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A study on the antimicrobial resistant patterns and molecular characterization of *Staphylococcus aureus* isolated from milk

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Abstract
Out of 400 milk samples collected from different sources, 374 samples were positive for *Staphylococcus aureus* by culture method and confirmed by biochemical tests and 191 isolates (51.07%) were positive by PCR method. All the coagulase positive *S. aureus* isolates (191) were positive for *nuc* gene. Out of 191 *S. aureus* isolates, 15.2% isolates were positive for *mecA* gene. Among the 374 isolates 191 isolates were confirmed as pathogenic *S. aureus* by a positive coagulase test. A pannel of 8 antibiotic discs were tested by using the standard disc diffusion method. Among the 191 isolates maximum resistance was observed for penicillin (74.9%) followed by gentamycin (27.2%), erythromycin (21%), cefoxitin (15.2%), and ciprofloxacin (11.5%), tetracycline (7.8%), cotrimoxazole (5.8%), chloramphenicol (0.5%).

Keywords: *Staphylococcus aureus*, molecular characterization, antimicrobial resistance

Introduction
Foodborne infection risk is low in the countries where pasteurization is applied to most milk products, but there exists a risk with raw milk and products made with raw milk. Enterotoxigenic strains need to grow to concentrations >105 cfu/g before the toxin is produced at detectable levels (EU regulation EC 2073/ 2005). Of note, enterotoxins are resistant to heat, freezing and irradiation. Hence, toxins produced before heat-treatment are extremely difficult to eliminate from foods and can cause intoxication. Foodborne infection risk is low in the countries where pasteurization is applied to most milk products, but there exists a risk with raw milk and products made with raw milk. Enterotoxigenic strains need to grow to concentrations >105 cfu/g before the toxin is produced at detectable levels (EU regulation EC 2073/ 2005). Of note, enterotoxins are resistant to heat, freezing and irradiation. Hence, toxins produced before heat-treatment are extremely difficult to eliminate from foods and can cause intoxication.

S. aureus infecting the mammary gland remains a major problem to the dairy industry worldwide because of its pathogenicity, contagiousness, persistence in the cow environment, colonization of skin or mucosal epithelia, and the poor curing efficacy of treatments. *S. aureus* also constitutes a threat to public health due to food safety and antibiotic usage issues and the potential for bidirectional transmission of strains between humans and dairy animals (cows and small ruminants) (Rainard *et al.*, 2018) [53]. *Staphylococci* have a reputation of rapidly developing resistance to virtually any antibiotic drugs (Pantosti *et al.*, 2007) [49]. In recent years, MRSA has been identified as an emerging pathogen in livestock (pigs, cattle and poultry) and companion animals (Antoci *et al.*, 2013; Cuny *et. al.*, 2013) [6, 17]. The methicillin resistance characteristic in *S. aureus* is due to the presence of altered penicillin binding protein (PBP2a) in the cell wall that has a reduced binding affinity to β-lactam antibiotics. PBP2a is encoded by *mecA* gene that is located in the large chromosomal cassette called staphylococcal chromosome cassette *mec* element (*SCCmec*) (Ganai *et al.*, 2016) [25].

The development of tetracycline resistance in *S. aureus* by two main mechanisms: active efflux, which is mediated by plasmid encoded *tetK* and *tetL* genes and ribosomal protection that is encoded by chromosomal or transposonal *tetM* or *tetO* genes (Trzcinski *et al.*, 2000; Chopra *et al.*, 2001) [65, 14]. The *tetK* and *tetL* genes confer resistance to tetracycline and chlortetracycline but neither to minocycline nor doxycycline.

The development of multiple antibiotic-resistant bacteria due to indiscriminate of antibiotics in animals and poultry production is well authenticated for pathogenic bacteria

(Hawkey *et al.,* 2009; Arenas *et al.,* 2017) [30, 9]. Contaminated food of animal origins with antibiotic-resistant bacteria can be a great threat to public health, and the antibiotic resistance determinants can be transferred from antibiotic-resistant bacteria to other bacteria affecting human (Threlfall *et al.,* 2000) [63]. Identical elements of antibiotic-resistant genes found in bacteria that affect both animals and humans have shown the role of raw foods in the dissemination of these resistance genes through the food chains (Teuber, 2001) [61] or through occupational contact with livestock (Leibler *et al.,* 2016) [39]. The multiple antibiotic-resistant bacteria were commonly isolated from food of animal origin such as raw milk and unpasteurized dairy products and meat products (Fawzy *et al.,* 2017) [23], the resistance genes can be transferred from antibiotic-resistant bacteria to the intestinal flora of humans through food products, and these bacteria can be a reservoir of resistant genes for pathogenic bacteria (Aarestrup *et al.,* 2008) [1]. Keeping in view of the public health significance this study was designed to study the antibiotic resistance profile of Staphylococcus aureus isolated from the milk samples collected in and around Tirupati.

