DR-2203
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RESEARCH ARTICLE Asian Journal of Dairy and Food Research

Resilient *Staphylococcus aureus* in Mastitis Milk: Insights into Antibiotic Resistance and Biofilm Formation

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10.18805/ajdfr.DR-2203

ABSTRACT

Background: *Staphylococcus aureus* is capable of producing a wide range of virulence factors. It is a deadly bacterium that is resistant to the majority of antibiotics that are typically provided. The ability of *S. aureus* to establish chronic, implant associated infections and our inability to cure them, is directly associated with its biofilm formation, creating an environment where bacteria can grow and persist while protected from the host immune response and antibiotic therapy. Antibiotic resistance of *S. aureus* is widely spreading with low cure rate.

Methods: A total of 62 *S. aureus* strains were isolated from animal pus, human pus, animal skin, human skin, mastitic milk, normal milk and unprocessed meat samples. The isolates were genotypically confirmed by 23S rRNA ribotyping for *S. aureus*. Using 14 antibiotics belonging to different generations were tested for their resistance pattern from various sources.

Result: All the mastitic milk, animal pus, human pus and unprocessed meat *S. aureus* isolates from all the places of sampling showed 100% sensitivity towards imipenem while isolates obtained from mastitic milk, human skin, animal skin showed 100% resistance towards ampicillin and ampicillin+sulbactum combination.

Key words: Biofilm, Imipenem, Mastitis Milk, Meat, *Staphylococcus aureus.*

INTRODUCTION

Staphylococcus aureus is a spherical, gram positive, non-motile, non-spore forming, facultative anaerobic bacterium causing the wide range of nosocomial infections (Nourbakhsh *et al*., 2016). It continues to be a dangerous pathogen for both community-associated and hospital-related infections. It is one of the most important human pathogens that contribute in variety of infections like skin, soft tissue, respiratory, bone, joint and endovascular infections including life-threatening cases of bacteremia, endocarditis and sepsis with substantial rates of morbidity and mortality (Engemann *et al*., 2003). Foodstuff contamination may occur directly from infected food-producing animals or use of unprocessed meat and it may result from poor hygiene during production processes, or the retail and storage of food, since humans may also harbour microorganisms. They have been implicated as potential sources for the transmission of the pathogen to humans (Normanno *et al*., 2007).

In healthy animals, *S. aureus* can colonize the skin, nasopharynx and the intestinal tract without any clinical findings and in some cases considered as a secondary invader for the wound. *Staphylococcus aureus* is reported to be one of the most common causative agents of food poisoning associated with the consumption of raw milk and milk products (Spanu *et al*., 2012). Beside that they have been implicated as potential sources for the transmission of the pathogen to humans (Normanno *et al*., 2007; Huong *et al*., 2010). Likewise, *S. aureus* is the most common cause of contagious mastitis and probably the most lethal agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured (Miles, 1992). The milk industry recognizes the importance of mastitis, which is considered the most common and expensive disease in dairy livestock, as well as the need for adopting methods for controlling and preventing the disease (Sinha, 2014).

To tackle *S. aureus* mediated infections, antibiotics are used by veterinary professionals but their therapeutic outcomes are nearly insignificant because of the stubborn nature of the pathogen (Li *et al*., 2009). Beside pathological factors, the resistance shown by this organism toward antibiotic is also a serious concern. Extensive and indiscriminate use of antibiotics have led to development of antibiotic resistance is this organism. This organism is

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How to cite this article: Nirwan, A., Khan, S., Kumari, M., Mistry, B. and Kataria, A.K. (2024). Resilient *Staphylococcus aureus* in Mastitis Milk: Insights into Antibiotic Resistance and Biofilm Formation. Asian Journal of Dairy and Food Research. DOI: 10.18805/ajdfr.DR-2203.

Submitted: 10-02-2024 **Accepted:** 17-04-2024 **Online:** 03-07-2024

also known to show multiple drug resistance (Lyon and Skurray, 1987) and showing antibiotic resistance throughout world by *S. aureus* isolates from various sources (Szweda *et al*., 2014). So keep these all aspects the objective of this purposed study was to examine multi drug antibiotic resistance or susceptibility of *S. aureus* isolated from different sources.

