Short communication

SCREENING OF STAPHYLOCOCCUS AUREUS ISOLATES FROM MASTITIS FOR ANTIBACTERIAL SUSCEPTIBILITY PATTERN AND BETALACTAMASE PRODUCTION

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ABSTRACT

Isolates of Staphylococcus aureus from bovine clinical and subclinical mastitis were tested for ß-lactamase production and antimicrobial susceptibility pattern. Beta-lactamase production was assessed by penicillin-phenol red acidometric test. Antimicrobial susceptibility was tested by measuring MIC by Resazurin dye broth microdilution method. Forty per cent of isolates tested were positive for â-lactamase production. The susceptibility of S. aureus isolates were: gentamicin (100%), enrofloxacin (70%), rifampin (70%) and tetracycline (40%). Complete resistance was observed for amoxycillin, ceftriaxone, nalidixic acid, erythromycin, sulphamethoxazole and trimethoprim. The results of the study indicate the widespread resistance of Staphylococcus aureus isolates against commonly used antimicrobials and also the utility of resazurin test and ß-lactamase test to study antimicrobial drug resistance.

Key Words: *Staphylococcus aureus- ß-lactamase- Antimicrobial resistance-Mastitis-resazurin dye test*

INTRODUCTION

Mastitis is the most important and expensive disease affecting dairy industry. It results in huge economic losses associated with a drop in milk production, high culling rate and treatment cost. Over 135 different microorganisms have been isolated from bovine mammary infections, but Staphylococci, Streptococci and Gram-negative bacilli are mainly responsible for majority of infections. Verma (1988) reported *S. aureus* as the most common bacteria from clinical and sub-clinical bovine mastitis.

Antimicrobial therapy is a primary tool for controlling staphylococcal mastitis. Widespread and indiscriminate use of antimicrobial agents has resulted in the emergence of resistant organisms. The way bacteria develop resistance to ß-lactam antibiotics is mainly through ß-lactamase

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enzymes. ß-lactamases render bacteria resistant to beta-lactam antibiotics by hydrolyzing the ß-lactam ring of penicillins and cephalosporins [Prescot *et al*., 2000]. Both Gram-positive and Gram-negative bacteria can become resistant to ß-lactam antibiotics via production of ß-lactamase.. There is wide correlation between ß-lactamase production of bacteria and resistance against penicillin. Therefore, measuring the ß-lactamase production in bacteria in conjunction with susceptibility testing can be a rapid useful method for detecting potential penicillin resistance in staphylococcal isolates from cases of bovine mastitis. (Gianneechini *et al.*, 2002).

Numerous studies have been conducted worldwide to determine the antibacterial susceptibility patterns of mastitis pathogens isolated from clinical studies. However, only limited information is available about the antimicrobial susceptibility of *S. aureus* isolated from bovine mastitis cases in Tamilnadu, India. The purpose of this study was to determine the *in vitro* activity of selected antimicrobial agents against strains of *S. aureus* isolated from bovine mastitis. Additionally, ß-lactamase production of the *S. aureus* strains was also determined.

MATERIALS AND METHODS

Bacterial Isolates

Ten *Staphylococcus aureus* isolates from bovine clinical and subclinical mastitis were used in this study. All strains were identified by standard procedures and maintained frozen at >20°C in the Dept. of Veterinary Pharmacology and Toxicology, Madras Veterinary College.

Drugs and Chemicals

Ten antimicrobials were employed in this study: Amoxycillin, ceftriaxone, enrofloxacin, trimethoprim, sulphamethoxazole, nalidixic acid, tetracycline, rifampin, gentamicin and erythromycin. All antibiotic powders were purchased from Hi Media Laboratories, Mumbai. Stock solutions (1 mg/ml) were prepared by initially dissolving 10 mg of antibiotics in respective solvent and further diluting it in distilled water. Ceftriaxone, enrofloxacin, trimethoprim and sulphamethoxazole were dissolved in 0.1N NaOH whereas nalidixic acid, tetracycline, rifampin and erythromycin were dissolved in 0.1N HCl. Amoxycillin was dissolved in Phosphate buffered saline while gentamicin was dissolved in distilled water.

Betalactamase detection

All isolates were tested for betalactamase production by penicillin-phenol red acidometric test (Banic, 1991). Penicillin G 10 IU was used for induction of betalactamase enzyme.