Materials and methods

For this study raw milk samples were collected by using sterile sampling tubes. A total of 400milk samples were collected aseptically from different sources in and around Tirupati, Andhra Pradesh (table 1). The source of the milk samples comprise of individual farmers and local street vendors. The milk samples were processed within 2 to 24 hours of collection.

Table 1: Source and number of raw milk samples collected

S. No.	Place of sample collection	No. of samples
1.	Gurava Reddy Nagar	19
2.	Tummalagunta	16
3.	Sri Nagar	36
4.	Rajeev Nagar	32
5.	VK puram	12
6.	Lingeswara Nagar	54
7.	Padmavathi Nagar	52
8.	Peruru	120
9.	Cherlapalli	24
10.	Ambedkar Nagar	35
	Grand Total	400

Tryptic soy broth was used for enrichment of inoculums. Baired Parker agar supplemented with 5% sterile egg yolk tellurite suspension was used for isolation of *S. aureus.* 1ml of milk sample was inoculated in 9ml of sterile enrichment broth and incubated for 24 h at 37 ^{0}C. Subsequently, a loop full of inoculum from enrichment broth was streaked on Baird Parker agar (HiMedia Pvt. Ltd., India). After 48 h of incubation at 37 ^{0}C, colonies appeared as jet black surrounded by a white halo were identified as characteristic growth of *S. aureus*. The typical colonies were selected and the smears prepared were stained by Gram's staining. The presence of Gram-positivecocci and showing bunch of grapes like appearance were presumed as *Staphylococci* spp. For confirmation of *S. aureus*, the biochemical test conducted were catalase test, oxidase test, coagulase test, IMViC tests and Triple sugar iron test.

The isolates were subjected to antibiotic resistance profile study against 8 antibiotics by using disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2016) [15] guidelines. *S. aureus* isolates were inoculated into BHI broth and incubated at 35 ^{0}C for 24h. A bacterial suspension with the turbidity adjusted to a 0.5 McFarland standard was seeded on Muller Hinton agar plates and spread by the sterilecotton swabs. Then the plates were allowed to dry and antibiotic discs were placed aseptically with sterile fine forceps. The plates were incubated at 35 ^{0}C for 16-18h and Cefoxitin is kept for 24h incubation. The diameter of the zone of inhibition was compared with interpretive standards for *S. aureus* given by CLSI (Table- 2). After the incubation period was over inhibition zones were measured.

Table 2: Interpretation chart for antibiotic sensitivity/resistance patterns (Hi-Media)

S. No	Antimicrobial agent	Disc Conc.	Diameter of zone of inhibition in mm		
			Sensitive	Intermediate resistant	Resistant
1.	Pencillin-G (P)	10 units	≥29	-	≤28
2.	Cefoxitin (CX)	30mcg	≥22	-	≤21
3.	Tetracycline (TE)	30 mcg	≥19	15-18	≤14
4.	Cotrimoxazole (COT)	25mcg	≥16	11-15	≤10
5.	Erythromycin (E)	15 mcg	≥23	14-22	≤13
6.	Gentamicin (GEN)	10 mcg	≥15	13-14	≤12
7.	Chloramphenicol (C)	30 mcg	≥18	13-17	≤12
8.	Ciprofloxacin(CIP)	5mcg	≥21	16-20	≤15

The turbidity standard for 0.5 McFarland standard was prepared by adding 0.5 ml of (1.17% w/v) Barium chloride dehydrate (BaCl2 2H2O) solution to 1% Sulphuric acid. The turbidity standard was placed in a tube identical to the one used for the broth sample and was stored in the dark at room temperature. The turbidity was equivalent to 108cfu/ml which is half the density of a Mac Farland 0.5 standard. The standard was agitated on a vortex mixer immediately before use. If the culture was found less turbid than the turbidity standard it was further incubated for 2-8 hours at 37 ^{0}C until turbidity was equivalent to the standard. If the turbidity exceeds that of the standard the culture solution was diluted

with tryptic soya broth to equitate with the standard.

