

METHICILLINE RESISTANT STATUS OF COAGULASE NEGATIVE STAPHYLOCOCCUS ISOLATED FROM BOVINE MASTITIS

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ABSTRACT: Mastitis continues to be a major economic issue for dairy producers all over the world. A total of 100 milk samples from mastitic buffaloes and cows were subjected to bacteriological examination in the department of Veterinary Microbiology for the isolation of methicillin-resistant *Staphylococcus aureus*. 68 staphylococci spp. were isolated on the basis of colony morphology, haemolytic pattern on blood agar plates and growth on mannitol salt agar plates. Gram's staining; catalase test, clumping factor test, coagulase test and mannitol fermentation tests were performed for the confirmation of *Staphylococcus aureus* and *Staphylococcus epidermidis*. 52 *Staphylococcus aureus* and 16 *Staphylococcus epidermidis* were purified on mannitol salt agar slants. Nitrocefin disks (Fluka) were used for the detection of beta-lactamase activity of these staphylococci. Out of 52 spp. of *Staphylococcus aureus*, 16 (30.76%) were found positive for beta-lactamase production; similarly, out of 16 spp. of *Staphylococcus epidermidis* 4 (25%) were found positive for beta-lactamase production. 29.41% of isolated staphylococci were found positive for beta-lactamase production through nitrocefin test. This beta-lactamase producer was subjected to antimicrobial susceptibility testing through disk diffusion test using oxacillin 1µg and cefoxitin 10µg antibiotic disks. Twenty two 22 (32.35%) isolates of staphylococci from a total of 68 were resistant to these disks hence declared as methicillin-resistant staphylococci. These methicillin-resistant staphylococci were subjected to antibiotic sensitivity testing through disc diffusion test using enrofloxacin, norfloxacin, chloramphenicol and amoxycillin discs. The results of in vitro sensitivity tests showed that enrofloxacin was most effective followed by norfloxacin and chloramphenicol. Amoxycillin was found ineffective. The aspect of methicillin-resistance should be kept in mind for the treatment of staphylococcal mastitis.

Key Words: Mastitis, Dairy Industry, *Staphylococcus aureus*, antibiotic, Pakistan

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INTRODUCTION

Mastitis is inflammation of the parenchyma of the mammary glands regardless of the cause and is characterized by physical, chemical and bacteriological changes in the milk and pathological changes in the glandular tissue. Clinically mastitis is recognized by abnormal milk, gland swelling and/or illness of the affected animal. (Radostits *et al.*, 2000). The epidemiology of bovine intramammary infections (IMI) has been characterized worldwide by an increase in prevalence of staphylococci. The staphylococci are the predominant pathogen in subclinical and chronic bovine mastitis all over the world. With *staphylococcus aureus* mastitis in dairy bovine the resulting damage to

developing mammary tissue can reduce milk production and cause the bovine to fail in reaching her maximum milk production potential. (Guidry *et al.*, 1998).

Beside this, coagulase negative staphylococci (CNS) are increasing in importance as cause of bovine IMIs throughout the world in recent years. The studies showed that CNS can cause an 8.7 % loss in milk production from a 305 day milk yield (Timms and Schultz, 1987). Beta-lactam antibiotics are frequently used in IMI therapy. Bacterial resistance to beta-lactam mechanisms includes production of beta-lactamases and production of a low-affinity penicillin-binding protein, PBP2a (Odd and Maeland, 1997). The latter, designated as methicillin-resistance (MR), precludes therapy with any of the currently available beta-lactam antibiotics, and

may predict resistance to several classes of antibiotics besides beta-lactams among all staphylococci, MR is encoded by the *mecA* gene. (Archer and Climo, 1994). Certain CNS isolates have the ability to form biofilms that may interfere with local defenses and impair the activity of bacteriostatic agents such as macrolides when temporary or permanent synthetic devices are implanted.

The National Committee for Clinical Laboratory Standards (NCCLS), now called the Clinical and Laboratory Standards Institute (CLSI), recommends the cefoxitin or oxacillin disc screen test for the detection of methicillin-resistant staphylococcus as standard methods along with PCR for the detection of *mecA* gene responsible for encoding penicillin binding protein in staphylococcus spp. (CLSI, 2005).

Buffaloes and cows are the major dairy animals in Pakistan. Field surveys of major livestock diseases in Pakistan have indicated that mastitis is the major problem in the country (Cady *et al.*, 1983; Ajmal, 1990). Unfortunately there is no estimated cost of losses due to mastitis but it is thought that the probable losses are much higher because mastitis control programs are not usually followed. Moreover, unjustified use of antibiotics have led to the development of resistant strains and resulted in the increase cost of mastitis therapy. The present study was aimed to;

- Find out the prevalence of CNS in sub-clinical and clinical cases of mastitis in bovine.
- Application of nitrocefin (chromogenic cephalosporin) test to check the MR status of CNS isolates.
- Evaluate the susceptibility of CNS against commonly prescribed drugs of choice under field conditions.