MATERIALS AND METHODS

Sample collection

A total of 180 samples were collected from various sources (Table 1) *viz* pus and skin of humans and animals, mastitic milk, normal milk and raw meat samples from different places in and around Bikaner (Rajasthan). Sterilized test tubes were used for sample collection and immediately transferred to the laboratory on ice for further processing.

Genotypic confirmation of *S. aureus*

Bacterial DNA isolation for PCR was done according to the method described with some modification. The genotypic confirmation of *S. aureus* based on 23S rRNA was carried out as per the described method Cowan and Steel (1974) and Quinn *et al.* (1994) by using following sequences for two primers, F-5-ACGGAGTTACAAA GGACGAC-3 and R-5-AGCTCAGCCTTAACGAGTAC-3. The reaction was carried out for 25 µL of the final volume of PCR. The volume of isolated DNA used was 3ìL. The primers were used at a concentration of 2 pmol each. The Thermo scientific DreamTaq™ Green PCR Master Mix (2X) was used for PCR. The PCR cycle included initial denaturation at 95°C for 1 min followed by 30 cycles of three steps (denaturation at 94°C for 90s, annealing at 55°C for 90s and extension at 72°C for 75s) and final extension at 72°C for 10 min. The amplify PCR product of 23S rRNA was analyzed by electrophoresis on 0.8% agarose gel, respectively.

Antibiogram study

To determine the antibiogram of the isolates against 14 different antibiotics the method of Bauer (1966) was followed (Table 2). In brief, the isolates were inoculated in sterile 5 ml nutrient broth tube, incubated for 18 h at 37°C and then the opacity was adjusted to 0.5 McFarland opacity standards (Quinn *et al*., 2000). The inoculum was well spread over the agar surface with the help of sterilized swab. Plates were allowed to dry for 10 min at 37°C and then antibiotic discs were carefully placed on the surface with enough space around each disc for diffusion of the antibiotic. Plates were incubated for 24 h at 37°C and the zone of inhibition of growth of the organism around each disc was measured in millimeters and compared with standard chart provided by disc manufacturer.

RESULTS AND DISCUSSION

Genotypic analysis

In this study, *S. aureus* could be identified by conventional methods in the present investigation but the genotyping with a PCR based method involving specific primer targeted against 23S rRNA gene revealed an amplicon of 1250 bp (Fig 1). Out of 180, 62 isolates confirmed to be *S. aureus* with 34.44% recovery rate. This method was demonstrated by Straub *et al*. (1999) and has been used by various researchers Sanjiv *et al.* (2008); Upadhyay and Kataria (2009); Khichar *et al.* (2012); Nathawat *et al.* (2015); Yadav *et al.* (2015b); Choudhary *et al.* (2018) from the same laboratory during the previous years for the identification of *S. aureus* from different sources (Table 3).

Antibiogram analysis

In the present investigation all the 62 *S. aureus* isolates were subjected to antibiogram studies using 14 antibiotics belonging to different categories and generations wherein wide variations were recorded in resistance patterns (Tables 4). All *S. aureus* isolates were most susceptible to five of 14 antimicrobials tested. Less resistance was observed against imipenem, cefotaxime, gentamicin, cefazolin and methicillin. One antibiotic namely imipenem was found 100% effective against all the isolates. Antibiogram of S. aureus isolates obtained from various sources on Mueller-Hinton agar (Fig 2).

In the present study all the mastitic milk (n=12), animal pus (n=9), human pus (n=8) and unprocessed meat *S. aureus* isolates (n=9) from all the places of sampling showed 100% sensitivity towards imipenem antibiotics (Fig 3, 4, 5 and 6). While except for imipenam the other five antibiotics which were cefepime, cefzolin and gentamicin found to be 100% effective against isolates from animal

Table 1: Detail of *S. aureus* isolates from various sources and places of sampling.

