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Resistance Pattern of *Staphylococcus aureus* Isolated from Subclinical Mastitis towards Beta-Lactam Group of Antimicrobials

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ABSTRACT

Antimicrobial resistance (AMR) is when organisms become irresponsive to the commonly used antimicrobials for which previously they were susceptible. Many organisms are responsible to cause mastitis in dairy animals. Beta-lactam group of antimicrobials are most widely used in the treatment of mastitis and other animal diseases. Therefore present study was planned to investigate selected beta-lactam antimicrobials susceptibility pattern against S. aureus organisms isolated from bovine subclinical mastitis. S. aureus grown on selective media and tested for susceptibility pattern on Muller-Hinton Agar. Total 168 S. aureus isolates were tested and showed different resistance patterns. Highest Resistance was observed to Ampicillin (57.14%) and Amoxicillin (56.54%). Greater sensitivity was observed to cephalosporins and other beta-lactams when used along with beta-lactamase inhibitors. It is suggested to observe the sensitivity pattern while treating animal diseases in order to minimize the resistance raised due to use of wrong and inadvertent use of these magic bullets.

Key words: *Resistance, Beta-lactams, S. aureus*

INTRODUCTION

Mastitis, or inflammation of the mammary gland, is predominantly due to the effects of infection by bacterial pathogens, although mycotic or algal microbes play a role in some cases. There are many causative agents of mastitits which includes *S. aureus, E. coli. Streptococci, pseudomonas* etc[1, 2 & 3]. *S. aureus* seems to be a major pathogen that can cause various forms of diseases varying from simple to life-threatening infection in human and animal population[4]. Bacteria are the most common cause of bovine mastitis. Several reports clarified that more than 137 microbes are considered as etiological agents of mastitis 5. The microbial causes of mastitis include a wide variety of microorganisms (aerobic and anaerobic bacteria, mycoplasma, yeasts and fungi). Because most pathogens involved in mastitis are ever-present, mastitis can be managed but not eradicated[6].

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Antimicrobial resistance (AMR) is when organisms become irresponsive to the commonly used antimicrobials for which previously they were susceptible. Since the discovery of penicillin in the late 1920s, hundreds of antimicrobial agents have been developed for anti-infective therapy. Antimicrobials have become indispensable in decreasing morbidity and mortality associated with a host of infectious diseases. The emergence of AMR was not an unexpected phenomenon and was predicted by Alexander Fleming, who warned in his Nobel prize lecture in 1945 against the misuse of Penicillin[7.]

Antibiotics are commonly used to treat mastitis cases. The most common treatment involves use of beta-lactam group of antimicrobials like ampicillin, amoxycillin, cephalosporins with or without the combination of clavulanic acid or sulbactam to treat staphylococcal infections. β-lactam antibiotics are among the most frequently prescribed antibiotics worldwide in the control of *S. aureus* infection. They act on peptidoglycan synthesis by molecularly acting on transpeptidases and carboxypeptidases thereby disrupting cell wall formation of the pathogen. However, the efficacy of antibiotics for therapy have suffered a setback due to the growing trend of multiply resistant strains observed in the organism to βlactam and other antibiotics [8,9,10]. Resistance to β-lactam group of antibiotics in *S. aureus* is mediated through a variety of β-lactamases or the expression of low-affinity penicillin binding protein PBP2a. The chromosomally mediated penicillin binding protein 2a initiates resistance to methicillin which confers a low affinity for all β -lactams and other unrelated group of antibiotics, thereby limiting choice for treatment[8, 10, 11]. In addition, selective pressure from excess antibiotic use accelerates the emergence of resistance. β-lactamase has been observed to be responsible for resistance in β-lactam, βlactamase inhibitors and extended spectrum cephalosporins[12,13.]

Antibiotic resistance in *S. aureus* have an adverse effect on healthcare management of infections. In response to the increasing rate of antibiotic resistance in *S. aureus*, this study aimed to analyse the antibiotics susceptibility patterns, the production of β-lactamase and its association to antibiotic resistance in *S. aureus* strains. Therefore the present study was undertaken to investigate the antimicrobial resistance of *S. aureus* with special reference to beta-lactam group.

