloxacin, Trimethoprim-sulphamethoxazole, Oxacillin, Vancomycin and Amoxyclav. The resistance genes targeted were *bla*Z (Penicillin); *erm*A, *erm*B, *erm*C (Erythromycin); *tet*K, *tet*L, *tet* M, *tet*O (Tetracycline); *aacA-aphD* (Gentamycin), *mec*A (Oxacillin). This study provides a bird view of antimicrobial susceptibility pattern of *Staphylococcus aureus* in Ludhiana and can help to form scientific basis for field Veterinarians to make therapy decisions empirically thus forming a pillar in combating the antibiotic resistance problem. This study also reiterates the usefulness of PCR based technique for detection of antibiotic resistance accompanying the phenotypic tests.

Introduction

Antibiotic resistance is a challenge world is facing in this era and the organism *S. aureus* is not barred from it. This organism has the capability to develop resistance to drugs rapidly indebted to the selective pressure imposed by antimicrobials (Diekema *et al.*, 2004). Antibiotic resistance is mediated by Chromosome or plasmid through transduction, transformation and conjugation (Chamber 1997).

Resistance to β-lactam antibiotics i.e. Penicillin and its derivatives is mediated by *blaZ*gene (Oslen *et al.*, 2006). Resistance to methicillin and other β-lactam antibiotics can be due to altered binding capacity of Phosphate binding protein (PBP) or synthesis of new PBP i.e. PBP2' which is encoded by *mecA* gene. Certain strains showing resistance are β-lactamase hyperproducers are termed as Boderline Oxacillin resistant *S. aureus* (Chamber, 1997). Resistance to tetracycline is attributed to the active efflux under the control of *tetK* and *tetL* genes located on the plasmid and ribosomal protection that is mediated by *tetO* or *tetM* genes present on transposon or chromosome (Warsa *et al.*, 1996). Resistance to aminoglycoside may be attributed to the

structural changes in the ribosomal proteins due to the mutations in the genes encoding so that it may no longer bind to the antibiotic thus inhibiting the therapeutic effect of the antibiotic. Aminoglycoside resistance may also be in response to the impermeability i.e. diminished uptake of the antibiotic or modification of antibiotic due to the cellular enzymes such as aminoglycoside acetyltransferases (AAC) or aminoglycoside phosphotransferases (APH) catalyzed by a bifunctional protein encoded by *aacA-aphD* gene (Lyon and Skurray 1987). Vancomycin intermediate resistance has been imputed to *vraSR* operon (Qureshi *et al.,* 2014) and *graS* gene (Howden *et al.,* 2008).VRSA could be due to the acquisition of *Enterococcivan*A gene. Exposure to vancomycin leads to the thickening of cell wall due to the altered peptidoglycan synthesis impairing the diffusion of drug inside the bacterial cell (Hanaki *et al.,* 1998). Inducible resistance to macrolide antibiotic is commonly due to *ermC* gene present on the plasmid (Shivakumar and Dubnau 1981). Resistance mediated by *ermA* gene can be due to chromosomal mutations (Lacey 1984). Constitutive resistance is mediated by *ermB*gene on plasmid. Less commonly *msrA, msrB, mphC, lnuA* genes are associated with resistance to this antibiotic.

Presence of drug resistant *Staphylococcus aureus* in the bovine milk indicates a threat to the community either on contact or on consumption of the milk or the milk products. Pasteurization of milk may help to reduce the bacterial load and prevent the possible consequences; minor skin infections to life threatening conditions such as bacteremia, endocarditis, necrotizing pneumonia, toxic shock syndrome, necrotizing fasciitis, necrotizing pneumonia, bone and joint infections accompanied by septic thromboembolic disease, purpura fulminans with or without Waterhouse-Friderichsen syndrome, orbital cellulitis and endophthalmitis, infections of the central nervous system(Moran *et al.,* 2006). However, the enterotoxins produced are heat stable and survive even after pasteurization and may result in food intoxication. They may also result in causing severe animal diseases; such as suppurative disease, mastitis, arthritis and urinary tract infections owing to the virulence factors, such as the production of extracellular toxins and enzymes **(**Waldvogel1990).In bovines, it can cause mastitis with moderate to serious local and systemic signs. The presence of resistant bacteria makes the disease more complicated by not responding to the drugs and may further aggravate the antibiotic resistance issue. Therefore, determining antimicrobial susceptibility is an essential part of routine surveillance inorder to update and provide susceptibility data in a particular region to provide scientific basis for field Veterinarians to make therapy decisions empirically. This may form a pillar in combating the antibiotic resistance problem. Both phenotypic and genotypic testing should be done to find the best option available for the therapeutic use as in many cases an isolate may show phenotypic susceptibility to a drug but genotypically it may carry the resistance gene.

