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Research Article

Antimicrobial Resistance Pattern of *Staphylococcus aureus* and *Streptococcus uberis* Causing Mastitis

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Abstract

It is very important to study and understand the antimicrobial resistance pattern of these infectious agents in order to provide best treatment regime. The present study is on antimicrobial resistance pattern of S. aureus and S. uberis causing mastitis. Disc diffusion test of S. aureus isolates showed that 69.35% of isolates were resistant to Penicillin G, followed by Ampicillin [50%], Erythromycin [30.64%], Methicillin [29.03%], Clindamycin [24.19%], Amoxycillin/Clavulanic acid [20.96%], Streptomycin [20.96%] and least resistance was shown to Gentamicin, Cotrimoxazole, Chloramphenicol, Tetracycline and Vancomycin. MRSA and MSSA has shown a great variation in antibiotic resistance pattern, MRSA was 2 to 4 times more resistant to all tested antibiotics. MRSA showed a high resistance to Penicillin G, Ampicillin, Clindamycin and Streptomycin. All MRSA isolates were biofilm producers which may be favouring to the increased antimicrobial resistance. All isolates were carrying blaZ gene and all except one isolate was carrying mecA gene. In vitro antibiotic sensitivity test of S. uberis revealed that 59% of the isolates were resistant to Ceftriaxone followed by Streptomycin, Erythromycin, Penicillin G, Tetracycline, Amoxycillin clavulanic acid, Enrofloxacin and most of the isolates were sensitive to Gentamicin, Chloramphenicol and Ampicillin/Sulbactam. Non biofilm forming S. uberis isolates were sensitive to Tetracycline, Chloramphenicol, Ampicillin/Sulbactam, Amoxycillin/Clavulanic acid, Gentamicin and Enrofloxacin whereas biofilm formers were resistant to these antibiotics by 30.76%, 30.76%, 2.56%, 25.64%, 15.38% and 25.64% respectively.

Keywords: Antimicrobial Resistance; S, aureus; S, uberis; Antibiotic Sensitivity Test; Methicillin Resistant S, aureus; Biofilm and Antimicrobial Resistance

Abbreviations

BHI Broth: Brain Heart Infusion Broth; MRSA: Methicillin Sensitive *Staphylococcus aureus;* MSA: Mannitol Salt Agar; MSSA: Methicillin Sensitive *Staphylococcus aureus;* PCR: Polymerase Chain Reaction; *S. aureus: Staphylococcus aureus;* SSB: Streptococcus Selective Broth; *S. uberis: Streptococcus uberis*

Introduction

Bovine mastitis is the foremost endemic infectious disease of dairy cattle worldwide, as well as in our country. It is the inflammation of the mammary gland, can be clinical or subclinical and caused by physical or chemical agents. Depending on the primary reservoir bacteria responsible, mastitis can be classified as environmental (*Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, and *Streptococcus uberis*) or contagious (*Staphylococcus aureus* and *Streptococcus agalactiae*) [1]. The most frequently isolated bacterial pathogen from bovine intramammary infections was identified to be *S. aureus* followed by *Streptococcus* species and Coliforms [2].

The common modes of treating mastitis are by administration of antibiotics, such as streptomycin, ampicillin, cloxacillin, penicillin, and tetracycline through intramammary infusion or parenteral

routes [3]. The clinical management of mastitis has become a concern to the veterinarians, as the conventional antibacterial therapy through intramammary route is largely associated with failures [4]. The success in treatment of mastitis relies on the antimicrobial susceptibility of the pathogens, the treatment regimen, the cattle breed, and the type of mastitis [5]. As resistance profiles are often herd specific, tackling drug resistance is a serious challenge for mastitis control [6]. Antimicrobial resistance is usually associated with the improper use of antimicrobial agents and it is important to monitor the antimicrobial sensitivity of mastitis pathogens [7]. Antibiotic therapy is becoming ineffective in the treatment of mastitis because of antibiotic resistance of biofilms [8]. The transformation of bactericide to the nontoxic form is mediated by enzymes that provide resistance to biofilm [9]. Epidemiological studies have revealed that following treatment with antimicrobials, cure rates vary between 0 and 80% but with no evidence of a significant loss of activity of the major classes of antibiotics licenced for the treatment of bovine mastitis [10]. Rather than using a single drug combination of more than one synergistic antimicrobial agent may give a fast and safe recovery [11,12].