Benzyl penicillin (Alembic limited, Vadodara) 10,00,000 U was dissolved in 4.5 ml of sterile triple distilled water. To this 0.5 ml of 0.5 % aqueous phenol red solution was added. The pH of the solution was adjusted to 8.5 using 1 M NaOH. One hundred microlitre of this solution was added to dense bacterial suspension of the test isolate prepared in 0.5 ml of normal saline and mixed well. Tube without bacteria was kept as control. A change in color from purple to yellow within 5 min was recorded as positive for betalactamase production (Fig. 1)

Antimicrobial susceptibility testing

The MICs of antimicrobials were determined by a Resazurin dye broth microdilution method (Sarker *et al*., 2007). Serial two fold dilutions of drugs in nutrient broth (50 µl) were prepared in 96 well ELISA plates. Resazurin dye (10 µl), nutrient broth (30 µl) and bacterial suspension (10 µl) adjusted to 5 x 10^6 CFU/ml were added in that order to all the wells. Sterility control without bacteria and growth control without drug were also included. Plates were wrapped and incubated at 37°C for overnight. The lowest concentration which inhibits visible growth (persistence of blue color) of bacteria was recorded as MIC. The MIC at which 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were inhibited were calculated. All the experiments were performed in triplicate

RESULTS AND DISCUSSION

The results indicate the utility of resazurin dye test and ß-lactamase production test to assess antimicrobial drug efficacy, which are simple, accurate and can be done for large no. of bacteria against many drugs in short time.

The MIC results of the isolates tested are presented in table 1 and fig. 2. Of the 10 isolates tested, 40% of isolates were positive for ß-lactamase production. Jones and Heath (1985) reported that 66.1% of the *S.aureus* isolates from mastitis were positive for ß-lactamase production whereas Oncel *et al.*, (2004) reported that 33.3% of *S. aureus* isolates were positive for ß-lactamase production in Turkey. Amsaveni *et al* (2007) recorded 45% of the isolates from Puducherry region were ß-lactamase producers.

All ß-lactamase positive *S. aureus* isolates were found to be gentamicin susceptible. Three ß-lactamase positive *S. aureus* isolates were found to be susceptible for enrofloxacin (75% susceptibility) whereas only 50% of ß-lactamase positive isolates were susceptible to rifampin. Watts and Salmon (1997) observed greater resistance against erythromycin among ß-lactamase positive *S. aureus* than ß-lactamase negative organisms. In this study, 100 % resistance was observed for erythromycin among *S. aureus* isolates.

In the present study, gentamicin was (100% susceptibility) highly sensitive followed by enrofloxacin (70% susceptibility), rifampin (70% susceptibility) and tetracycline (40% susceptibility). Complete resistance was observed for amoxicillin, ceftriaxone, nalidixic acid, erythromycin, sulphamethoxazole and trimethoprim. Oliviera *et al.* (2000) reported MIC value of ≤0.06 to 64.0 µg/ml and 0.125 to >64.0µg/ml for enrofloxacin and erythromycin, respectively, against 811 *Staphylococcus aureus* strains isolated from bovine mastitis from 11 countries. Martin *et al.*, (2003) observed 38 % resistance against amoxicillin whereas resistance values for enrofloxacin and gentamicin were below 8% levels. In this present study, we observed 100% resistance to amoxycillin.

Gianneechini *et al*. (2002) observed MIC values of ≤1µg/ml, ≤ 0.5 µg/ml and ≤0.25 µg/ml for gentamicin, erythromycin and enrofloxacin, respectively, against *S. aureus*. In the present study, almost all antibiotics except enrofloxacin, gentamicin, rifampin were found to be resistant to *S. aureus* tested. Indiscriminate and continuous use of antimicrobials has led to the emergence of multiple drug resistance which

had led to a black scenario which makes the management of mastitis very difficult for the practicing veterinarian. Antibiotic susceptibility test and MIC studies should be continuously carried out in particular geographical location to assess the resistance among pathogens. This can also be accompanied by ß-lactamase detection test for detection of potential penicillin resistance, which correlates well with resistance against antibiotics such ß-lactams and macrolides.

Though studies with larger sample size would be desirable to arrive at a database on antimicrobial drug resistance, the results of the present study present a preliminary evidence on multiple drug resistance among *S. aureus* causing mastitis. This also highlights the urgent need for creating awareness about the indiscriminate use of antibiotics among farmers and veterinarians.