Materials and Methods

Two hundred and Forty milk samples were collected from different areas of Punjab. Within 4-5 hours of collection of samples they were processed for isolation of *Staphylococcus aureus.* The protocol, recommended by Bacteriological Analytical Manual (BAM 2016), with necessary modifications was followed for the isolation of *S. aureus.* Ten ml milk sample was mixed in 90 ml buffered peptone water (BPW) and incubated at 37˚C overnight for pre-enrichment. One ml of pre-enriched sample was transferred to 9 ml Tryptone Soya broth (TSB) containing 2%Nacl (Enrichment broth

and incubated at 37˚C overnight for enrichment. Turbidity in the broth indicated presence of an organism in the sample. A loopful of enriched milk sample in TSB was streaked onto a Baird-Parker agar (BPA) supplemented with egg-yolk tellurite emulsion (Hi media, Mumbai).The plate was incubated overnight at 37˚C for 24 - 48 hours. Samples positive for *S. aureus* showed colonies with typical morphology showing smooth, circular, jet black to grey colonies, surrounded by opaque halo which is due to the lecithinase activity. Two to three typical colonies were selected from each sample. Each colony was further inoculated in TSB for overnight incubation at 37˚C and further streaked on BPA for purification. These plates were kept for overnight incubation at 37˚C. Purified colonies from the sub cultured plates were subjected to gram staining. Slides showing gram-positive cocci as a bunch of grapes were characteristic for *S. aureus*. The isolates showing characteristic colony morphology were stored in TSB containing 20% (v/v) glycerol at -20˚C. The presumptive *S. aureus* colonies were further subjected to biochemical testing for conformation using HiStaph^{TM}Identification Kit (HiMedia Labs, Mumbai). Antibiotic E-test strips (Epsilometer test) *viz.* oxacillin, penicillin, tetracycline, chloramphenicol, Co-Trimoxazole, enrofloxacin, clindamycin, gentamicin, erythromycin, and vancomycin and discs Amoxyclav and Oxacillin (HiMedia Lab, Mumbai) were used for determining the susceptibility pattern as per Clinical Laboratory Standards Institute guidelines (CLSI, 2011) on 40 confirmed *S. aureus* isolates from 31 positive samples out of the 240 samples collected from Ludhiana, Punjab.*S. aureus* strain ATCC 25923 was used as a control strain for AST.

The test was performed by applying bacterial inoculums (1.5×10^8 CFU ml^{-1}) having same turbidity as the 0.5 McFarland (HiMedia Lab, Mumbai) standard to the surface of a Muller Hinton Agar plates. For testing sensitivity to oxacillin MHA with 2% Nacl was used. The antimicrobial strips were placed on the inoculated agar surface and were incubated at 35°C for 18 hours for all the antibiotics; except Oxacillin, for which the incubation period was 24 hours. The MIC value of antibiotic was read where zone of inhibition (ellipse) intersected with the MIC scale on the strip and the isolates were classified as susceptible, resistant as per the criteria mentioned by the manufacturer.

According to Bauer-Kirby (1999) disc diffusion method, antibiotic amoxicillin/clavulinic acid disc was used to check the β-lactamase hyperproduction among those isolates which showed resistance to oxacillin but were *mec*A gene negative (Fluit *et al.,* 2001). Test inoculum with turbidity matching 0.5 McFarland (HiMedia Lab, Mumbai) was mopped on with the swab on the MHA plate with 2% NaCl. Oxacillin and Amoxclav discs were placed, at an appropriate distance (24 mm), on plate. Following incubation of 24 hours, the zone size was measured to the nearest mm using a ruler; including the diameter of the disk.

Isolates showing resistance to erythromycin but susceptible to clindamycin were subjected to D-test for testing the Inducible Clindamycin Resistance due to*erm* or *msrA* gene. Erythromycin and Clindamycin strips were placed approximately 12 mm apart on a Mueller-Hinton agar plate that had been inoculated with a*S. aureus* isolate; the plate was then incubated for 16 to 18 h. A clear, D-shaped susceptible zone of inhibition around the Clindamycin strip was designated as positive for induced resistance.

Molecular detection of antibiotic resistant genes was carried out using PCR in all the *S. aureus* isolates. They were tested for the

presence of following antibiotic resistance genes: *mec*A, *Bla*Z, *aac*A-*aph*D, *erm*(*erm*A, *erm*B, *erm*C*), tet (tet*K, *tet*L, *tet*M, *tet*O*)* and *van*A, encoding for Oxacillin, Penicillin, Gentamicin, Erythromycin, Tetracycline and Vancomycin resistance, respectively. Primers used for amplification in this study were procured from Xcleris (India) (Table 1).