This study is sought to identify antibiotic resistance pattern in *S. aureus* obtained from milk samples of the buffaloes from Mumbai region. The present study is also carried out with aim to further enrich the knowledge of dairy scientists and Veterinarians in order to assist in the effective control and treatment of mastitis.

MATERIAL AND METHODS

Collection of Samples: A total of 750 buffaloes were screened by California Mastitis Test (CMT) from the six different farms from the western suburbs of Mumbai which included Goregaon, Palghar and Virar and Out of 750 milk samples, 310 were positive for CMT, thus the overall prevalence of SCM recorded was 41.33 percent. Individual mammary quarter milk samples were aseptically collected into sterile vials immediately before milking, after discarding the first three milking streams. The milk samples were transported to laboratory and procedures of isolation and identification were performed following Koneman *et al*[14].

Isolation of *Staphylococcus aureus*: *Staphylococcus aureus* was isolated from the milk of asymptomatic subjects. A loopful of milk was streaked on MSA plate and incubated at 37ºC for 24 h. Golden yellow colonies that grew on MSA were presumptively selected as *S. aureus* (Plate.1). The presumptive *S. aureus* was further confirmed by Gram staining of the golden yellow colonies from MSA and minimal biochemical tests for *S. aureus* identification (catalase, coagulase) as earlier described[15].

Antibiotic Susceptibility Testing: Antibiotic susceptibility testing of the bacterial isolates was carried out using Kirby-Bauer disk

diffusion method as modified by Clinical and Laboratory Standards Institute[16] Briefly four (4) colonies of the isolates were transferred into 5 ml of sterile normal saline (0.9 g NaCl, distilled water to 100 ml) in a tube such that the turbidity of the bacterial suspension was equivalent to 0.5 McFarland Standard. The sterile swab was dipped in the bacterial suspension and streaked on MHA and each antibiotic disc was aseptically placed with a sterile pair of forceps on the surface of the inoculated MHA plate. The plate was incubated at 37ºC for 24 h. The diameter of the zone of inhibition was measured using meter rule and the result was interpreted in accordance with the susceptibility break point as earlier described[16].

Beta-lactam Antibiotics tested: Specific antimicrobial discs obtained from Hi-media Mumbai were tested for the resistance pattern of *S. aureus* such as Amoxicillin (30 µg), Amoxicillin + Clavulanic acid (20/10 µg), Ampicillin (10 µg), Ampicillin + Sulbactam (10/10 µg), Cefotaxime (30 µg), Cefotaxime + Clavulanic acid (30/10), Cefepime (30 µg) Cefepime + Clavulanic acid (30/10 µg), Ceftazidime (30 µg), Ceftazidime + Clavulanic acid (30/10 µg), Methicillin (5 µg) and Oxacillin (5µg).

RESULTS AND DISCUSSION

A total of 750 buffaloes were evaluated by California Mastitis Test from the six different farms from the western suburbs of Mumbai which included Goregaon, Palghar and Virar and Out of 750 milk samples, 310 were positive for CMT, thus the overall prevalence of SCM recorded was 41.33 percent. From the 310 CMT positive milk samples overall 168 showed presence of *S. aureus* after culture on MSA agar as well as from biochemical tests and gram staining.

Antibiogram: When all the 168 *S. aureus* were tested for its sensitivity pattern with above listed beta-lactam antimicrobials (Table. 1). It was observed that, maximum sensitivity of the *S. aureus* for Cefepime + Clavulanic acid (77.38%), followed by Cefotaxime + Clavulanic acid (73.80%), Amoxicillin + Clavulanic acid combination (73.21%), Methicillin (70.23%) , Oxacillin (60.04), Ampicillin+ Sulbactam combination (76.78%), Cefotaxime (61.30%), Cefepime (56.54%), Amoxicillin (43.45%) and Ampicillin (42.85%) **(Plate 2 and 3). Antibiotic sensitivity is also shown in graph (Fig.1)**