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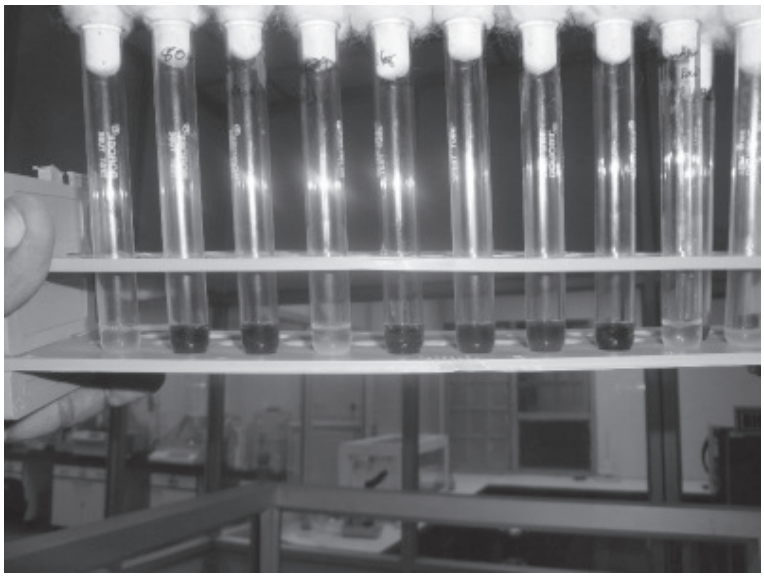
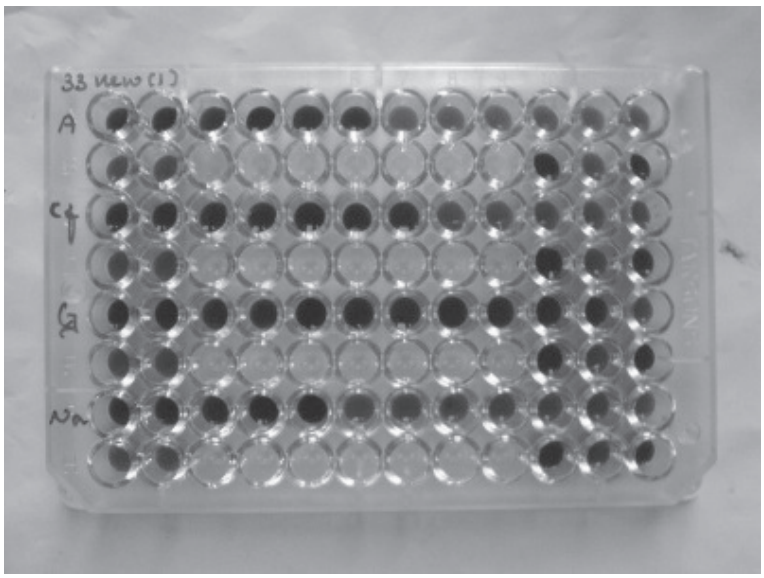
Fig 1 Test for ß-lactamase Production in *Staphylococcus aureus* isolates



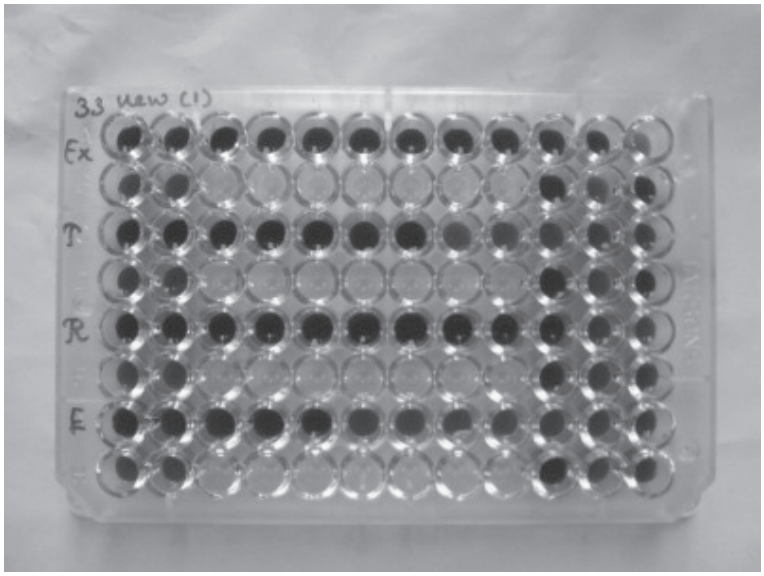
Yellow color indicates betalactamase positive