The isolation of genomic DNA from *S. aureus* was done by using boiling and chill method, with slight modifications (Ivanov and Bachvarov 1987). *S. aureus* isolates from glycerol stock were streaked on TSA plates and incubated at 37°C for 24 hours. After incubation, 2-3 colonies were dispensed in 300 µl nuclease free water (NFW) in a 2 ml centrifuge tube and kept for boiling at 99°C for 10 mins. Immediately after boiling, cold shock was given by placing it on ice for 10-15 mins. After cold shock treatment, tube was centrifuged at 10000 rpm for 10 mins. The supernatant containing the DNA was aliquoted in a sterile tube for further use.

Multiplex PCR was carried out for detection of antibiotic resistant genes. The composition of the PCR reaction volume was same, for each PCR. A reaction volume of 25 µl was set, containing 12.5 µl EmeraldAmp® GT PCR Master Mix (Takara Clontech, Japan), 10 pmol/µl of each primer set containing forward and reverse primers, 0.01 µg- 0.2 µg template and sterilized nuclease free water to make up the reaction volume. All the reactions were performed in a Mastercycler Gradient Thermocycler (Applied Biosystems, USA). The multiplex PCR for the detection of tetracycline resistant genes (*tet*K, *tet*L, *tet*Mand *tet*O) was performed with cycling conditions (Zehra *et al.*, 2017) consisting of initial denaturation of DNA at 94°C for 5 minutes; followed by 35 cycles each of denaturation at 94°C for 30 seconds; annealing at 55°C for 30 seconds; extension at 72°C for 30 seconds; followed by a final extension at 72°C for 4 minutes and hold at 4°C. All the four primer setsof *tet*K, *tet*L, *tet*M and *tet*O were added to reaction volume.

PCR assay was performed for the detection of erythromycin and gentamicin resistant genes. All the four primers set *erm*A, *erm*B, *erm*C, *aac*A-*aph*D were added to the reaction volume. The cycling conditions of PCR included an initial denaturation of DNA at 94°C for 5 minutes; followed by 35 cycles each of denaturation at 94°C for 30 seconds; annealing at 55°C for 30 secs; extension at 72°C for 30 secs, followed by a final extension at 72°C for 4 minutes and hold at 4°C (Zehra *et al.*, 2017).

PCR amplification was performed for the detection of *bla*Z genes. *bla*Z primer sets were added to the reaction volume. The cycling conditions of PCR included an initial denaturation of DNA at 94°C for 45 seconds; followed by 30 cycles each of denaturation at 94°C for 20 seconds; annealing at 55°C for 15 seconds; extension at 70°C for 15 seconds; followed by a final extension at 72°C for 2 minutes and hold at 4°C (Zehra *et al.*, 2017).

The methodology of Saha *et al.*, (2008) was followed for the amplification of *van*A gene. The PCR amplification mixture contained the following components: 12.5µl master mix EmeraldAmp® GT PCR Master Mix, 2 µM each primer, 0.1 µg template DNA. The amplification conditions were initial denaturation at 98°C for 2 minutes; followed by 35 cycles each of denaturation at 98°C for 10seconds; annealing at 50°C for 1 minute; extension at 72°C for 60 seconds and final extension at 72°C for 5 minutes.

Results and Discussion

Out of 240 samples 31 samples were positive for *Staphylococcus aureus*from which 40 isolates were isolated. Resistance to Penicillin was seen in 100% isolates, 82% isolates showed resistance to Erythromycin, 65%

isolates were resistant to Ciprofloxacin, 35% isolates resistant to Clindamycin, 30% isolates resistant to Tetracycline, 25% isolates resistant to Oxacillin, 12.5% isolates resistant to Gentamicin, 7.5% isolates resistant to Trimethoprim- Sulfamethoxazole (TSH), 10% isolates resistant to Clindamycin and none to Vancomycin. None of the isolates showing erythromycin resistance showed inducible clindamycin resistance on D-test.

Out of 38 *S. aureus* isolates, showing resistance phenotypically to penicillin with MIC≥ 0.25 µg/ml, 35 were positive for β-lactamase gene (*blaZ*) as they yielded a 173bp product by PCR. Three isolates were negative for *blaZ* gene but showed phenotypic resistance to Penicillin on AST (Table 2).

Twelve isolates were phenotypically resistant to tetracycline MIC ≥ 16. Out of these 12 isolates 11 carried *tetK* gene (amplification reaction yielded 360bp) and 1 carried *tetM* gene (158 bp amplicon size). However 3 isolates carrying *tetK* gene and 6 isolates carrying *tetM* gene were phenotypically sensitive to tetracycline (Table 3).