After identification, biochemical characterization and detection of antibiotic resistance profile of the isolates the pure cultures were streaked on Nutrient agar slant and incubated for 24 h at 37 ^{0}C and then stored at 4 ^{0}C for further analysis.

Molecular characterization of the isolates for *nuc* gene and mecA gene was carriedout by Polymerase Chain Reaction. The reference strains for Staphylococcus aureus (MTCC3103) and for Methicillin Resistant *Staphylococcus aureus* (ATCC 33591) were obtained from Deapartment of Veterinary Public Health & Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh. For the extraction of DNA Suspensions of the bacterial colonies maintained on the nutrient agar slant were prepared in 1.5 ml microcentrifuge tubes in 250µl of sterile double distilled water by gentle mixing. The samples were boiled for 10 min, cooled on ice for 10 min and centrifuged at 10,000rpm for 10 min. 4µl of the supernatant was used as the template for each polymerase chain reaction (PCR) (Islam *et al.,* 2016) [32]. The primers used in the study were custom synthesized by Eurofins genomics India Private Limited, Bangalore. The details of the primers are given in Table.3.

Table 3: Details of oligonucleotide primers used in this study

Target gene	Primer sequence (51 -31)	Amplicon size (bp)	Reference
Nuc	F- GCG ATT GAT GGT GAT ACG GTT	270	Bharathy *et al.* (2015) [11]
	R- AGC CAA GCC TTG ACG AAC TAA AGC		
mec A	F- AAA ATC GAT GGT AAA GGT TGG C	533	Arafa *et al.* (2016) [7]
	R- AGT TCT GCA GTA CCG GAT TTG C		

Initial experiments to optimize PCR reaction conditions for *S. aureus* template involved the empirical variation of annealing temperature (53 ^{0}C – 56 ^{0}C), concentration of primer (5 – 15 p mol), template volume (2µl - 5µl) and the cycling conditions. Optimal results were obtained using 5 µl of bacterial lysate or 20ng of diluted DNA as template in a master mix (2X). Amplification for *nuc* gene was carried by 5 min initial denaturation at 94 °C followed by 35 cycles of denaturation at 94 ^{0}C for 1min, annealing at 55 ^{0}C for 40sec and extension at 72 ^{0}C for 1 min and a final extension for 10 min at 72 ^{0}C. Amplification of *mecA* gene was carried by 5 min initial denaturation at 94 ^{0}C followed by 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 55 ^{0}C for 1 min and extension at 72 ^{0}C for 1 min and a final extension for 10 min at 72 ^{0}C. The given PCR conditions was found to be optimum for obtaining the desired PCR amplification of 270 bp from *nuc* gene and 533 bp from *mecA* gene of *S aureus*.

A simplex PCR assay was developed for the detection of species specific gene of *S. aureus* (*nuc* gene) and gene responsible for methicillin resistance of *S. aureus* (*mecA* gene) was used in our study according to Bharathy *et al.* (2015) [11] and Arafa *et al.* (2016) [7] with slight modifications. The simplex PCR protocol for each gene was followed for the detection of various genes using standard protocols. Following initial trials with varying concentrations of components, the reaction mixture for each gene was optimized as below indicated in the following tables-4&5.

Table 4: Optimized reaction mixture for detection of *nuc* gene

Name	µl per reaction
PCR Master mix(2X)	12.5
nuc gene(Forward primer) (10pmol)	1.0
nuc gene(Reverse primer) (10pmol)	1.0
Template DNA	4.0
nuclease free water	6.5
Total	25.0

Table 5: Optimized reaction mixture for detection of*mecA* gene

Name	µl per reaction
PCR Master mix(2X)	12.5
*mecA*gene(Forward primer) (10pmol)	1.0
*mecA*gene (Reverse primer) (10pmol)	1.0
Template DNA	4.0
nuclease free water	6.5
Total	25.0

DNA amplification was performed in a Thermal cycler with a pre-heated lid. The cycling conditions for detection of all genes were depicted in the table-6. The resultant PCR products were stored at 4 ^{0}C in TE buffer for further analysis by 1.5% agarose gel electrophoresis stained with ethidium bromide. PCR was run for 35 cycles with initial denaturation at 94 ^{0}C for 5 minutes and final extension at 72 ^{0}C for 10 min for all the oligonucleotide primer sets. (Bharathy *et al*., 2015 and Arafa *et al.,* 2016) [11, 7].

Table 6: Cyclic conditions used for simplex PCR assay targeting *nuc* gene and *mecA* gene.