The effective prevention and control of mastitis related to prompt identification and understanding of the diversity of the associated pathogens. However, in near future the control is anticipated to become troublesome owing to the rapid increase in antibiotic-resistant pathogens [11]. Consumption of unpasteurised or raw milk may cause the transmission of foodborne pathogens and antimicrobial resistant mastitis pathogens to humans could [13,14]. The widespread use of antibiotics for treatment of mastitis greatly increases the risk of installing and transmitting antibiotic resistance to consumers, which requires a scientific redefinition of antibiotic therapies taking into account of the intersection of animal welfare with public health concerns [15].

Materials and Methods

Milk samples were collected from bovine mastitis cases from Veterinary Hospitals and farms in Krishna, Guntur and West Godavari districts, Andhra Pradesh during the period from October 2017 to March 2018. Milk samples were collected according to the guidelines of National Mastitis Council [16]. Udder quarters were washed with tap water and dried. Before sampling, the first streams of milk were discarded and teat ends were disinfected with cotton swabs soaked in 70% alcohol and allowed to dry [17]. Approximately 10 ml of milk was collected aseptically from clinical cases into sterile vials. Collected samples from each quarter were transported on ice and immediately cultured or stored at 4ºC until cultured/enriched.

Cultural isolation

Milk samples were centrifuged at 2000 g at 37°C for 10 minutes, supernatant was discarded and 5 ml of BHI broth was added to the sediment and incubated at 37°C for 24 h [3]. After incubation of milk samples in BHI broth, the morphology of the organisms was studied by Gram's staining and cultural characters of the isolates was studied using different media such as MSA and *Streptococcus* selection agar enriched with blood. The tentatively identified organisms were further subjected for biochemical characterization.

For isolation of *Streptococcus* species 0.9 ml of SSB was inoculated with 0.1 ml of milk sample and incubated at 37°C in an anaerobic jar for 24 hr. After incubation of milk samples in SSB, the broth was examined for the presence of *Streptococcus* species by Gram's staining and further inoculated on to Edward's medium.

Molecular confirmation of bacterial isolates by PCR

Growth pattern and morphological examination of microorganisms on specific media are given in table 1. The isolates were subjected to various biochemical tests as per the methods described by Cruickshank., *et al*. [18]. Bacterial DNA was extracted by High salt method according to Anand Kumar [19] and re suspended in 40 µl sterile distilled water and stored at -20°C till use. *S. aureus,* and *S. uberis* were screened using species specific oligonucleotide primers in PCR. Oligonucleotide primers and thermal cycling conditions required for detecting the genes responsible for molecular characterization of *S. aureus, S. uberis* and antibiotic resistance of *S. aureus* isolates [blaZ, mecA,] are shown in table 2.

Table 1: Growth pattern and morphology of microorganism.

Medium	MSA	Edward's medium
Colony characteristics	Yellow coloured small colonies	Pin point brown colonies
Morphology on Gram's staining	Gram positive cocci in bunches	Gram positive cocci in chains
Organism identified	*Staphylococcus* species	*Streptococcus* species