Sample	No.of samples	Place of sampling
Pus of human	25	PBM Hospital OPD center, Fort dispensarySPMC microbiology department
Pus of animal	30	TVCC, CVAS, Bikaner, Polyclinic, Bikaner
Skin of human	15	Apex centre students, RAJUVAS, From shopkeepers of sweet shops, From family member, CVAS clinic Bikaner
Skin of animal	21	Street dog, cow, Pets, Untreated animals of CVAS clinic
Mastitis milk	27	CVAS clinic, Polyclinic Bikaner, LRS, Kodemdesar, Laxminath ji dairy farm, Beechwal dairy farm
Normal milk	40	Local daries, Pugal Road dairy farm, Udasar dairy farm, milk supplied by vendors
Unprocessed meat	22	Kote gate slaughter house, Jaipur Road slaughter house
Total	180	

skin (n=7) samples (Fig 7). All the nomal milk (n=10) and human skin (n=7) isolates were found 100% sensitive toward impenam and gentamicin (Fig 8, 9).

Mastitic milk, human skin, animal skin isolates showed 100% resistance towards ampicillin and ampicillin+ sulbactum. While normal milk, animal pus, human pus and unprocessed meat isolates were 100% resistant to only ampicillin antibiotic as displayed in Table 4 and Fig 4, 5, 6 and 9. Mastitic milk isolates showed least resistance towards imipenam and vanomycin as represented in figure 3 while isolates from normal milk showed least resistance towards cefotazime, imipenam, methicillin and gentamicin,

Table 2: List of antibiotics used for antibiogram study against *S. aureus* isolates obtained from various sources.

Antibiotic/Symbol (interpretation zone in mm- R/I/S)	Disc content (mcg)
Ampicillin/AMP^{10} (28/-/29)	10
Ampicillin+sublactum/A/S$^{10/10}$(11/12-14/15)	10/10
Cefzolin/CZ30 (14/15-17/18	30
Cefaclor/ CF^{30} (14/15-17/18)	30
Cefotaxime + Clavulanic AcidCEC30/10 18/-/22	30/10
Cefepime/CPM^{30} (14/15-17/18)	30
Cefotaxime/CTX^{30} (14/15-22/23)	30
Ciprofloxacin/CIP5 (15/16-20/21)	05
Cloxacillin/COX10 (23/24-33/34	10
Gentamicin/ HLG120 (12/13-14/15	120
Imipenem/ IPM10 (13/14-15/16)	10
Levofloxacin / LE5 (15/16-18/19)	05
Methicillin/MET^{5} (9/10-13/14)	05
Vancomycin/ VA30 (14/15-16/17)	30

Table 3: Detail of recovery of *S. aureus* isolates from various sources.

Source	No. of samples	Total isolates	Recovery (%)
Mastitis milk	25	12	48%
Normal milk	30	10	33.33%
Animal pus	15	9	60%
Human pus	21	8	38.09%
Skin of animal	27	7	25.92%
Skin of human	40	7	17.5%
Unprocessed meat	22	9	40.90%
Total	180	62	34.44%

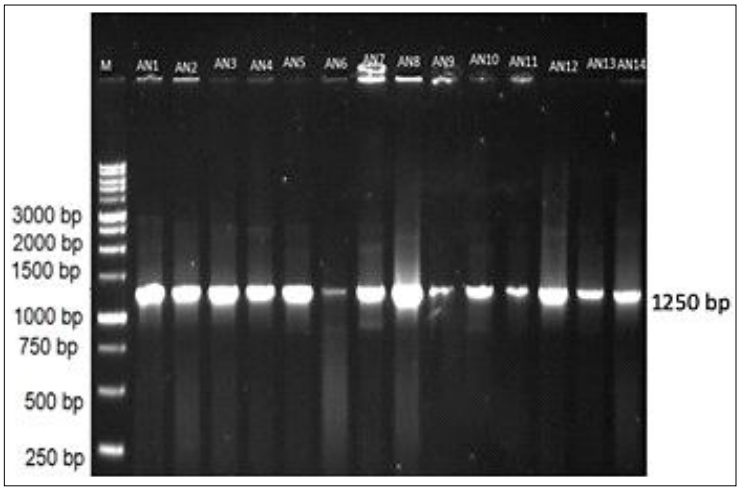

Fig 1: Agarose gel electrophoresis of PCR product of 23S rRNA ribotyping of *S. aureus* isolates; M- Molecular marker (1250 bp); AN1- AN14: Isolates from various sources.

from animal pus isolates cloxacillin, imipenam, from human pus isolates cefaclor, cefotaxime and imipenam, from human skin isolates cefotaxime, gentamicin and imipenam ,from unprocessed meat isolates cefepime, cefzolin and imipenam as displayed in Fig 9.