Table 1: Antimicrobial Sensitivity and resistance pattern of *S. aureus*

Antimicrobials	Susceptible % (n)	Intermediate % (n)	Resistant % (n)
Ampicillin	42.85 (72)	-	57.14 (96)
Ampicillin + Sulbactam	65.47 (110)	3.57(6)	30.95 (52)
Cefepime	64.28 (108)	17.85 (30)	17.85 (30)
Cefepime + Clavulanic acid	77.38 (130)	7.14 (12)	15.47 (26)
Cefotaxime	61.30 (103)	15.47 (26)	22.02 (37)
Cefotaxime + Clavulanic Acid	73.80 (124)	10.11 (17)	16.07 (27)
Amoxicillin	43.45 (73)	-	56.54 (95)
Amoxicillin + Clavulanic Acid	73.21 (123)	-	26.18 (45)
Methicillin	70.23 (118)	5.95 (10)	23.80 (40)
Oxacillin	69.04 (116)	0	30.95 (52)

The reported percentage of β- lactam resistant *S. aureus* in cases of bovine mastitis world over detected by disc diffusion method are variable which was between 20 and 80 % [17,18,19,20, 21]. From India Penicillin resistant *S. aureus* strains procured from bovine mastitis were isolated by maximum researchers *viz.*, 28.9 percent by Kumar *et al*.[22], 45 percent[23] by Sumathi *et al.*, 100 percent [24] by Thaker *et al*., 76.77 percent[25] by Kaliwal *et al.*, 83.3 percent[26] by Chandrasekaran *et al*., 63.5 percent[27] by Chandrasekaran *et al.*, and 82.35 percent[28] by Mohanty *et al*.

In the present study resistance shown towards Ampicillin was 57.14%. Ampicillin is active against many Gram-positive and Gram-negative bacteria. It was the first 'broad spectrum' Penicillin with activity against Gram-positive bacteria including *Streptococcus pneumoniae*, *Streptococcus*

pyogenes and some isolates of *S. aureus* (but not Penicillin-resistant or Methicillin-resistant strains). Its spectrum of activity is enhanced by co-administration of Sulbactam, a drug that inhibits β- lactamase, an enzyme produced by bacteria to inactivate Ampicillin and related antibiotics[29].

Reported resistance of *S. aureus* for Ampicillin by various researchers was 54.54 percent [30] by Bernabe *et al*., 70.59 percent [26] by Kaliwal *et al*., 75.8 percent[31] by Adwan, 63.3 percent [32] by Guler *et al*., 53.68 percent [33] by Nichita *et al*., 96 percent [34] by Akindele *et al*., and 3.94 percent [35] by Mubarak *et al*.

In the present study the resistance to Ampicillin and Ampicillin + Sulbactam was 57.14 and 30.95 percent, respectively. It was also observed from the previous reports that high variation in resistance percentage to Ampicillin could be due to different geographical areas and previous exposure to antimicrobials.

As the introduction and use of Ampicillin alone started in 1961 many organisms became resistant due to production of β-lactamase enzyme[36]. The introduction of Sulbactam since 1987, combined with Ampicillin made β-lactamase producing bacteria more susceptible. This is observed in the present study where greater percent of *S. aureus* (65.47 percent) was sensitive to the combination as compared to Ampicillin alone (42.85 %).

The results in the present study suggest that inclusion of Sulbactam enhanced the sensitivity and lowered the resistance percentage. However, 100 percent sensitivity could not be achieved indicating that other mechanisms of resistance development might be involved like alterations in PBP and decreased permeability of antimicrobials.

Similarly the degree of sensitivity of *S. aureus* towards Cefotaxime and its combination with Clavulanic acid in present study was 61.30 and 73.80 percent, respectively whereas intermediate sensitivity of 15.47 and 10.11 percent showed effectiveness of Cefotaxime than other antimicrobials. Therefore overall sensitivity to Cefotaxime and Cefotaxime + Clavulanic acid could be considered as 76.77 and 83.91 percent, respectively. In contrast to the our results, Rajaduraipandi and associates from Tamilnadu interpreted 63.2 percent resistance to Cefotaxime in human isolates of *S. aureus*[37]. In 2011 Chakraborty and associates from Bangalore, India interpreted Cefotaxime resistance in human pus samples as 26.67 percent[38].