Out of the 40 isolates taken for study, gentamicin resistance gene (*aac*A-*aph*D) amplification (227 bp amplicon size) was seen in 13 *S. aureus* isolates out of which only four isolates showed phenotypic resistance to gentamicin with the MIC value ≥ 16. However,9 isolates although possessed *aac*A-*aph*D gene but were sensitive to gentamicin phenotypically (Table 4). Out of the 9 isolates which were resistant to Erythromycin and 24 isolates which showed intermediate resistance to Erythromycin with MIC value ≥ 1, seven isolates and 1 isolate possessed only *erm*C (gene evident by amplified product size of 295bp) respectively (Table 8). *erm*B and *erm*A was not evident in any of our isolates (Table 5). Eleven isolates were phenotypically resistant to Oxacillin with MIC≥2. Out of them only 6 isolates carried *mec*A gene (amplified PCR product size 532bp) and other 5 lacked *mec*A gene (Table 6).

Five oxacillin resistant but *mec*A negative *S. aureus* isolates were further tested in order to verify β-lactamases hyper production using amoxyclave (amoxicillin-clavulinic acid) disk diffusion test. All these isolates were β-lactamases hyper producers with inhibition zone more than 20 mm and considered Border-line Oxacillin Resistant *Staphylococcus aureus* (Table 7; Fig. 1–7).

Penicillin and Erythromycin could be attributed to the common use of the antibiotics for treatment purpose of diseases in bovines in this region, (Based on the information from the field vets). The ease of access and cost efficacy can be the possible reasons for the misuse of these antibiotics.The Oxacillin resistant isolates from milk (n=6) also showed resistance to Clindamycin, Ciprofloxacin and Trimethroprim- Sufamethoxazole.

This is the cause of concern, as there is a possible chance of transmission of such isolates from animals to humans. Therapeutic options available for the treatment of this organism are less, so it becomes important to prevent the spread of this organism. This can be done by taking simple preventive measures during milking and handling the food by adopting hygienic practices. Higher relationship between phenotypic and genotypic expression of resistance to antibiotic penicillin was observed in our study.

Table.1 Primers used for detection of various genes in *S. aureus*

Gene	Oligonucleotide sequence (5`-3`)	Amplicon size (Base pairs)	Reference
***mec*A**	F: AAA ATC GAT GGT AAA GGT TGG C R: AGT TCT GCA GTA CCG GAT TTG C	532	Strommenger *et al.*, (2003)
***erm*(A)**	F: AAG CGG TAA ACC CCT CTG A R: TTC GCA AAT CCC TTC TCA AC	190	
***erm*(C)**	F: AAT CGT CAA TTC CTG CAT GT R: TAA TCG TGG AAT ACG GGT TTG	299	
***aac*A-*aph*D**	F: TAA TCC AAG AGC AAT AAG GGC R: GCC ACA CTA TCA TAA CCA CTA	227	
***tet*K**	F: GTA GCG ACA ATA GGT AAT AGT R: GTA GTG ACA ATA AAC CTC CTA	360	
***tet*M**	F: AGT GGA GCG ATT ACA GAA R: CAT ATG TCC TGG CGT GTC TA	158	
***tet*L**	F: GTC GTT GCG CGC TAT ATT CC R: GTG AAC GGT AGC CCA CCT AA	696	Huys*et al* (2005)
***tet*O**	F: AAT GAA GAT TCC GAC AAT TT R: CTC ATG CGT TGT AGT ATT CCA	781	
***van*A**	F: ATG AAT AGA ATA AAA GTT GC R: TCA CCC CTT TAA CGC TAA TA	1032	Saha*et al* (2008)
***erm*(B)**	F:CTA TCT GA TTG TTG AAG AAG GAT T R:GTT TAC TCT TGG TTT AGG ATG AAA	142	Martineau *et al* (2000)
***bla*Z**	F: ACT TCA ACA CCT GCT GCT TTC R: TGA CCA CTT TTA TCA GCA ACC	173	

Table.2 Relationship between the phenotypic and genotypic antibiotic resistance to penicillin

blaZ gene presence	No. of strains tested by E strip (MIC µg/ml)		
	Sensitive (≤0.12)	**Intermediate (0.12-0.25)**	**Resistant (≥ 0.25)**
Positive	0	1	34
Negative	0	0	3

Table.3 Relationship between the phenotypic and genotypic antibiotic resistance to tetracycline

tet gene presence		No. of strains tested by E strip (MIC µg/ml)		
*tet*K	***tet*M**	**Sensitive (≤ 4)**	**Intermediate (8)**	**Resistant (≥ 16)**
11[a]	1[a]	0	0	12
3[b]	6[b]	9	0	0

a: phenotypically also resistant to tetracycline; b: phenotypically sensitive to tetracycline