Fig 2: Antibiogram study of *S. aureus* isolates obtained from various sources on mueller-hinton agar plate.

The present study is in agreement to that of Sanjiv and Kataria (2006) who recorded 13 antibiotics effective against 90% or more isolates with similar sensitivity pattern. The resistant pattern recorded was 66% for oxytetracycline and 49% for penicillin.

Kumar *et al*. (2011) reported 100% isolates from bovine mastitis susceptible to vancomycin, 81.3% to ofloxacin and clindamycin as observed in present study but unlike the present study highest resistance was observed to streptomycin (36.4%), oxytetracycline (33.6%) gentamicin and ampicillin (29.9%), penicillin-G (28.9%), chloramphenicol and ciprofloxacin (26.2%). Similar to present findings Suleiman *et al.* (2012) recorded 100% isolates from subclinical mastitis susceptible to vancomycin.

Hussain *et al*. (2012) reported higher sensitivity of isolates of *S. aureus* from mastitis towards oxytetracycline (95.65%), amoxicillin (86.95%), ampicillin (82.60%), ciprofloxacin (82.60%) and chloromphenicol (82.60%) and lower sensitivity towards enrofloxacin (69.56%) and gentamicin (86.95%) as compared to present study.

Present study observed a high level of resistance to beta lactam antibiotics such as ampicillin. Similar results were reported by Schmidt (2011) with 47.8 % of the isolates

Table 4: Antibiotic resistance activity exhibited by *S. aureus* isolates obtained from various sources.

Source name	Mastitis milk (12)		Normal milk (10)		Pus of human (9)		Pus of animal (8)		Skin of animal (7)		Skin of human (7)		Unprocessed meat (9)	
AMP	S	0	S	0	S	0	S	0	S	0	S	0	S	0
	R	12	R	10	R	8	R	9	R	7	R	7	R	9
AS	S	0	S	0	S	0	S	0	S	0	S	0	S	0
	R	12	R	9	R	7	R	7	R	7	R	7	R	8
CF	S	9	S	6	S	7	S	6	S	4	S	5	S	7
	R	1	R	2	R	0	R	2	R	1	R	1	R	1
CPM	S	9	S	8	S	5	S	6	S	7	S	6	S	8
	R	2	R	1	R	2	R	2	R	0	R	1	R	0
CEC	S	9	S	9	S	6	S	8	S	6	S	6	S	7
	R	3	R	1	R	2	R	1	R	1	R	1	R	2
CTX	S	5	S	9	S	6	S	4	S	5	S	4	S	3
	R	1	R	0	R	0	R	2	R	0	R	0	R	2
CZ	S	9	S	8	S	5	S	6	S	7	S	6	S	8
	R	2	R	1	R	2	R	2	R	0	R	1	R	0
CIP	S	2	S	0	S	1	S	2	S	1	S	0	S	1
	R	7	R	9	R	5	R	3	R	4	R	5	R	5
COX	S	0	S	0	S	0	S	0	S	0	S	0	S	0
	R	2	R	2	R	1	R	0	R	3	R	2	R	3
GEN	S	10	S	10	S	7	S	8	S	7	S	7	S	8
	R	2	R	0	R	1	R	1	R	0	R	0	R	1
IPM	S	12	S	10	S	8	S	9	S	7	S	7	S	9
	R	0	R	0	R	0	R	0	R	0	R	0	R	0
LE	S	2	S	3	S	1	S	5	S	2	S	2	S	2
	R	3	R	3	R	5	R	1	R	2	R	2	R	2
MET	S	9	S	9	S	5	S	6	S	6	S	5	S	7
	R	1	R	0	R	1	R	2	R	0	R	1	R	1
VA	S	0	S	0	S	0	S	0	S	0	S	0	S	0
	R	0	R	6	R	2	R	5	R	4	R	2	R	3

resistant to penicillin and 65.6% resistant to ampicillin. Nam *et al*. (2011) found over 66% of the *S. aureus* isolates from bovine mastitic milk resistant to penicillin and Memon *et al.* (2013) reported 91% *S. aureus* isolates from bovine mastitis resistant to ampicillin. Xu *et al.* (2015), Wang *et al*. (2015) and Parth *et al*. (2016) reported 82.1%, 90.4% and