On similar grounds Sonth *et al* from Bagalkot, Karnataka reported 52.6 percent resistance to Cefotaxime from the *S. aureus* collected from human samples. As a β-lactam antibiotic in the third-generation class of cephalosporins, Cefotaxime is active against numerous Gram-positive and Gram-negative bacteria, including several with resistance to classic β-lactams such as penicillin. It is active against *S. aureus* but not MRSA, S. *epidermidis, Str. pneumoniae, S. pyogenes* and *E. coli* etc[39].

In the present study resistance of *S. aureus* to Amoxicillin and Amoxicillin + Clavulanic acid was 56.54 and 26.18 percent among the tested samples. Amoxicillin is a moderate-spectrum, bacteriolytic, β-lactam antibiotic in the aminopenicillin family used to treat susceptible Gram-positive and Gram-negative bacteria. França *et al*. from Brazil, reported that resistance of *S. aureus* samples obtained from bovine mastitis to Amoxicillin alone was 50 percent[40]. Elizabeth *et al*. studied AMR of *Staphylococcus* spp. isolates from cases of mastitis in buffalo in Brazil and reported 49.2 percent resistance to Amoxicillin [41]. However, very low resistance was reported by Ashraf *et al*. in *S. aureus* isolated from chickens. As Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, which are resistant to a narrow spectrum of β-lactam antibiotics, such as Penicillin, it is combined with Clavulanic acid, a β-lactamase inhibitor. This drug combination is commonly called Co-Amoxiclav[42].

The sensitivity of *S. aureus* isolates towards Cefepime and Cefepime + Clavulanic acid in present study was 64.28 and 77.38 percent, respectively. Cefepime is a fourth-

generation Cephalosporin antibiotic having extended spectrum of activity against Gram-positive and Gram-negative bacteria, with greater activity against both types of organisms than third-generation agents.

From Brazil, Santos et al. reported *in-vitro* antibiotic resistance and susceptibility of 34 *S. aureus* isolates obtained from bovine mastitis which were 32.4 and 67.60 percent, respectively[43].

There are several reports published about the increased sensitivity to cephalosporins if used in combination with β-lactamase inhibitors. Moreover several authors mentioned that, β- lactam resistance can be due to the expression of inducible β-lactamases encoded by the *blaZ* gene, which causes hydrolysis of β - lactam ring of Penicillin[44, 45, 46].

In the present study resistance observed to Methicillin and Oxacillin was 23.80 and 30.95 percent, respectively. The first report on the ability of *S. aureus* to metabolize Penicillin was published in 1940, a year before the antimicrobial was introduced for therapeutic use. However the discovery of Penicillin dramatically reduced the incidence of bacterial infections around the world. This single antibiotic was effective against a large number of bacteria for many years, until *S. aureus* developed the ability to produce β-lactamase, an enzyme that destroys Penicillin. *S. aureus* develops resistance to antibiotics through plasmid-mediated genetic mutations [47]. These mutations confirmed that, S. *aureus* has the remarkable ability to adapt to changing antibiotic environments. The resiliency of *S. aureus* motivated pharmacologists to create a class of semi-synthetic Penicillins that could withstand β-lactamase. These antibiotics became known as β-lactam Penicillins, with Methicillin as the prototype. For years, infections with *S. aureus* were reliably eradicated with Methicillin and its analogs, Nafcillin and Cloxacillin. However, the resourceful bacterium soon became able to resist these β-lactam antibiotics, and the first strain of MRSA was identified in 1961. Since the mid-1980s, antibiotic resistance among nosocomial *S. aureus* isolates has been increasing appreciably.