Table.4 Relationship between the phenotypic and genotypic pattern of antibiotic resistance to gentamicin

*aac*A-*aph*D gene presence	No. of strains tested by E strip with gentamicin (MIC µg/ml)		
	Sensitive (≤ 4)	**Intermediate (8)**	**Resistant (≥ 16)**
5[a]	0	1	4
9[b]	9	0	0

a: phenotypically resistant to gentamicin; b: phenotypically sensitive to gentamicin

Table.5 Relationship between the phenotypic and genotypic antibiotic resistance to erythromycin

erm (ABC) gene presence			No. of strains tested by E strip (MIC µg/ml)		
*erm*A	***erm*B**	***erm*C**	**Sensitive (≤ 0.5)**	**Intermediate (1-4)**	**Resistant (≥ 8)**
0	0	8	6	24	10

***Plate.1 Staphylococcus aureus* colony on Baird Parker Agar**

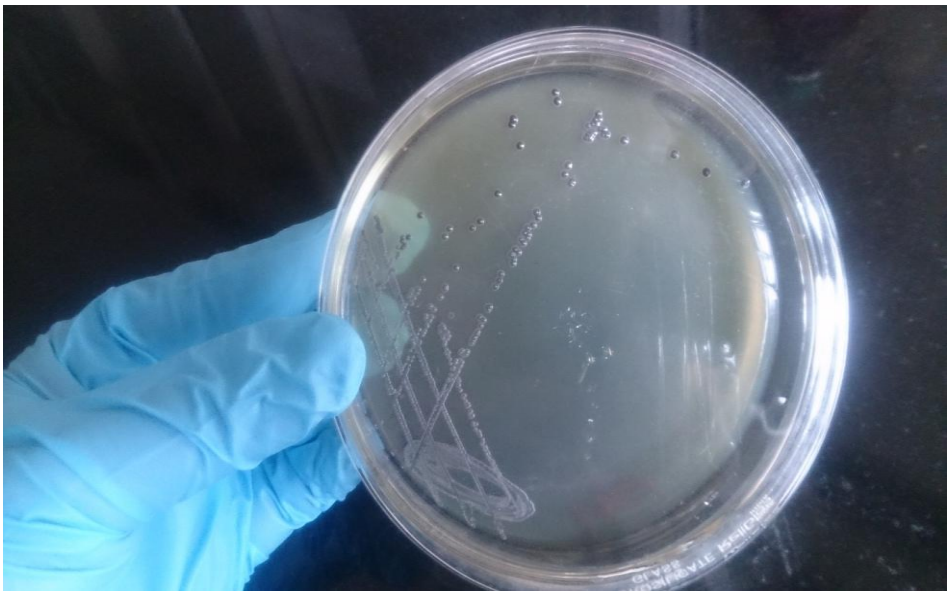


Table.8 MIC values (µg/ml) of Group B *S. aureus* isolates

SAMPLE	PEN	OXA	TET	CLI	VAN	TSH	CPH	ERY	CHL	GEN	
L31	6	1.5	2.5	8	2	0.5	4	2.5	6	0.25	MDR
L34	16	2	12	0.38	1	0.5	1	1	4	1	MDR
L32	6	0.5	12	0.19	1.5	0.75	38	1	6	0.25	MDR
B2	10	1	4	0.19	2	6	4	0.75	6	0.38	MDR
L42	12	2	12	0.38	2	6	4	1	3	0.38	MDR
B1	16	2	24	8	2	2	64	1	6	0.38	MDR
M18	12	4	1.5	0.094	1.5	6	24	0.5	2	0.25	MDR
110A	8	4	1.5	0.5	2	0.5	0.5	1	3	0.19	MDR
M10	12	0.5	2	0.5	1.5	0.75	64	0.75	8	0.38	MDR
M22	32	6	0.125	25	2	0.75	64	0.75	3	0.38	MDR
45C	32	1	0.75	0.5	2	0.75	24	0.75	4	0.38	MDR
40C	12	1	2	0.75	1.5	0.5	48	0.19	3	0.5	MDR
M12	32	2	16	0.125	2	1	32	0.75	3	0.25	MDR
47C	6	1	1	0.125	2	1	1.5	1	4	0.5	MDR
M9	16	1	0.25	0.5	1.5	1	16	0.75	2	0.38	MDR
FT2C	32	4	0.064	0.25	2	0.5	96	0.75	2	24	MDR
L24	32	6	256	0.75	2	1	8	256	4	0.25	MDR
3Mec	1.5	4	0.064	0.064	1	0.38	0.38	0.25	0.5	0.38	
5MecB	32	6	0.94	256	2	1	256	48	1.5	1.5	MDR
L33	32	2	0.125	1	1.5	0.75	1.5	12	1.5	1.5	MDR
38R	6	0.5	4	0.25	1.5	0.38	0.19	0.75	1.5	0.25	
L22	32	1.5	16	0.19	2	0.5	0.94	0.75	3	16	MDR
27R	6	2	0.25	0.75	2	0.5	0.25	1.5	12	0.38	MDR
L52C	1.5	1.5	0.032	0.38	1	0.5	0.125	0.75	12	0.25	MDR