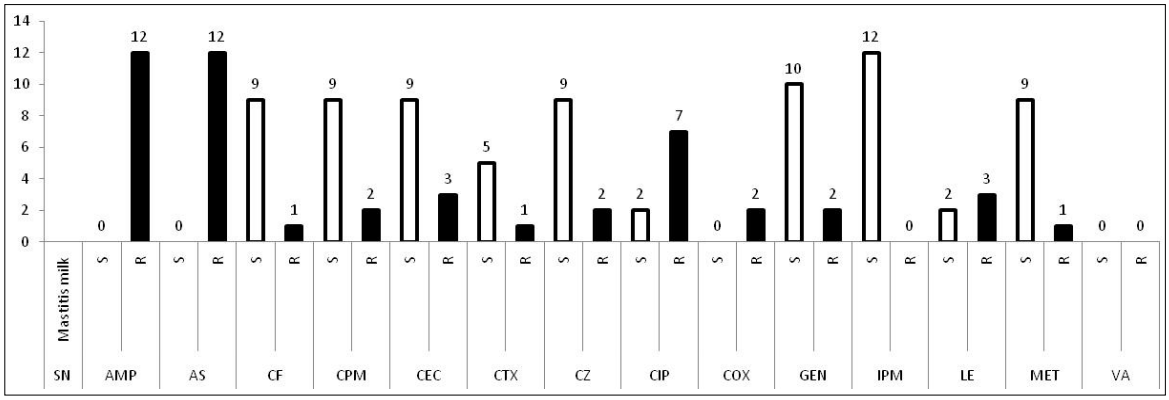

Fig 3: Multi drug resistance pattern of *S. aureus* isolates from mastitis milk.

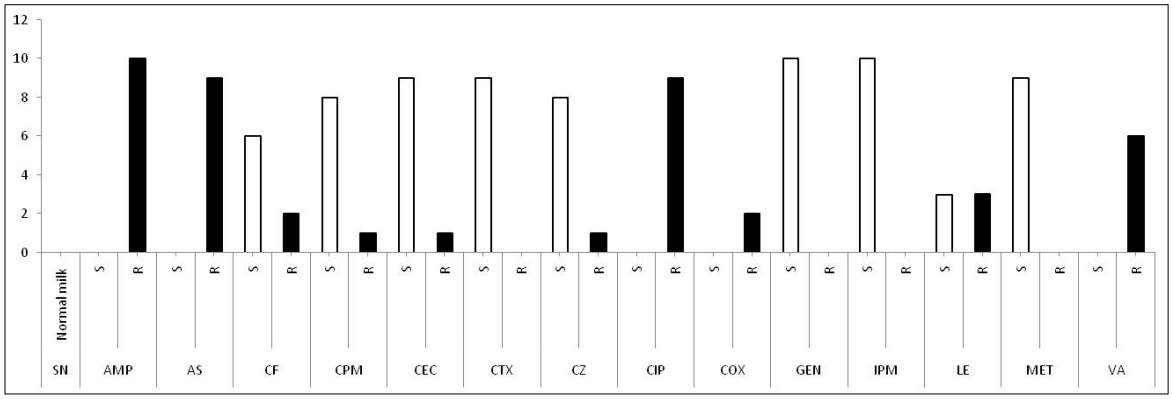

Fig 4: Multi drug resistance pattern of *S. aureus* isolates from normal milk.