Primer/ Gene	Sequence	Product size	Initial denaturation		Denaturation		Annealing		Extension	
			Temp	Time	Temp	Time	Temp	Time	Temp	Time
S. uberis 23srRNA [Sub 306 and Sub 396]	F- CGA AGT GGG ACA TAA AGT TA R- CTG CTA GGG CTA AAG TCA AT [Riffon., *et al.* 2001]	94 bp	94°C	2 min	94°C	30 Sec	53°C	30 Sec	72°C	30 Sec
S. aureus 23srRNA [Staur 4 and Staur 6]	F- ACG GAG TTA CAA AGG ACG AC R- AGC TCA GCC TTA ACG AGT AC [Straub., *et al.* 1999 and Riffon., *et al.* 2001]	1250 bp	94°C	2 min	94°C	45 sec	64°C	1 min	72°C	2 min
blaZ	F- AAG AGA TTT GCC TAT GCT TC R- GCT TGA CCA CTT TTA TCA GC [Vesterholm-Nielsen., *et al.* 1999]	517 bp	94°C	4 min	94°C	1 min	56°C	1 min	72°C	1 min
mecA	F- TCC AGA TTA CAA CTT CAC CAG G R- CCA CTT CAT ATC TTG TAA CG [Stegger., *et al.* 2012]	162 bp	94 °C	5 min	94°C	45 sec	50°C	30 sec	72°C	30 sec

Table 2: Oligonucleotide primers and thermal cycler conditions of PCR for detection of *Staphylococcus* species, *S. aureus* and antibiotic resistance genes [blaZ and mecA] in *Staphylococcus* species.

PCR was run for 35 cycles and Final extension step was maintained at 72°C for 10 min for all the oligonucleotide primer sets.

The PCR tests were carried out in Proflex PCR system, Applied Biosystems. All the reactions were carried out in a volume of 25 µl in 0.2 ml PCR tubes. The PCR amplicons were analysed by electrophoresis on 1.7% agarose gel stained with 0.5 µg of ethidium bromide/1 ml in Tris-Borate EDTA [TBE] buffer. Electrophoresis was carried out at 90V for 120 min in submarine gel electrophoresis unit [BIORAD, UK] and the PCR products were visualized in BIORAD molecular imager XR+. The sizes of PCR products were verified by comparison with quantitative DNA ladder [SRL, Mumbai]. Negative control with sterile distilled water was maintained in each PCR.

Biofilm production by the *S. aureus* isolates were studied by microtitre plate assay according to Dhanawade., *et al*. [20]. The ability of *S. uberis* strains to form biofilms *in vitro* on an abiotic surface was determined with a method previously described by others [21,22] with minor modifications by Moore [23].

Antibiotic disc diffusion test

The modified disc diffusion method of Kirby-Bauer was employed and Antibiotic susceptibility testing was performed using antibiotic test discs [Hi media, Mumbai] and interpretation was done according to 2007 CLSI guidelines. The disc diffusion test was done for each isolate on Mueller-Hinton agar. The antibiotic discs and their concentrations which are used in this study were given in the table 3 and 4. Antibiotic discs were aseptically applied on the inoculated plates with the help of forceps. These plates were then placed in an incubator at 37ºC for 24 hours in inverted position. After 24 hours, plates were examined for the zone of inhibition and the zone was measured [24].z

Table 3: Antibiotic discs used in disc diffusion test for *Staphylococcus aureus* isolates.

Sl. no	Name of the Antibiotic disc	Symbol	Concentration [µg]
1.	Penicillin G	P	10 IU
2.	Tetracycline	TE	30
3.	Chloramphenicol	C	30
4.	Ampicillin	AMP	10
5.	Methicillin	MET	5
6.	Amoxycillin/Clavulanic acid	AMC	20/10
7.	Gentamicin	GEN	10
8.	Streptomycin	S	10
9.	Cotrimoxazole	COT	25
10.	Clindamycin	CD	2
11.	Erythromycin	E	10
12.	Vancomycin	VA	30

Table 4: Antibiotic discs used in disc diffusion test for *Streptococcus uberis* isolates.