A32	2	4	1.5	0.5	2	1	1.5	8	32	0.38	MDR
M1	32	4	0.38	0.75	2	0.38	0.38	256	8	0.38	MDR
G18C	32	6	256	4	2	1	8	24	12	8	MDR
40C1	12	2	0.19	0.25	2	1	48	1.5	4	0.38	MDR
L51C	32	2	0.125	2	2	2	4	12	3	2	MDR
V17	32	2	1.25	2	2	1	32	6	2	2	MDR
4MecB	1.5	1.5	0.125	12	2	0.75	16	1	0.5	3	MDR
B3	12	1.5	16	0.75	1	0.75	1.5	12	4	0.38	MDR
H2	12	1.5	0.25	0.25	0.75	0.5	32	1	8	0.38	MDR
K1	0.75	2	32	0.19	1.5	0.19	1	0.75	8	16	MDR
K2	2	2	32	0.25	1	0.5	1	0.5	6	16	MDR
H7	0.023	1	1.5	0.38	0.5	0.75	0.38	24	0.5	6	MDR
H8	1.5	1.5	0.75	0.38	1	0.19	0.75	0.25	6	0.94	
H12	0.19	1.5	2	0.125	1	0.75	1.5	0.75	6	0.19	MDR
H1	1.5	1.5	0.75	0.5	1	0.125	0.5	0.25	4	0.94	
H3	12	1.5	16	0.75	1	0.75	1.5	12	4	0.38	MDR

Green, yellow and pink colour in the table signifies the isolate as sensitive, intermediate resistant and resistant, respectively

Table.6 Relationship between the phenotypic and genotypic antibiotic resistance to oxacillin

mecA gene presence	No. of strains tested by E strip (MIC µg/ml)		
	Sensitive (≤2)	**Intermediate (2-4)**	**Resistant (≥4)**
5	0	6	4

Table.7 Characteristics of the isolates resistant to oxacillin but negative for *mecA* gene by PCR

Source	Isolate	Oxacillin MIC (µg/ml)	Inhibition zone* (mm)	β-lactamase hyper producers
Milk	L24	6	30	+
Milk	G18-C	6	24	+
Milk	A(3)2	4	30	+
Milk	M1	4	28	+
Milk	M18	4	30	+

*The diameter of the inhibition zone with amoxyclave (amoxicillin-clavulinic acid: 20 and 10µg respectively) exceeding 20mm considered β-lactamases hyper producers.

Biochemical test using HiStaph Identification Kit (Himedia)

Fig.1 MHA with E strips showing zone of inhibition as eclipse intersecting the strip at MIC value: D-Test

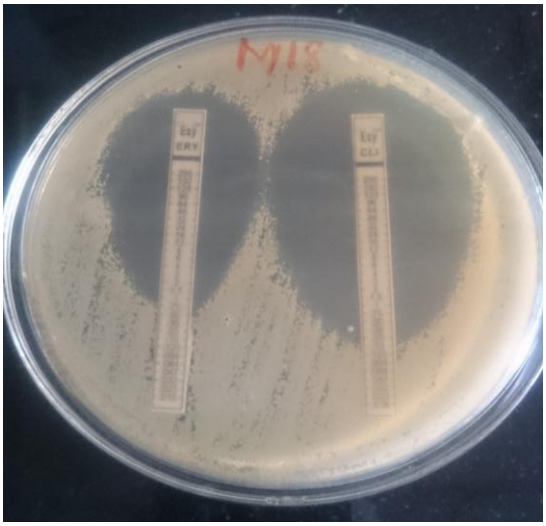


Fig.2 Gel electrophoresis picture of *16SrDNA* (420bp), *femA* (132bp), *mecA* (532bp)
L-Gene ruler; S- Positive control; N-No template control
Lane 1-13: test isolates