	Gene Denaturation		Annaeling		Extension	
	Tempeatue (^{0}C)	Time (Seconds)	Tempeatue (^{0}C)	Time (seconds)	Tempeatue (^{0}C)	Time (seconds)
nuc	94	60	55	40	72	60
mecA	94	60	55	60	72	60

The resultant PCR products were subjected to 1.5% agarose gel electrophoresis as described by Sambrook and Russel (2001) [56]. Agarose gel (1.5%) was prepared by boiling 0.6 gm of agarose in 40 ml of 1X TBE buffer. After cooling, ethidium bromide was added to the agarose solution to a final concentration of 0.5µg / ml. The molten agarose was poured into a gel casting tray fitted with acrylic comb was kept undisturbed till the gel has solidified. After solidification of the gel, a few ml of 1X TBE buffer was added and the comb was removed carefully and then the tray containing the gel was placed in a submarine horizontal electrophoresis unit filled with 1X TBE buffer up to a level of 1mm above the gel surface.

About 5µl of each PCR product was mixed with 1µl of gel loading dye (6X) and loaded into each well. Electrophoresis was performed at 5V/cm and the mobility was monitored by the migration of the dye. After sufficient migration, the gel was observed under UV transillumination using Alpha innotech gel documentation system to visualize the bands. The PCR product size was determined by comparing with a

standard molecular weight marker.

Results & Discussion

In the present investigation, a total of 400 raw milk samples were collected in and around Tirupati city, Andhra Pradesh. All the milk samples were subjected to isolation and identification by conventional methods and from a total of 400 milk samples 374 (93.5%) samples were positive for staphylococcal species by culture methods. For confirmation of *S. aureus,* all the 374 isolates were subjected to different biochemical tests like catalase test, oxidase test, indole test, methyl red test, Voges Proskauer test, citrate utilization test and triple sugar iron agar test. All the 374 isolates were found catalase positive, oxidase negative, indole test negative, methyl red test positive, Voges Proskauer test positive, citrate utilization test negative and triple sugar iron test positive by yellow discolouration of slant and butt without production of H2S gas. The present findings were in agreement with Reddy *et al.* (2015) [55], Habib *et al.* (2015) [26] and Ramya *et al.* (2017) [54] who have subjected the isolates of *staphylococci* to same biochemical tests and found similar type of reactions.

Multi drug resistance is now the norm among the Gram Positive bacteria like pneumococci, enterococci and staphylococci. *S. aureus* is perhaps the pathogen of concern because of its intrinsic virulence, its ability to cause a diverse array of life threatening infections in humans and in various animal species and its capacity to adapt to different environmental conditions. The evolution of increasingly antimicrobial resistant bacteria stems from a multitude of factors including the strong selective pressure caused by the wide spread and sometimes inappropriate use of antimicrobial agents. The increase in regional and international travel and the relative ease with which antimicrobial resistant bacteria cross geographic barriers (Lowy, 2003) [40] During the present study, all *S. aureus* isolates were found variably resistant to the antibiotics tested (Fig. 1, 2 and 3). Among the 191 *S. aureus* isolates maximum resistance was observed for penicillin-G (74.9%) followed by gentamycin (27.2%), erythromycin (21%), cefoxitin (15.2%), ciprofloxacin (11.5%), tetracycline (7.8%), cotrimoxazole (5.8%) and chloramphenicol (0.5%).

In the present study, *S. aureus* isolates have showed highest resistance to penicillin (74.9%) which was in agreement with the reports of Hanson *et al.* (2011) [28] and Fawzy *et al.* (2017) [23] who have found 77.7% and 73.6% resistance respectively. The findings of the present study with regarding the resistance of *S. aureus* to penicillin differ from earlier report of Khakpoor *et al.* (2011) [35], Thaker *et al.* (2013) [62], and Jahan *et al.* (2015) [35] who have recorded 100% resistance to penicillin among *S. aureus* isolates. Slightly higher percentage of resistance to penicillin than the findings of this investigation was observed from the findings of Abera *et al.* (2010) [2], Tigabu *et al.* (2015) [64], Awad *et al.* (2016) [10], Feng *et al.* (2016) [24], Elemo *et al.* (2017) [20], Can *et al.* (2017) [12] and Yadav, (2018) [69], who have reported 94.4%, 83.5%, 83.3%, 84.09%, 87.3%, 81.81% and 82.23% of resistance respectively.