Sl. no	Name of the Antibiotic disc	Symbol	Concentration [µg]
1.	Penicillin G	P	10 IU
2.	Tetracycline	TE	30
3.	Chloramphenicol	C	30
4.	Ampicillin + Sulbactam	A/S	10/10
5.	Amoxycillin/Clavulanic acid	AMC	30/15
6.	Gentamicin	GEN	10
7.	Streptomycin	S	10
8.	Ceftriaxone	CTR	30
9.	Erythromycin	E	10
10.	Enrofloxacin	Ex	10

Results and Discussion

A total of 91 Bovine mastitic milk samples were subjected to cultural examination in the present study. Out of these 75 (82.41%) samples were found to be positive for *S. aureus* and *S. uberis* isolates. No bacterial pathogens were isolated from 16 (12.08%) milk samples.

Morphological and biochemical characterization

The golden yellow coloured colonies on MSA were examined by Gram's staining for *S. aureus* isolates. Gram positive cocci in clusters were identified as *Staphylococcus* species. The isolates were subjected to biochemical tests *viz.,* catalase test, coagulase test, Voges-Proskauer test, mannitol fermentation, DNase production and Gelatin liquefaction test. Out of 62 isolates of *S. aureus* 58 [93.54%] were coagulase positive and 4 [6.45%] were coagulase negative.

For isolation of *S. uberis* the samples with Gram positive cocci in chains on Gram's staining from *Streptococcus* selection broth were inoculated on to Edward's medium. The cultures showing greyish, pinpointed colonies and/or aesculin hydrolysis were tentatively identified as *Streptococcus* species. The suspected isolates of *Streptococcus* species were further identified by various biochemical tests *viz.,* catalase test, ninhydrin test, sodium hippurate hydrolysis test and type of haemolysis on 7% sheep blood agar. The haemolysis pattern observed were 86.9% isolates showed α- haemolysis, 2.1% isolate was β- haemolytic and 10.8% isolates were non-haemolytic.

Molecular characterization of the isolates by PCR

After biochemical characterization, the isolates were further confirmed by PCR using genus specific and species-specific primers for each pathogen. Sixty two isolates were confirmed as *S. aureus* using the oligonucleotide primers [Staur4 and Staur6] specific for *S. aureus* with product size of 1250 bp [Figure 1]. Fourty four isolates were found to be *S. uberis* with product size of 94 bp [Figure 2] using oligonucleotide primers [Sub302 and Sub396]. Genetic determinants of beta lactam resistance [blaZ] and methicillin resistance [mecA] were determined by PCR which are given in figure 3 and figure 4 respectively.

(Figure 1-4)

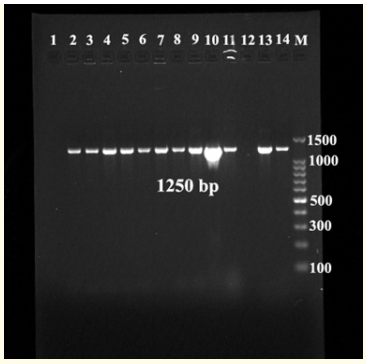

Figure 1: PCR amplification product of Staur 4 and Staur 6 oligonucleotide primers for S. aureus.

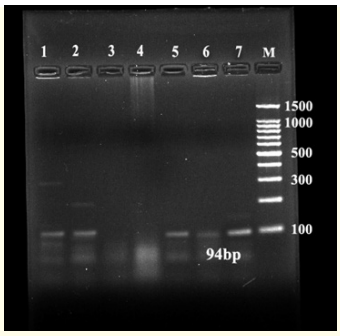

Figure 2: PCR amplification product of Sub 302 and Sub 396 oligonucleotide primers for S. uberis.

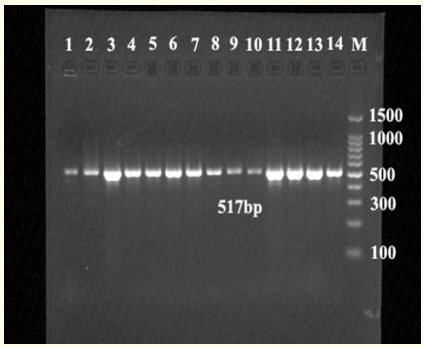

Figure 3: PCR amplification product of blaZ gene.