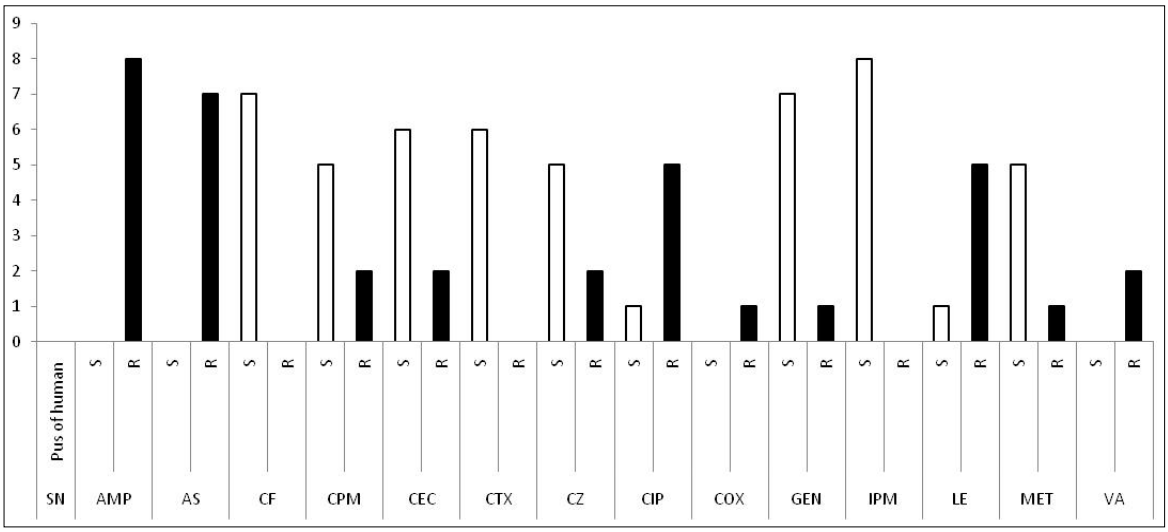

Fig 5: Multi drug resistance pattern of *S. aureus* isolates from pus of human.

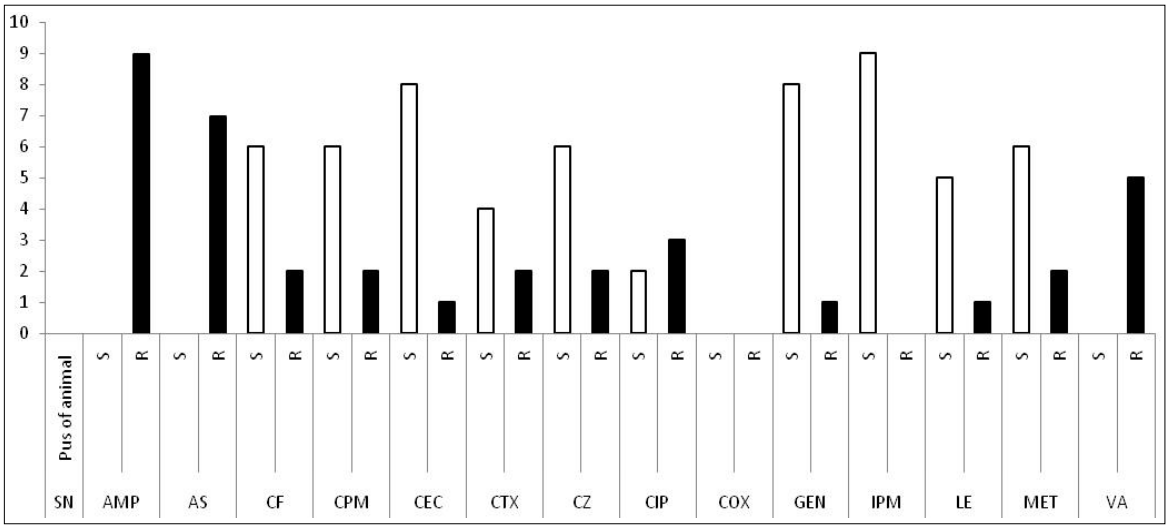

Fig 6: Multi drug resistance pattern of *S. aureus* isolates from pus of animal.