Resistance to cefoxitin was found 15.2% in *S. aureus* isolates of the present study which was in agreement with the report of Shamila-Syuhada *et al.* (2016) [57] who found 15% resistance in Penang, Malaysia. Higher resistance to cefoxitin by the isolates of *S. aureus* compare to the present finding was observed in Tigabu *et al.* (2015) [64], Özdemir and Keyvan *et al.* (2016) [47], Ammar *et al.* (2016) [5], and Elemo *et al.* (2017) [20] who have reported 53.2%, 22.8%, 55%, and 58.1% of resistance respectively. In contrast to the present findings lower resistance to cefoxitin by *S. aureus* was observed by Mashouf *et al.* (2015) [41] and Can *et al.* (2017) [12] who have observed 5.1% and 9.09% of resistance respectively.

In the present investigation resistance to tetracycline was found to be a 7.8% among the isolates of *S.auereus* which was in agreement with the report of Shamila-Syuhada *et al.* (2016) [57] who found 5% resistance to tetracycline among the isolates of *S. aureus* in Penang, Malaysia. Higher resistance compared to the present finding was observed for tetracycline resistance among the isolates of *S. aureus* in the findings of Mirzaei *et al.* (2012) [44], Mubarack *et al.* (2012) [46], Jackson *et al.* (2013) [33], and Feng *et al.* (2016) [24] who have reported 23%, 11.84%, 25%, and 15.91% of resistance respectively. Only 1% of resistance for tetracycline was observed among the isolates of *S. aureus* in the findings of Wang *et al.* (2018) [68] which was found to be lower than the findings of the present investigation.

Resistance to gentamicin in the present study by the isolates of *S. aureus* was 27.2% which was in agreement with the reports of Momtaz *et al.* (2013) [45] and Mashouf *et al.* (2015) [41] who found 29.26% and 27.6% of resistance in the isolates of *S. aureus* respectively. Higher resistance compared to the present findings was observed by Jackson *et al.* (2013) [33] who reported 33.3% resistance in Georgia. Lower resistance to gentamycin compared to the present findings was observed by Thaker *et al.* (2013) [62], Tigabu *et al.* (2015) [64], Feng *et al.* (2016) [24] and Wang *et al.* (2018) [68] who have reported 10%, 2.8%, 9.09%, and 1% respectively.

The isolates of *S. aureus* in present study have shown 21% of resistance to erythromycin which was in agreement with the report of Feng *et al.* (2016) [24] who found 20.45% resistance in Northwest China.Higher resistance to erythromycin by the isolates of *S. aureus* compared to the present finding was observed in Mashouf *et al.* (2015) [41] and Fawzy *et al.* (2017) [23] who reported 30.6% and 26.3% resistance respectively. Lower resistance to erythromycin compare to the present finding was observed by Hanson *et al.* (2011) [28], Tigabu *et al.* (2015) [64], Can *et al.* (2017) [12], Hoque *et al.* (2018) [31] and Wang *et al.* (2018) [68] who have observed 14.8%, 4.6%, 16%, 8.2% and 5.2% of resistance respectively.

Resistance to cotrimoxazole was found to be a 5.8% by the isolates of *S. aureus* in the present study which was in agreement with the report of Tigabu *et al.* (2015) [64] who found 4.6% resistance among the isolates of *S. aureus* in central highlands of Ethiopia. Higher resistance compared to the present finding towards the cotrimoxazole by the isolates of *S. aureus* was observed in the findings of Mashouf *et al.* (2015) [41] and Pourtaghi *et al.* (2015) [51] who have reported 14.3% and 11.18% resistance respectively in Iran. Lower resistance to cotrimoxazole than the present findings was observed by Mirzaei *et al.* (2012) [44], Özdemir and Keyvan *et al.* (2016) [47] and Ammar *et al.* (2016) [5] who have observed 3%, 3.5%, and 2.5% resistance respectively.

The isolates of *S. aureus* have shown resistance to ciprofloxacin and it was found as 11.5% which was in agreement with the report of Ammar *et al.* (2016) [5] and Awad *et al.* (2016) [10] who have observed 10% and 14.3% resistance in the isolates of *S. aureus* in Egypt. Higher resistance compare to the present finding was observed by Kumar *et al.* (2011) [37], Kreausukon *et al.* (2012) [36], Pati *et al.* (2016) [50], Wang *et al.* (2018) [68] and Yadav, (2018) [69] who have reported 26.2%, 24.5%, 37%, and 18.8% and 42.1%

of resistance respectively among the isolates of *S. aureus*. Lower resistance to ciprofloxacin compared to the present findings was observed in the findings of Tigabu *et al.* (2015) [64] who reported 3.7% of resistance in his study.

