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RESEARCH ARTICLE

Application of Nitrocefin Test for the Direct Detection of Methicillin Resistant *Staphylococcus aureus* from Bovine Mastitis Milk Samples

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ABSTRACT

Mastitis continues to be a major economic issue for dairy producers in most parts of the world including Pakistan. *Staphylococcus (S.) spp.* are frequently isolated from bovine and bubaline mastitis. The indiscriminate use of antibiotics has led to the development of resistant strains of staphylococci and this factor contributed to the difficulties in its elimination from dairy herds leading to heavy economic losses to the industry. Milk samples were collected from mastitic buffaloes and cows from livestock farms around Faisalabad, Pakistan and one hundred were selected on the basis of surf field mastitis test. Isolation, identification and bacteriological examination were carried out through growth on blood agar, Grams' staining, catalase, clumping factor, coagulase and mannitol fermentation test. The purification of staph isolates was done on mannitol salt agar. Disc diffusion (oxacillin & cefoxitin) and nitrocefin tests were used to detect the production of β-lactamase by staphylococci and hence called methicillin-resisitant staphylococci (MRS). The MRS were subjected to antimicrobial susceptibility testing through disc diffusion method against antibiotics including enrofloxacin (5µg), norfloxacin (10µg), amoxycillin (25µg) and chloramphenicol (10µg). Out of 100 mastitis smaples, 68 were carrying staph *spp.* including *S. aureus* (76.47%) and *S. epidermidis* (23.53%). Nitroceffin test detected 30.76% as MRS. *aureus* (MRSA) and 25% coagulase negative methicillin resistant *S. epidermidis*. The disc diffusion test detected 32.69% as MRSA and 37.5% as MRSE. Nitrocefin discs applied directly to 25 milk samples, could detect ~25%, while, disc diffusion test could identify 40% as MRS. Antimicrobial sensitivity test revealed that enrofloxacin was most effective followed by norfloxacin and chloramphenicol. While, amoxycillin was found resistant. The use of nitrocefin test proved better than disc diffusion method for the detection of MRS. Furthermore, aspect of methicillin-resistance must be considered while suggesting the treatment of mastitis due to *Staphylococcus spp* to minimize the losses.

INTRODUCTION

Bovine mastitis, a multi-factorial disease, is characterized by physical, chemical and microbiological changes in the milk and pathological changes in the glandular tissue of udder. The infectious agents causing bovine mastitis are commonly classified into two categories as contagious and environmental mastitis. Staphylococcus (*S.) aureus* falls under the category of major contagious pathogen (Kaung and Kwon, 2011). Elimination of this organism from dairy herds requires treatment of infected mammary glands

with antimicrobial agents and aggressive involuntary culling of refractory animals (Matthews et al., 1992; McDowell et al., 1995). It imposes severe economic losses for the livestock farmers (Pitkala et al., 2004; Pyorala and Taponen, 2009; Thorberg et al., 2009). According to Ratafia (1987), worldwide annual losses caused by this disease were nearly 35 billion US dollars (Khan and Muhammad, 2005). In Pakistan, the losses due to mastitis are even higher because of not practicing the techniques of mastitis prevention like teat dipping and dry period antibiotic therapy (Arshad, 1999).

S. aureus is an important opportunistic pathogen both in humans and in dairy cattle. It is also a common cause of mastitis in dairy cows (Trinidad et al., 1990; Waage et al., 1990). The use of antimicrobial agents on dairy farm animals is a major concern in the emergence of resistant zoonotic bacterial pathogens (Piddock, 1995).

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates are frequently multidrug resistant (Cosgrove, 2006) and has been associated with infections in human and animals (Witte et al., 2007). First incidence of MRSA was recorded in animals (Devriese and Hommez, 1975). The presence of MRSA in bovine mastitis is a potential risk to other exposed cattle and farm workers including veterinarians (Juhász-Kaszanyitzky et al., 2007).

Beta-lactam antibiotics are frequently used in mastitis therapy and the resistance to this is due to production of beta-lactamases and low-affinity penicillin-binding protein, PB2a (Brakstad and Maeland, 1997). *S. aureus* isolates are often resistant to other classes of antibiotics (through different mechanisms) (CLSI, 2005, 2006) making treatment options limited. Such types of isolates are designated as Methicillin-resistant *S. aureus* (MRSA). Clinical and Laboratory Standards Institute (CLSI), recommends cefoxitin or oxacillin disc screen test for the detection of MRS as standard method along with PCR for the detection of *mec A* gene responsible for encoding PBP 2α in staphylococcus spp. (CLSI, 2005). Nitrocefin test has been applied with reliability for the detection of beta-lactamase producing *Staphylococci* (Montgomery et al., 1979; O'Callaghan et al., 1972), Haemophilus spp. (Skinner and Wise, 1977).