S. aureus usually shows limited host specificity, and transfer between different host species may occur[48]. The transmission of milk-associated *S. aureus* strains between cows and humans was suggested. The risk for spread of MRSA from bovine sources into the human population is low. Generally, persons are not at risk as long as raw milk is not consumed. However, persons in close contact with MRSA infected cattle, including Veterinarians, farmers, milkers, and persons working at slaughterhouses, may become colonized from the bovine source. In addition to Methicillin, strains of *S. aureus* have developed resistance to other antibiotics. MRSA is resistant to Cephalosporins, Erythromycin, Clindamycin, Gentamicin, Trimethoprim-Sulfamethoxazole, and Ciprofloxacin. Vancomycin, a glycopeptide antibiotic, was relied upon until recently to eradicate MRSA infection. As expected, strains of Vancomycin-resistant *S. aureus* (VRSA) have been isolated and are fast becoming a new treatment challenge[49].

Impaired treatment response is associated with Penicillin resistance of *S. aureus* strains. Importance of prolonged β-lactam associated resistance in *S. aureus* and higher MIC values demonstrated that Ampicillin and Penicillin are consistently the antimicrobial agents to which the *S. aureus* are most commonly resistant Highest number of Penicillin resistant *S. aureus* and sensitive to cephalosporins were also observed in the study of Onyenwe *et al*. and also revealed that 80 percent of bovine isolates were producing β-lactamases[50].

Penicillin predicts resistance to other β- lactam group of antimicrobials like Ampicillin. *S. aureus* β- lactamase will be resistant to Penicillin and Cephalosporins. In the present study Methicillin and Oxacillin was included for detection of MRSA where 23.80 and 23.21 percent strains were resistant to Methicillin and Oxacillin.

Oxacillin resistance is reported to show resistance to all β- lactam antimicrobial agents. Thus, such high degree of resistance to

Oxacillin in the present study is alarming. Oxacillin resistance was not detected by Gianneechini *et al*[18]. In contrast to the present study no MRSA strains were detected from bovine mastitis originated *S. aureus* isolated in Argentina by Gentillini *et al*[17].

There are several factors other than antimicrobial usage which influence the susceptibility pattern of mastitis pathogens and the general recommendation is to cull all animals with chronic *S. aureus* intra mammary infections (IMI)[51]. The control of IMI sustained by *S. aureus* should involve the best management practices and selective antimicrobial usage. Unfortunately most antimicrobial agents used in veterinary medicine still rely on interpretive criteria developed for humans and the validity of these criteria for categorizing veterinary pathogen as susceptible or resistant has not been established. Thus, the usefulness of susceptibility data is limited to monitoring the percentage of *S. aureus* with MIC above threshold value and to predict efficacy[51].