Resistance to chloramphenicol by the isolates of *S. aureus* in the present investigation was found only 0.5% which was in agreement with the report of Wang *et al.* (2018) [68] who found 1% resistance in his isolates of *S. aureus* in Beijing, China. Higher resistance compared to the present finding was observed in the findings of Ammar *et al.* (2016) [5], Pati *et al.* (2016) [50] and Özdemir and Keyvan *et al.* (2016) [47] who have reported 12.5%, 9% and 5.2% resistance respectively. No resistant isolates for chloromphenicol were observed in the findings of Abera *et al.* (2010) [2], Dittaman *et al.* (2017) [18], Elemo *et al.* (2017) [20]. Due to reduced usage of chloramphenicol in the field condition, negligible amount of resistance was observed in the present study.

Although it is extremely difficult to explain these conflicting data with regards to both time and place of study, the variation is probably due to differential clonal expression and drug pressure in community.

Several workers have used PCR with varied success for detection of MRSA from clinical samples using specific gene primers for targeting. Of the specific gene sequences *nuc* and *mecA* genes have been most frequently targeted for PCR based detection of *staphylococcus* and its methicillin resistance respectively. The *nuc* gene has been designated as species specific gene for *Staphylococcus aureus*, because *S. aureus* strains produce an extra cellular thermostable nuclease (thermonuclease, TNase) with a frequency similar to that as which they produce coagulase enzyme (Madison *et al.*, 1993). The TNase protein has been well characterized and its gene the *nuc* gene has been cloned and sequenced (Tucker *et al.*, 1978) [66]. Thus the *nuc* gene which encodes the thermostable nuclease that is highly specific for *S. aureus* (Saha *et al.*, 2008).

The isolates positive for *S. aureus* by culturing and biochemical tests were further confirmed by PCR targeting for species specific gene of *S. aureus* (*nuc* gene) (figure 4). *nuc* gene codes for thermostable nuclease (TNase) which is specific for *S. aureus* (Bharathy *et al.*, 2015) [11].

In the present study, out of 400 samples screened, 47.75% (191/400) *S. aureus* isolates were positive for *nuc* gene. This was nearly in agreement with findings of Medvedová *et al.* (2014) [43] and Awad *et al.* (2017) [10] who found 44% and 42% of the isolates were *S. aureus* respectively. Higher incidence compared to the present findings was reported by Cremonesi *et al.* (2007) [16], Falaki and Mahdavi *et al.* (2017) [22], Pati *et al.* (2016) [50] and Bharathy *et al.* (2015) [11] who have found 100%, 85%, 83.8% and 65.57% of *S. aureus* isolates from their study and were characterized by *nuc* gene. Lower incidence of *nuc* gene compared to the present findings was reported by Tanzin *et al.* (2016) [60], Srednik *et al.* (2018) [58] and Mashouf et al. (2015) [41] who have reported 35.29%, 28.1% and 23.52% of *S. aureus* isolates were *nuc* gene respectively.

The *mecA* gene has been designated as the gene for methicillin resistance of *S. aureus*, because MRSA produce a novel penicillin binding protein (PBP) in addition to the usual PBPs. This is the primary mechanism of *staphylococcal* methicillin resistance and is referred to as intrinsic resistance (Hartman *et al.*, 1984) [19]. PBP2a has a low affinity for β-lactam antibiotics and is thought to function in their presence to confer resistance to the bacteria. MRCoNS also become resistant by acquisition of PBP2a encoding gene *mecA* (Chambers, 1987) [13]. *mecA* is a chromosomally derived gene that has been cloned and sequenced (Matsuhasi, *et al.*, 1986) [42]. It has a very high level of homology in MRSA and MRCoNS and is absent from methicillin susceptible staphylococci isolates (Predari *et al.*, 1991) [52]. Additionally, the *mecA* gene is virtually identical in all staphylococcal strains and thus is a useful molecular marker of methicillin resistance (Archer *et al.*, 1994) [8].