MATERIAL AND METHOD

According to the National Mastitis Council guidance the milk samples for the culturing and isolation of staphylococci were collected from the mastitic udders of bovine determined positive by Surf Field Mastitis Test (SFMT). A total of 100 milk samples were collected from various private livestock farms around Faisalabad.

The blood agar was used as a general purpose medium and was prepared by using the procedure illustrated by Sullia&Shantharam, (1998). The agar was autoclaved at 121°C for 15 minutes at 15 lbs. It was cooled to 45-50°C, then aseptically added 50 ml of 5 % sterile defibrinated ovine blood. After gentle mixing, the media was dispensed in sterilized Petri plates for further use. The mannitol salt agar was prepared as a selective as well as differential medium for the isolation and purification of staphylococci (Sullia&Shantharam, 1998). The medium was autoclaved at 121°C for 15 minutes at 15 lbs. The inoculated blood agar plates were wrapped in plastic bags and incubated at 37°C for 24-48 hrs. The colony morphology and hemolytic pattern of various bacterial growths were studied. Gram staining were

performed for micrographic identification. Catalase test, coagulase test were also performed for biochemical test according described by NMC, 2004.

Coagulase negative staphylococci were tested for B-lactamase production by the nitrocefin test. A loopful of milk sample was smeared on the surface of nitrocefin disc and the results were recorded as colour change from pale yellow to pink or red.

Oxacilline (OX-1, 1µg oxoid,U.K.) was included for detection of methicilline resistant because it is more stable than methicilline and provide more reliable results of CNS (NCCLS,1999). Other discs used for the antimicrobial susceptibility of methicillin-resistant CNS were

1. Enrofloxacin (ENR-5, 5µg OxoidU.K.)
2. Amoxycillin (AMOX -25, 25µg OxoidU.K.)
3. Chloramphenicol (CHLOR-10, 10 µg OxoidU.K.)
4. Norfloxacin (NOR-10, 10µg OxoidU.K.).

After screening of Methicilline resistant CNS, they were subjected to antimicrobial disk diffusion testing using the same technique and antibiotic discs as mentioned. The zones of inhibition of each antibiotic disc were recorded and interpreted referring to Zone Diameter Interpretive Standards of CLSI and the organisms were reported as susceptible or resistant for each antibiotic disc.

RESULTS AND DISCUSSION

Mastitis is primarily associated with bacterial infection of various types but other infections like mycoplasma, mycotic or algal may also be involved. Staphylococci species are the most common cause of contagious mastitis. (Bagley, 1997).

Mastitis is common in dairy cows causing significant losses to the dairy industry and effects milk hygienic and sanitary features. Prevalence in dairy cattle approaches or exceeds 25% of quarters at any time. Staphylococci are gram-positive cocci (0.5-1.5µm) that occur singly, in pairs, tetrads, short chains and irregular grape-like clusters. They are non-motile and non-spore forming. On blood agar plates, staphylococci produce golden yellow colony pigmentation and haemolytic zone of beta Hemolysis. This is due to the beta toxin produced by staphylococcus cell. But the spp. of *s. epidermidis* does not show haemolysis on surface of ovine blood agar plates. Mannitol salt agar is used as a selective as well as differential medium for isolation of staphylococci. Only staphylococci can tolerate 7.5 % NaCl concentration in the medium. The colonies of mannitol-fermenter are golden yellow and they change the colour of the agar from red to golden yellow. Most of CNS isolates are mannitol non-fermenter and they give white colonies on mannitol salt agar. In this study similar pattern of growth was observed by the isolated staphylococci on blob agar

and mannitol salt agar as described by Freeman, (1979) and Power, (1988).

Gram's staining reveals the morphological characteristics of isolated species of staphylococci. The cocci form that was much more uniform in size occurring in irregular often grape like clusters and was stained

darkly by crystal violet was considered as *S. aureus*. Those isolates that were found singly, in pairs or in bunch and were stained lightly are thought to be of CNS. The results are in accordance with those described by Leslie *et al.* (1998).

Table 1. Efficacy of Enrofloxacin against Methicillin-Resistant Staphylococci.

| Characteristic | Methicillin-Resistant <i>Staphylococcus aureus</i> (MRSA) | Coagulase-Negative <i>Staphylococci</i> (CNS) |
|--------------------------------------|--|--|
| Prevalence in dairy cattle | 0.25 | 0.25 |
| Appearance on blood agar | Golden yellow colonies with a hemolytic zone | White colonies |
| Mannitol salt agar | Golden yellow colonies that change the color of the agar from red to golden yellow | White colonies |
| Gram staining | Irregular grape-like clusters of darkly stained cocci | Singly, in pairs, or in bunches of lightly stained cocci |
| Catalase production | Positive | Positive |
| Coagulase production | Positive | Negative |
| Resistance to β -lactam agents | Resistant | Variable |
| Disk diffusion test | Incubation at 35°C for 18 hours | Incubation at 33°C for 24 hours |
| Antimicrobial susceptibility | Resistant to penicillin family antibiotics | Resistant to amoxycillin |
| Beta-lactamase production | Positive | Positive |