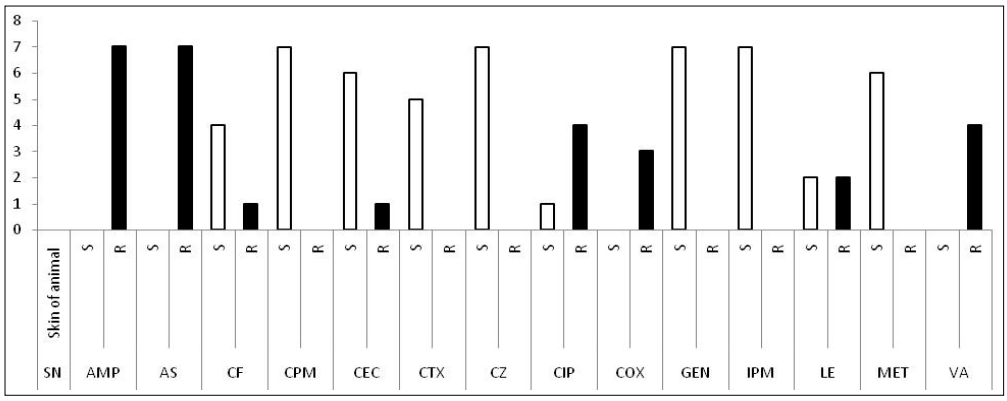

Fig 7: Multi drug resistance pattern of *S. aureus* isolates from skin of animal.

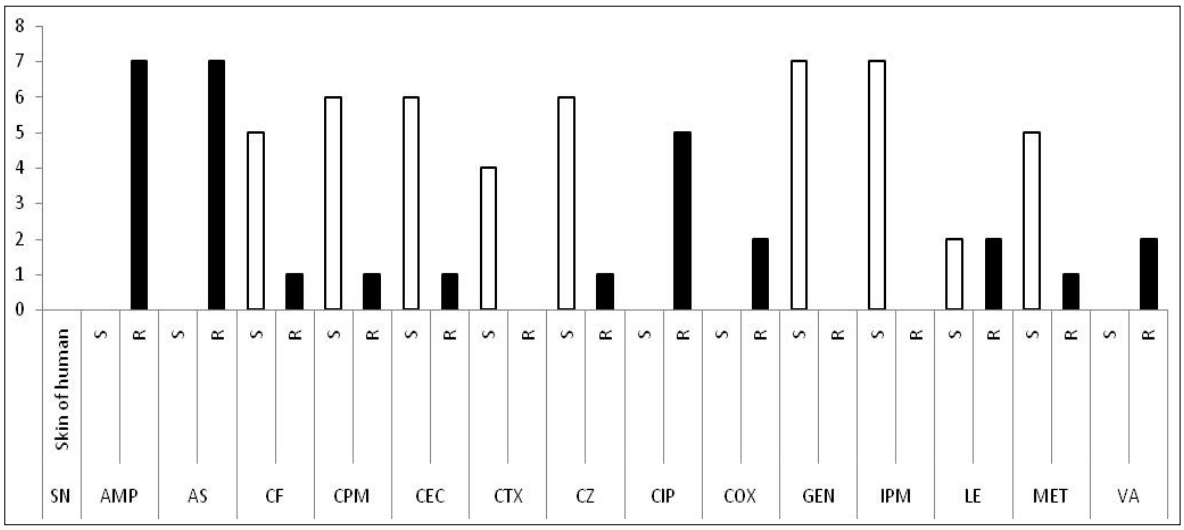

Fig 8: Multi drug resistance pattern of *S. aureus* isolates from skin of human.