In the present study, for all the cefoxitin resistant *S. aureus* isolates PCR assay was conducted for targeting *mecA* gene. Out of 191 *S. aureus* isolates, 15.2% isolates were positive for *mecA* gene (figure 5). Almost similar findings were recorded by Al-Ruaily and Khalil *et al.* (2011) [4], Hamid *et al.* (2017) [27] who have found 15% and 16.6% of isolates have shown *mecA* gene respectively. Higher incidence compared to the present finding was observed by Ullah *et al.* (2012) [67], Mirzaei *et al.* (2012) [44], Ganai *et al.* (2016) [26], Al-Ashmawy *et al.* (2016), Ammar *et al.* (2016) [5] and Hoque *et al.* (2018) [31] and who have observed 46%, 28.99%, 44.1%, 53%, 19.8% and 20% of *mecA* gene in their isolates respectively. While lower percentage of *mecA* gene compared to the present finding was observed by Lee et al. (2003) [38], Kumar *et al.* (2011) [37], Suleiman *et al.* (2012) [59], Kreausukon *et al.* (2012) [36], Ektik *et al.* (2017) [19] and Wang et al. (2018) [68] who have reported that 3.6%,13.1%, 7.6%, 4.4%, 5.88% and 1.4% of their isolates have shown *mecA* gene respectively.

Antibiogram Representation by Graphs

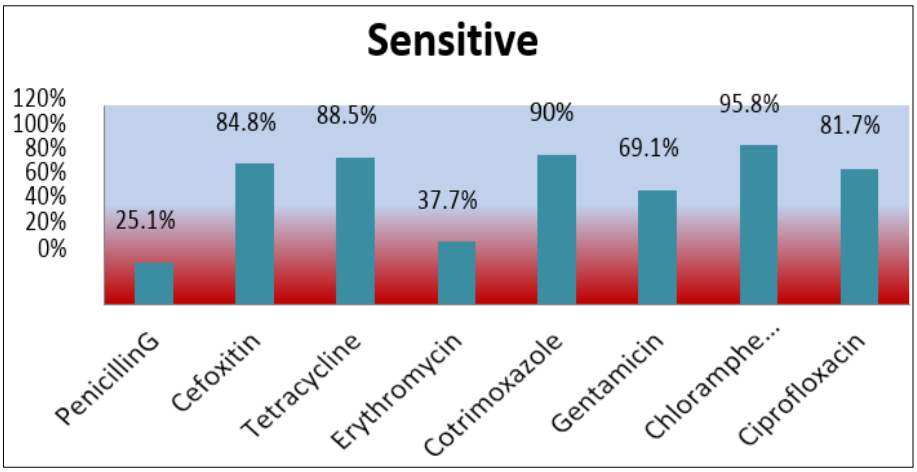

Fig 1: *S. aureus* showing sensitivity patterns to different antibiotics

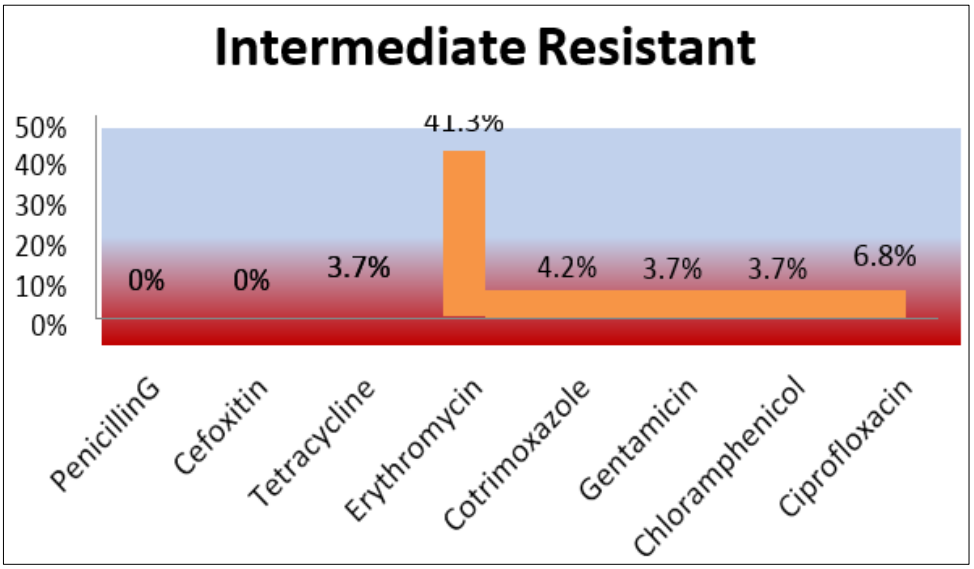

Fig 2: *S. aureus* showing intermediate resistance patterns to different antibiotics