Red color indicates betalactamase production negative

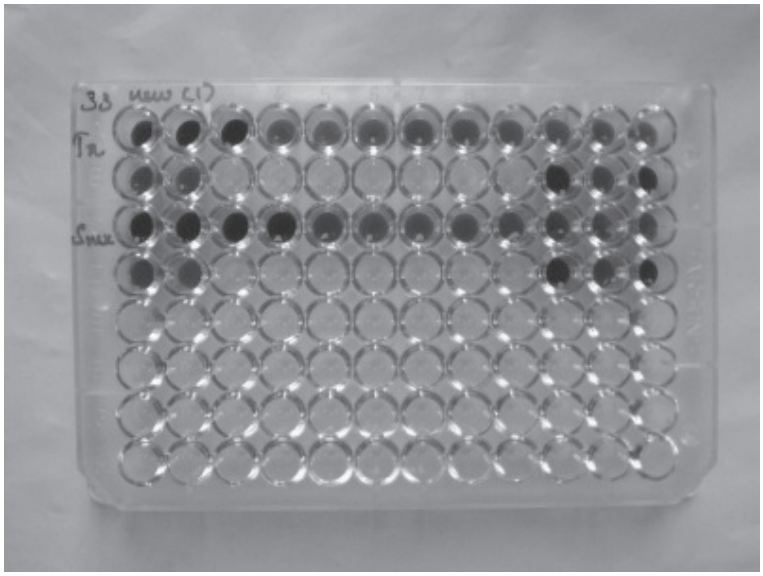
Fig 2 MIC of *Staphylococcus aureus*



(C1 sterility control, C2 bacteria control, C3 drug control
A - Amoxicillin, Cf - ceftriaxone, G -Gentamicin ,NA -Nalidixic acid)



Ex - Enrofloxacin, T - Tetracycline, R – Rifampin, E - Erythromycin



Tr - Trimethoprim and Smwx - Sulphamethoxazole

Table 1 MIC (µg/ml) for *Staphylococcus aureus*

Isolate No.	ß-lactamase	AMX	CF	GEN	NA	ENR	TET	RFM	ERY	SM	TM
1	-	78.125	78.125	9.76	625	1.22	2.44	1.22	156.25	1250	625
2	+	78.125	78.125	4.88	312.5	0.61	4.88	0.61	156.25	2500	625
3	+	156.25	156.25	4.88	625	2.44	39.06	9.76	78.125	1250	625
4	-	156.25	78.125	4.88	312.5	2.44	39.06	4.88	156.25	1250	625
5	+	156.25	156.25	2.44	312.5	1.22	39.06	4.88	625	1250	625
6	-	39.06	39.06	0.61	39.06	0.305	2.44	2.44	156.25	2500	625
7	-	78.125	78.125	4.88	625	0.61	9.76	1.22	39.06	2500	625
8	-	78.125	156.25	9.76	312.5	2.44	39.06	4.88	78.125	2500	625
9	-	39.06	156.25	4.88	312.5	2.44	78.125	2.44	156.25	1250	625
10	+	156.25	78.125	4.88	625	0.305	39.06	2.44	78.125	2500	625
MIC_{50}		78.125	78.125	4.88	312.5	1.22	39.06	2.44	78.125	1250	625
MIC_{90}		156.25	156.25	9.76	625	2.44	39.06	4.88	156.25	2500	625
MIC range		39.06 – 156.25	39.06 – 156.25	0.61 – 9.76	39.06 – 625	0.305 – 2.44	2.44 – 78.125	0.61 – 9.76	39.06 – 625	1250– 2500	625
Susceptibility breakpoint		≥0.5 µg/ml	≥8.0 µg/ml	≥16.0 µg/ml	≥32 µg/mL	≥2 µg/ml	≥16 µg/ml	≥4 µg/mL	≥8 µg/ml	≥512 µg/m	≥4 µg/ml

AMX: Amoxycillin, CF: Ceftriaxone, GEN: Gentamicin, NA: Nalidixic acid, ENR: Enrofloxacin, TET: Tetracycline, RFM: Rifampicin, ERY: Erythromycin, TM: Trimethoprim, SM: Sulphamethoxazole;

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