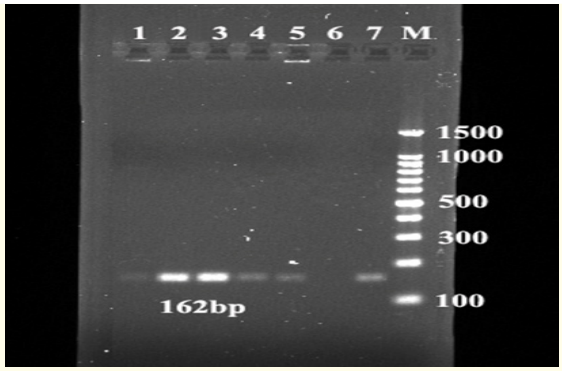

Figure 4: PCR amplification product of mecA gene.

According to this study, *S. aureus* were the most prevalent accounting for 58.49% and followed *by S. uberis* 41.5%. This finding is almost in close agreement with the study by Mohanty., *et al*. [25] who reported the prevalence of *Staphylococcus* species (47%) followed by *Streptococcus* species (32%) from bovine mastitis. The higher incidence of *S. aureus* indicates unhygienic handling during hand milking [26]. The results of current study are also in agreement with the findings. In contrary to the present study, a higher prevalence of *S. dysgalactiae* was reported by Tuteja [27]. However, Davies., *et al*. [28] reported similar prevalence of *S. uberis* (38%).

Reasons for variation in the results amongst studies can be due to variations in the breed, herd size, management, nutrition, and sanitary conditions during the dry period and at calving, environmental and climatic factors, criteria used for the inclusion of cows in the study, sampling methodology and schedules and number of samples [29].

Antibiotic resistance pattern of S. aureus

The isolates were subjected for antibiotic sensitivity test by disc diffusion method using the antibiotics mentioned in table 3 and 4. The results of *in vitro* antibiotic sensitivity test revealed that most of the *S. aureus* isolates were resistant to Penicillin G (69.35%) followed by Ampicillin (50%), Erythromycin (30.64%), Methicillin (29.03%), Clindamycin (24.19%), Amoxycillin/Clavulanic acid (20.96%), Streptomycin (20.96%), Gentamicin (8.06%), Cotrimoxazole (8.06%), Chloramphenicol (4.83%), Tetracycline (3.22%) and Vancomycin (3.22%). The overall antibiotic resistance/sensitivity pattern of the isolates was given in table 5. All the isolates of *S. aureus* were found to be biofilm producers except one which was

Sl. No	Antibiotic disc	S	I	R
	Penicillin G	30.65	0	69.35
	Ampicillin	50	0	50
	Methicillin	51.61	19.35	29.03
	Chloramphenicol	95.16	0	4.83
	Tetracycline	93.54	3.22	3.22
	Erythromycin	37.09	32.25	30.64
	Gentamicin	90.32	1.61	8.06
	Streptomycin	70.9	8.06	20.96
	Clindamycin	53.22	22.58	24.19
	Vancomycin	96.77	0	3.22
	Amoxycillin/Clavulanic acid	74.19	4.83	20.96
	Cotrimoxazole	85.48	6.45	8.06

Table 5: Antibiotic resistance pattern of *S. aureus* isolates in percentage [n = 62].

Only one of the *S. aureus* isolates was non biofilm former, which was sensitive to all tested antibiotics.

found sensitive to all the antibiotics tested. The findings are almost in close association with Ebrahimi and Taheri [30], reported 87% resistance to Penicillin, 62.5% to Ampicillin and susceptibility of all *S. aureus* isolates to Gentamicin, Tetracycline and Chloramphenicol. The isolates were sensitive to common antibiotics like Gentamicin and Tetracycline which was reported by Muhamed., *et al*. [31]. The resistance to β-lactam antibiotics in *S. aureus* isolates was 49.5% which is in accordance with Ramani Pushpa., *et al*. [32] and Basappa., *et al*. [33] who reported 58.8% resistance to β-lactam in India and abroad.