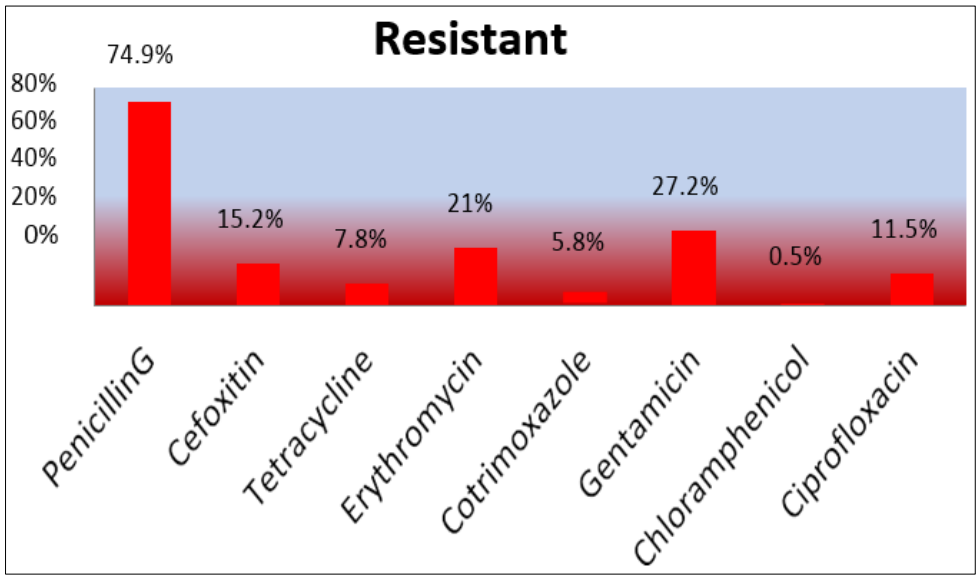

Fig 3: *S. aureus* showing resistance patterns to different antibiotics

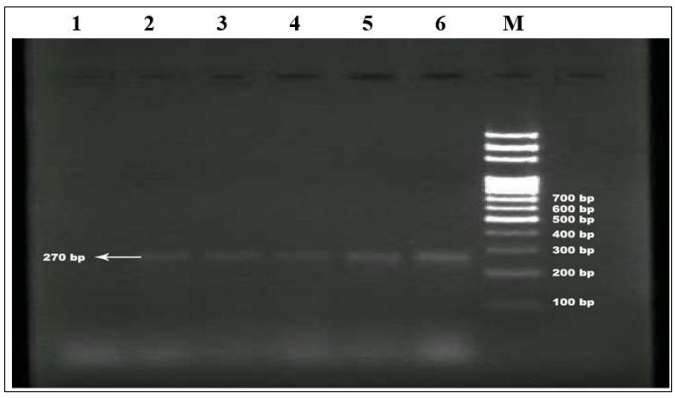

Fig 4: *S. aureus* isolates showing *nuc* gene

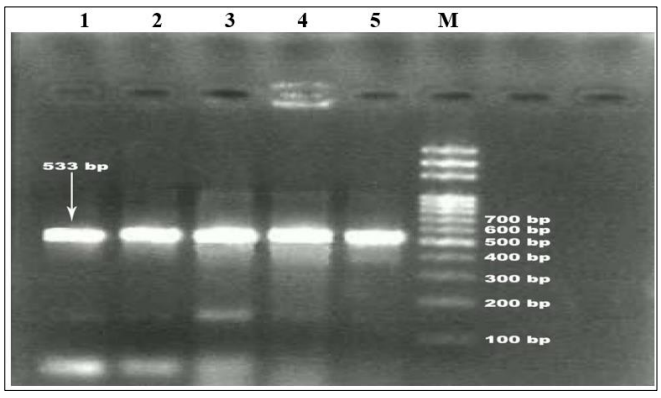

Fig 5: *S. aureus* isolates showing *mecA* gene

Lane M	Molecular marker(100bp)
Lane 2, 3, 4, 5 and 6	*S. aureus* isolates carrying *nuc* gene(270bp)

Lane M	Molecular marker(100bp)
Lane 1, 2, 3, 4 and 5	*S. aureus* isolates carrying *mecA* gene(533bp)

Conclusion

The present investigation revealed that there is an increased trend in the presence of antibiotic resistant *Staphylococcus aureus* in the milk samples collected from the vendors. As the antimicrobial resistant microflora is having greatest public health threat, necessary precautions have to be taken with regarding the administration of antibiotics through feed and indiscriminate use of antibiotics in the treatment should also be curtailed. Bringing awareness among the public about the harmful effects of multi drug resistant microflora is another important objective for the scientists to protect the humans from these super bugs.3

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