All the isolated staphylococci produced Catalase enzyme when their colonies were mixed with H₂O₂ on clean glass slides. This character of staphylococci has also been described by Bisen and Kavita, (1998). Mannitol fermenter produces coagulase enzyme which converts soluble fibrinogen of rabbit plasma into insoluble fibrin resulting in its coagulation and other do not produce coagulase enzyme are called coagulase negative staphylococcus (Cruickshank *et al.*, 1975). Similar finding has also been obtained in this study.

Methicillin/oxacillin-resistant staphylococci are heterogeneous in their expression of resistance to β -lactam agents and the test conditions have a major effect on the expression and therefore the detection of resistance. Some rapid and/or automated methods are also available, including latex agglutination techniques for the detection of PBP_{2a}. The gold standard method for the detection of resistance mediated by *mecA* is PCR, which is most commonly used as a reference method at present. It was further reported that varying test conditions had major effects on the detection of resistance. This confusion is partly because there are marked differences between strains, and different populations of strains have been included in different studies.

The early evaluations of the single-disk agar diffusion tests described by Bauer *et al.* (1959) and by Ericsson (1960) were conducted before methicillin was commercially available. Hence, methicillin-resistant *Staphylococcus aureus* strains were not included in the preliminary studies of the disk diffusion test. Disk diffusion tests were incubated at 35 and 37°C for 18 and 48 h. The authors found that all isolates were correctly categorized as methicillin-resistant if plates were

incubated for 18 h at 35°C and results were not accurate when incubated at 37°C.

Sixty eight isolates of staphylococci (48 *S. aureus* and 20 of CNS) from bovine mastitis were evaluated in the present study through disk diffusion method for the detection of methicillin resistance using oxacillin disks. All the plates were incubated at 33° C for 24 hrs as recommended by Brown, 2001. The efficacy of results for detection of methicillin resistance improved. The results obtained in this study are in line with the observations of Boyce *et al.* (1984) and Boubaker *et al.* (2004). They compared two Oxacillin disc methods with a cefoxitin disc diffusion test for detection of methicillin resistant staphylococci using PCR for *mecA* as reference method. Testing with both Oxacillin and cefoxitin discs would give better sensitivity (100%) than the cefoxitin test alone, but at the expense of specificity (99.1%).

In the present study enrofloxacin was found most effective against methicillin-resistant staphylococci. The results obtained are similar to those obtained by Ganiere *et al.* (2001) who examined and compared the minimal inhibition concentrations (MICs) of enrofloxacin against 393 *Staphylococcus intermedius* strains isolated in France from canine pyoderms during three different years, 1995 (174 isolates), 1997 (101 isolates) and 1999 (118 isolates). Despite the increased use of fluoroquinolones, none of the strains were found resistant in 1997 and only two strains were found resistant in 1999. The resistance of amoxycillin to methicillin-resistant staphylococci was found in the present study; similar results were obtained by Lee, (2003). Antimicrobial susceptibility tests of *mecA*-positive MRSA strains were performed by the disk diffusion

method. All isolates were resistant to members of the penicillin family, such as ampicillin, oxacillin, and penicillin.

Nitrocefin is the chromogenic cephalosporin developed by Glaxo Research Limited. (Coded 87/312; 3-(2, 4 dinitrostyl) - (6R, 7R-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer). This compound exhibits a rapid distinctive colour change from yellow (max at pH 7.0 = 390nm) to red (max at pH 7.0 = 486nm) as the amide bond in the beta-lactam ring is hydrolyzed by a beta-lactamase, it is sensitive to hydrolysis by all known lactamases produced by Gram-positive and Gram-negative bacteria (Callaghan *et al.*, 1972). Nitrocefin discs were used for the detection of beta-lactamase production by staphylococcus isolated from bovine IMI by Watts and Salmon(1997) and Gentilini *et al.* (2002).they used the isolated CNS for antibiotic sensitivity against various antibiotics. Similar method was adopted in this study and almost similar results were obtained. Mastitis milk sample were also directly used for detection of beta-lactamase produce by coagulase negative staphylococci and satisfactory results were obtained.

Conclusions: Methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci (CNS) are both common causes of mastitis in dairy cows. MRSA is more resistant to antibiotics than CNS, but both species can produce beta-lactamase, which can break down β -lactam antibiotics. The disk diffusion test is a reliable method for detecting methicillin resistance in staphylococci. Enrofloxacin is the most effective antibiotic against MRSA, while amoxycillin is the least effective. Nitrocefin discs can be used to detect beta-lactamase production in staphylococci.

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