This study shows that 29.03% of the *S aureus* isolates were Methicillin resistant and 51.61% were Methicillin sensitive. It is observed that there is a great variation in antibiotic resistance pattern of MRSA and MSSA, MRSA was found to be showing 2 to 4 times more resistance to all tested antibiotics. MRSA showed a high resistance to Penicillin G (83.33%), Ampicillin (77.77%), Clindamycin (66.66%) and Streptomycin (44.44%) whereas the resistance pattern of MSSA was observed as Penicillin G (56.25%), Ampicillin (25%), Clindamycin (3.12%) and Streptomycin (9.37%). MRSA and Methicillin resistant Coagulase negative *S. aureus* were also found more resistant to other antibiotics than Methicillin susceptible Staphylococci [34]. Also, this study found that all MRSA isolates were biofilm producers which may be favouring to the increased antimicrobial resistance, whereas 96.87% of MSSA were biofilm producers. The positive correlation between biofilm production and antimicrobial resistance was widely studied in India and abroad [35-37]. Out of 62 *S. aureus* isolates 18 were found to be Methicillin resistant, 32 were MSSA and 12 were showing intermediate sensitivity. Antibiotic resistance pattern of MRSA and MSSA is given in table 6.

Table 6: Antibiotic resistance pattern of MRSA and MSSA [%].

	Antibiotic disc	S	I	R	S	I	R
1	Penicillin G	16.66	0	83.33	43.75	0	56.25
2	Ampicillin	22.22	0	77.77	75	0	25
3	Amoxycillin/ Clavulanic acid	83.33	0	16.66	78.12	0	21.87
4	Chloramphenicol	88.88	0	11.11	100	0	0
5	Tetracycline	83.33	11.11	5.55	100	0	0
6	Erythromycin	50	11.11	38.88	46.87	28.12	25
7	Gentamicin	77.77	5.55	16.66	96.87	0	3.12
8	Streptomycin	50	16.66	33.33	87.5	3.12	9.37
9	Clindamycin	11.11	22.22	66.66	65.62	31.25	3.12
10	Vancomycin	88.88	0	11.11	96.87	3.12	0
11	Cotrimoxazole	77.77	0	22.22	90.62	6.25	3.12

Antibiotic resistance pattern of Streptococcus uberis

In vitro antibiotic sensitivity test of *S. uberis* revealed that 59.09% of the isolates were resistant to Ceftriaxone followed by Streptomycin (34.09%), Erythromycin (31.81%), Penicillin G (29.54%), Tetracycline (27.27%), Enrofloxacin (20.45), Amoxycillin clavulanic acid (22.72%), Gentamicin (13.63%), Chloramphenicol (4.54%) and Ampicillin/ Sulbactam (2.27%). The detailed sensitivity/resistance pattern is given in table 7.

The results were in agreement with Elango., *et al*. [38] but the resistance to Ceftriaxone was a contradiction from the study which showed only 7.34% resistance. In favour of the present study Jain., *et al*. [39] also reported high sensitivity of *Streptococcus* isolates to Gentamicin and Ampicillin. [40] examined 223 *S. uberis* isolates and found that 82.02% were resistant to tetracycline, followed by ceftiofur (19.30%) and erythromycin (19/228, 8.33%). Among the isolates biofilm formers showed a high degree of resistance to antibiotics than non biofilm formers.

Sl. No	Antibiotic disc			
		S	I	R
	Penicillin G	25	4.5	29.54
	Ampicillin/Sulbactam	95.45	2.27	2.27
	Amoxycillin/Clavulanic acid	77.27	0	22.72
	Ceftriaxone	27.27	13.63	59.09
	Chloramphenicol	81.81	13.63	4.54
	Tetracycline	59.09	13.63	27.27
	Erythromycin	36.36	31.81	31.81
	Gentamicin	79.54	6.81	13.63
	Streptomycin	47.72	18.18	34.09
	Enrofloxacin	70.45	9.09	20.45

Table 7: Antibiotic resistance pattern of *S. uberis* in percentage [n = 44].