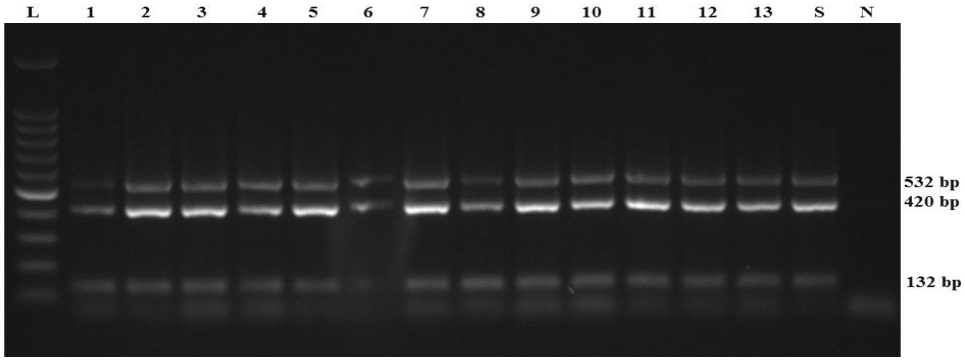


Fig.3 Gel electrophoresis picture of *coa* (between 700bp) and *blaZ* (173bp) gene
L-Gene ruler; S- Positive control; N-No template control
Lane 1-11: test isolates

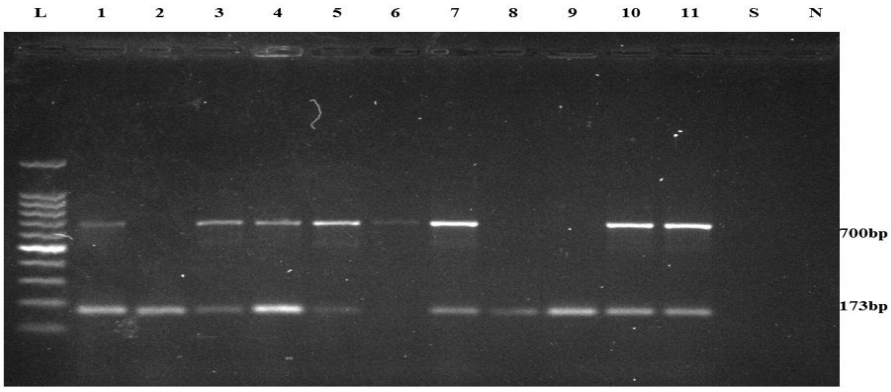


Fig.4 Gel electrophoresis picture of *tetM* (150bp), *tetK* (360bp)
Lane 1-6; test isolates

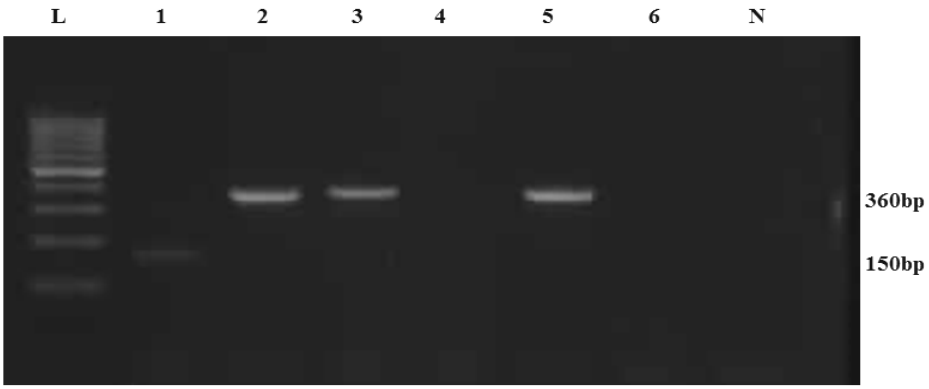


Fig.5 Gel electrophoresis picture of *aacA-aphD*gene (227bp)
L-Gene ruler; S- Positive control; N-No template control
Lane 1-8: test isolates