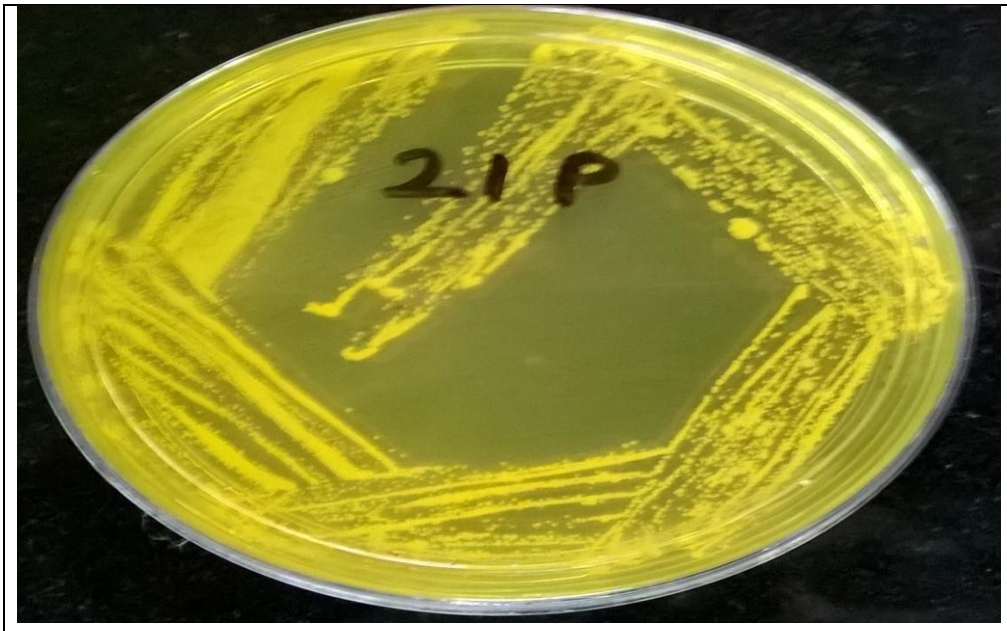

Plate 1: *S. aureus* showing golden yellow colonies on mannitol salt agar

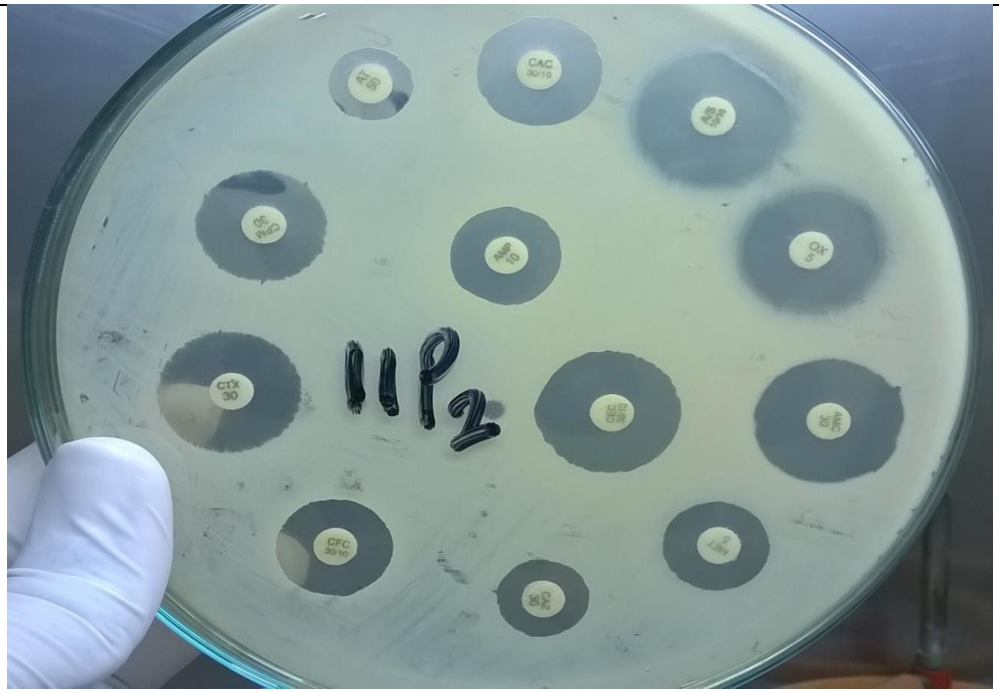

Plate 2: *S. aureus* showing susceptibility different β-lactam antimicrobials on Muller Hinton agar