S - Sensitive, I - Intermediate, R - Resistant, n - Number of Isolates.

Antibiotic resistance among biofilm formers and non biofilm formers

In the present study out of 62 *S. aureus* isolates all except one isolate were found to be biofilm producers. The non biofilm former was sensitive to all the antibiotics used for sensitivity testing. Among 44 *S. uberis* isolates, 37 were biofilm producers and 7 were non biofilm producers. Among non biofilm forming *S. uberis* all isolates were sensitive to Tetracycline, Chloramphenicol, Ampicillin/Sulbactam, Amoxycillin/Clavulanic acid, Gentamicin and Enrofloxacin whereas biofilm formers were resistant to these antibiotics by 30.76%, 30.76%, 2.56%, 25.64%, 15.38% and 25.64% respectively. Antibiotic resistance to Ceftriaxone, Streptomycin and Penicillin G by non biofilm formers was 28.57%, 14.28% and 14.28%, respectively. The details are graphically represented in figure 5.

The increased antibiotic resistance among biofilm formers may be attributed to the interchange of extra chromosomal DNA which is accountable for antibiotic resistance, virulence dynamics and environmental persistence at enhanced rates in biofilm [41] or other perspectives like delayed penetration of the antimicrobial agent through the biofilm matrix, altered growth rate of biofilm forming organisms and other physiological changes due to the biofilm mode of growth [42].

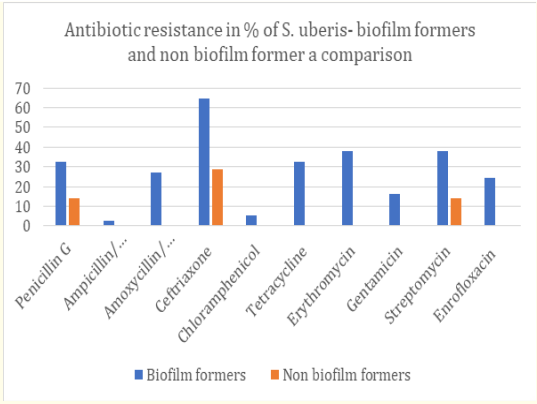

Figure 5: Antibiotic resistance pattern of biofilm forming and non biofilm S. uberis in percentage.

Genetic determinants of methicillin resistance (mecA) and beta lactam resistance (blaZ) were determined by PCR. Out of 62 isolates of *S. aureus*, 61 were carrying mecA gene and all isolates were carrying blaZ gene, but only 18 (29.03%) were found to be Methicillin resistant carrying mecA gene, and only 43 (69.35%) isolates were β-lactamase producers harbouring blaZ gene. This can be explained by the possible involvement of other genes in the process of ß-lactam resistance which can affect the expression of mecA gene [43,44]. Some strains produce low levels of Penicillin Binding Protein 2a and escape classic detection and phenotypically misidentified as methicillin sensitive despite having the mecA gene [45,46]. The results were almost in accordance with Asfour and Darwish [47] reported 20/25 (80%) carrying mecA gene and was in contrary to the prevalence of blaZ gene, only 17 out of 25 (68%) were carrying the gene.

Conclusion

Antimicrobial resistance varies with area and time. Biofilm forming microbes were highly resistant to antibiotics when compared to nonbiofilm formers, irrespective of the species studied. Genetic determinants for Methicillin resistance and beta lactam resistance were found in 99% of the *S. aureus* isolates, which points the upcoming risk in treating the disease conditions. MRSA was observed to be more resistant to treated antibiotics than MSSA, among that biofilm forming MRSA was possessing high resistance.

Conflict of Interest

The authors declare that they have no competing interests.

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