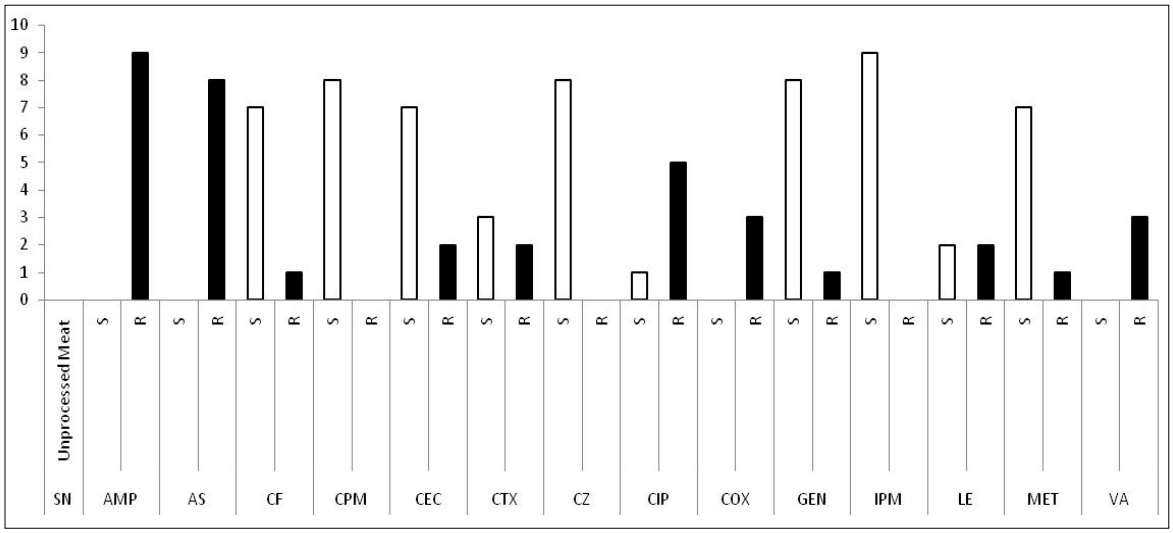

Fig 9: Multi drug resistance pattern of *S. aureus* isolates from unprocessed meat.

94.34% isolates from bovine mastitis resistant to penicillin respectively. In contrast to these findings, Corti *et al*. (2003) recorded low resistance of isolates towards penicillin.

Contrary to present findings of low resistance towards oxytetracycline (6.5%), some workers observed higher resistant rate to tetracycline *i.e*. Mekuria *et al*. (2013) 66.7%; Memon *et al.* (2013) 59% Jamali *et al*. (2014) 76.7%; Wang *et al*. (2015) 74.4%; Tassew *et al*. (2017) 63.41% and Hoque *et al*. (2018) 74.5%. Other workers have also reported similar intermediate sensitivity to streptomycin *i.e.* 30% by Thaker *et al.* (2013) and 33.84% by Shichibi *et al*. (2017). High sensitivity to gentamicin and vancomycin similar to present study has been reported by Thaker *et al.* (2013) and Shichibi *et al*. (2017).

In the present study gentamicin, methicillin, oxacillin and vancomycin were found to be highly effective against majority of the *S. aureus* isolates from different sources and places of sampling which is contrary to the study of other workers from other parts of the world. Mekuria *et al*. (2013) reported resistance to oxacillin (33.3%), gentamicin (19.6%) and vancomycin (3.9%) in *S. aureus* isolates from milk samples of dairy cows and nasal swabs of farm workers. Xu *et al.* (2015) recorded resistance to gentamicin (32.1%) in *S. aureus* isolates from mastitis milk samples. Similarly, Sharma *et al.* (2015) observed resistance to vancomycin (88.89%), methicillin (66.67%) and gentamicin (22.2%) which is in contrast to present results.

Resistance pattern to gentamicin (59.37%) and vancomycin (56.75%) was seen by Tassew *et al*. (2017); to oxacillin (56.4%) by Elemo *et al*. (2017) and to oxacillin (55.9%) and gentamicin (17.9%) by Hoque *et al*. (2018).

Similar antibiogram was reported by Yadav *et al.* (2015a) from present area of study for 32 *S. aureus* isolates from bovine mastitis against 33 antibiotics belonging to different categories and generations. Antibiotics such as gentamicin, methicillin and tobramycin were more effective against all isolates.

CONCLUSION

In conclusion, because of the use of different antimicrobial agents, methods, sample sources, number of isolates, as well as isolates obtained from different geographical locations, it is difficult to determine the prevalence of antimicrobial resistance of *S. aureus* from various sources cases based on a simple comparison. Different geographical regions may have different *S. aureus* strains. As a result, comparisons between studies from different countries may not be of great value. Hence it is recommended to record drug sensitivity and resistance patterns for different classes of antibiotics frequently used in a given geographical area against *S. aureus* associated cases. This may help in understanding and tracking the development of multidrug resistance in *S. aureus* strains in a particular geographical area.

ACKNOWLEDGEMENT

Authors duly acknowledge Head of Department of Veterinary Microbiology and Animal Biotechnology, College of Veterinary and Animal Science, Bikaner (CVAS, Bikaner) for providing facilities for carrying out the research work.

Conflict of interest

There is no competent conflict between authors.

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