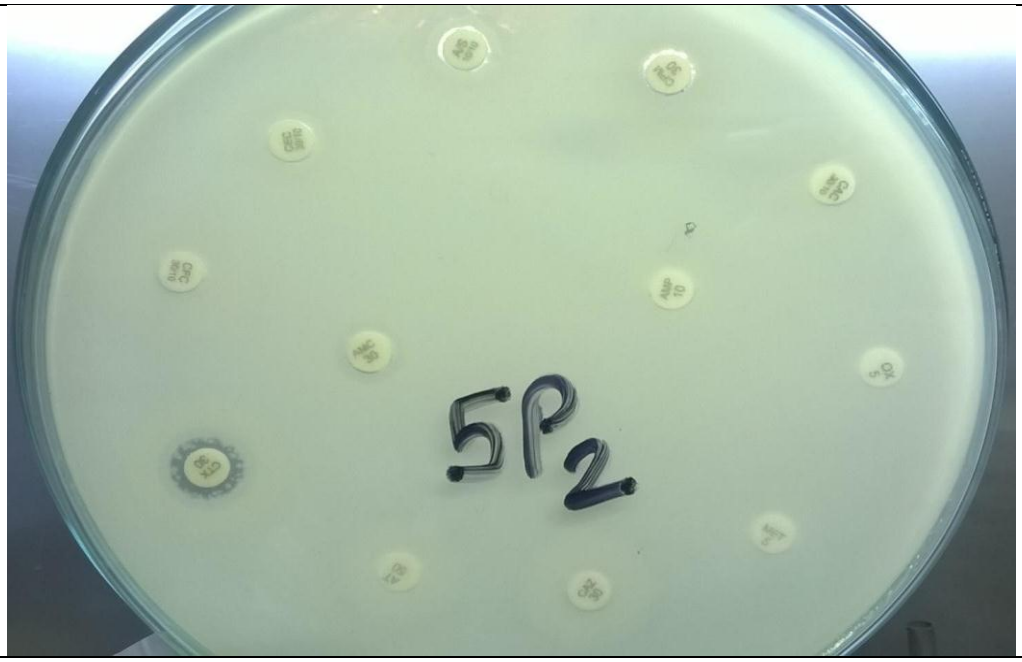

Plate 3: *S. aureus* showing resistance to different β-lactam antimicrobials on Muller Hinton agar

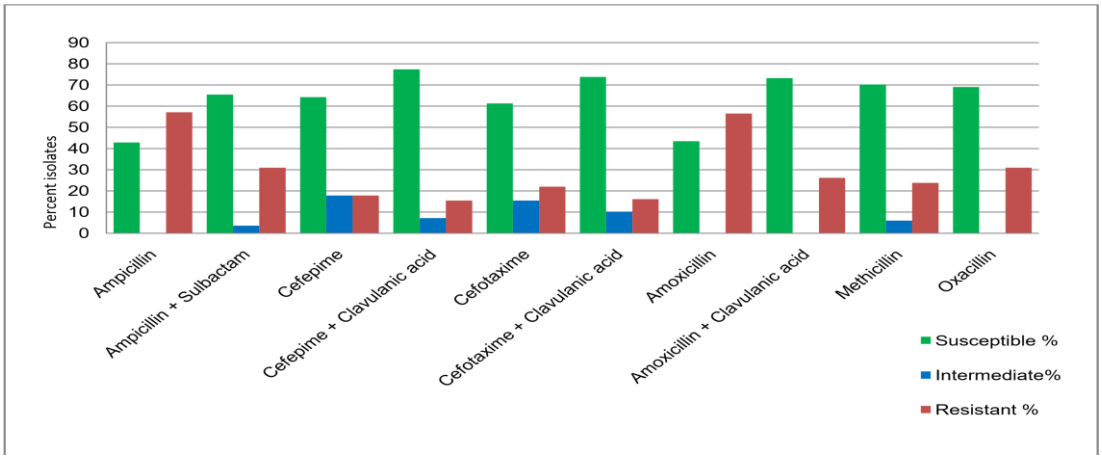

Fig. 1 : Antibiogram for S. aureus against different Beta-lactams used.

CONCLUSIONS

Beta-lactam resistance was found to be widely spread among *S. aureus* isolates. The susceptibility of oxacillin-resistant strains to beta-lactamase inhibitor association pointed to the implication of beta-lactamase production in the detected resistance.

The high rate of β-lactam resistance amongst *S. aureus* from milk of bovine mastitis is likely due to the wide use of intramammary preparations containing combinations of different antibiotics and broad-spectrum antimicrobials including Penicillin. Numerous factors can influence the overall susceptibility pattern of mastitis pathogens. It is strongly recommended that, the use of beta-lactam antimicrobials after confirming beta-lactamase production should be with combination of sulbactam or clavulanic acid. The present study recommends that treatment of animal pathogens must be undertaken after susceptibility testing in order to avoid problem of resistance

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