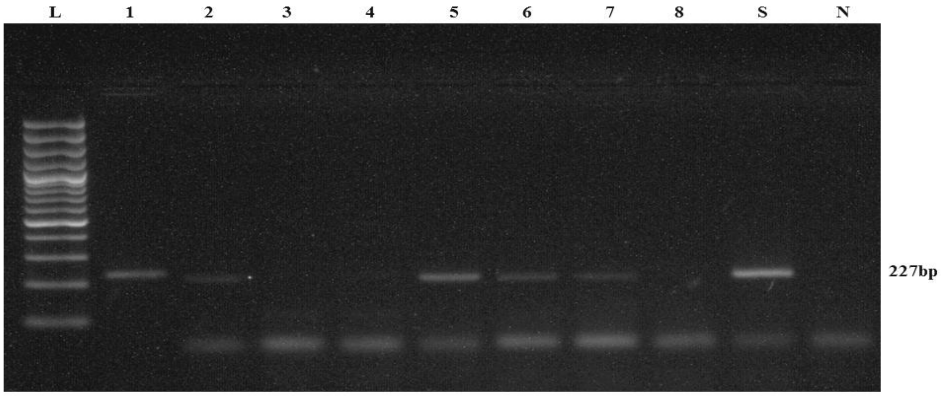
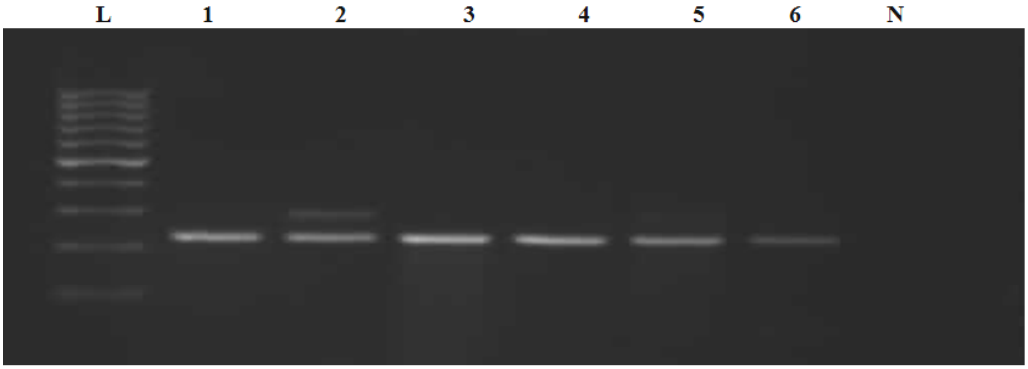


Fig.6 Gel electrophoresis picture of *emcC*gene (295bp) and *aacA-aphD*(227bp)
L-Gene ruler; N-No template
Lane 1-6: test isolates



In this bird view study on the presence of drug resistant *Staphylococcus aureus*, majority (90%) of the isolates showed multidrug resistance with maximum resistance to Penicillin, Erythromycin and Ciprofloxacin. High rate of resistance to

The phenotypic expression of penicillin resistance in these isolates in absence of *blaZ* gene could be due to tolerance in *S. aureus* to the bactericidal effect of β-lactam antibiotic (Sabath, 1979; Sabath, 1982) or presence of additional protein having a reduced affinity for the β-lactam antibiotics (Brown and Reynold, 1980). Although in presence of *tetK* and *tetM* gene isolates showed sensitivity to antibiotic phenotypically. Same was the case with 9 other isolates which possessed *aac*A-*aph*D gene but were sensitive to gentamicin phenotypically. This can be attributed to the less expression of *tet*M and *aac*A-*aph*D gene (Huys *et al.,* 2005; Emaneini*et al*2013; Martineau *et al.,* 2000, Choi *et al.,* 2003).Twenty - four isolates showed intermediate resistance to Erythromycin phenotypically and one isolate complete resistance to Erythromycin but lacked *erm* gene. This may be accounted to other genes like *msrA*or novel gene *ermTR* (Martineau *et al.,* 2000).

There was a positive relationship between phenotypic and genotypic expression of antibiotic resistance to Oxacillin. Isolates lacking *mec*A gene but showing resistance phenotypically, tested by amoxyclav and Oxacillin disc showed that they were β-lactamases hyper producers with inhibition zone more than 20 mm and were considered Border-line Oxacillin Resistant *Staphylococcus aureus* (Tambekar *et al.*, 2007; Pereira *et al.*, 2009 and Martineau *et al.*, 2000) have also reported *S. aureus* isolates which showed phenotypic resistance to Oxacillin but lacked *mecA* gene. Martineau *et al.*, (2000), Wyke *et al.*, (1982) and Laurent *et al.*, (2012) reported that such antibiotic resistance pattern may be because of variant *mecA* gene i.e. *mecC* gene, production of normal PBP with altered binding capacity or β-lactamases hyper production.

In conclusion, antibiotic resistant isolates are present in the milk samples in Ludhiana, Punjab, India. The isolates are showing resistance to the most commonly used antibiotics and six isolates are resistant to Oxacillin. The presence of antibiotic resistance from the food of animal origin is a concern as this may act as a source of infection for the consumers on contact or on consumption of raw milk or milk products. So it is important to carry the Antimicrobial susceptibility testing prior to the treatment in order to combat the issue of antimicrobial resistance. Genotypic testing along with the phenotypic testing is important to form the basis for the empirical therapeutic decisions at field level.

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