Buffaloes and cows are 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). 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. The present study was designed for isolation and identification of *S. aureus* from mastitis milk samples. The MRSA detection through nitrocefin and cefoxitin & oxacillin disc tests would help in finding the animals infected with MRSA or MRSE and highlighting the appropriate treatment options for reducing the losses to dairy industry of Pakistan.

MATERIALS AND METHODS

Collection of milk samples

One hundred milk samples were collected from mastitic cows and buffaloes from private livestock farms present in surroundings of Faisalabad. The selection of samples was based on clinical signs California mastitis Test (CMT). Seventy five samples were collected on the basis of clinical signs and 25 on CMT. The guidelines of NMC were followed for sample collection, transportation, culture and isolation of staphylococci. The samples were then transported on ice to the Bacteriological laboratory, Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan.

Culture isolation and identification of *Staphylococcus aureus*

Blood agar medium (Sullia and Shantharam 1998) and MSA (Sullia and Shantharam, 1998) were prepared in the laboratory and used as general purpose and selective medium respectively for the growth of bacteria present in the samples. The colony morphology and haemolytic pattern of RBCs on blood agar plates due to various bacterial growths were observed and recorded. Cultured bacteria were put to battery of tests for identification. Heat fixed smears on grease free glass slides were prepared and stained by Gram's method as described by (Awan and Rehman, 2002). The guidelines of (NMC, 1981) were followed for detection of catalase, clumping and coagulase forming ability of isolated staphylococci. For tube coagulase test positive control of *staphylococcus aureus* ATCC 25923 was incorporated for reliability of the tests (NMC, 1981).

Nitrocefin test

Nitrocefin (chromogenic cephalosporin) discs (Fluka 49862) were used for the detection *mec A* mediated resistance of MRSA. For quality control of each test, S. aureus strains positive (ATCC 25923) and negative (ATCC 29213) controls were used (Watts and Salmon, 1997; Broekema et al., 2009). The change in colour of disc from blue to maroon or red was recorded and considered as positive. Nirocefin disc were also applied directly to mastitis milk samples to assess the presence of MRSA.

Oxacillin and cefoxitin disc diffusion test

Oxacillin 1 µg disc (OX-1, Oxoid, UK) and cefoxitin 10 µg disc (FOX-10, Oxoid, UK) were also used to identify *mec A* mediated resistance of staphylococci through disc diffusion method (CLSI, 2006; Broekema et al., 2009). For the purpose, Mueller-Hinton agar plates were used with pre added 2% NaCl and incubation temperature was 33 ^{o}C for 24 hrs. Zones of inhibition were measured and interpreted as (CLSI,

2007). Oxacillin and cefoxitin disc diffusion test was also applied directly to mastitis milk samples to assess the presence of MRSA.

Antibiotic susceptibility test

Antimicrobial susceptibility testing was carried out at equivalent to 0.5 McFarland turbidity standard by agar disc diffusion method on Mueller-Hinton agar plates following the guidelines of (CLSI, 2007). For the screening of MRS, antibiotic discs were used (Kircher et al., 2004; Skov et al., 2005). Commercially available antibiotic discs (Oxoid, UK) were used in the study viz Enrofloxacin (ENR-5, 5µg), Amoxicillin (AMOX-25, 25µg), Chloramphenicol (CHLOR-10, 10µg) and Norfloxacin (NOR-10, 10µg). The sizes of the zone of inhibition were recorded and interpreted according to (CLSI, 2007) and were reported as either bacteria were susceptible or resistant to the exposed agent.

RESULTS

Isolation and identification of mastitis culture isolates

Clinical milk samples showed 37.3% (28/75) positivity through nitrocefin test, while 24% (6/24) of the subclinical milk samples were identified as carrying MRSA positive status. Bacterial cultures (Staphylococci) from mastitic samples were obtained on blood agar and mannitol salt agar plates. The colonies of haemolytic staphylococci were smooth, circular, moist, 1-2 mm in diameter, pinhead, raised, convex having entire margins and yellow or creamy gold in color. The colonies of non-haemolytic staphylococci were circular, pinhead, raised, convex with entire margins and white in color. Both non- and haemolytic colonies were selected and streaked on blood and MSA plates for isolation of pure cultures. Various identification tests were applied on these cultures and out of 100 milk samples, 52 isolates were identified as *S. aureus* and 16 as *S. epidermidis* giving the total of 68. Hemolytic zones on sheep blood agar plates were detected in 76% (52/68) while remaining samples i.e. 24% (16/68) were non-hemolytic. Among 68 isolated staphylococci, 52 (76%) were mannitol fermenter and 16 (24%) were mannitol non-fermenter. The colonies of staphylococci that fermented mannitol were suspected of *S. aureus* and that of mannitol non-fermenter were *S. epidermidis*. The brief summary of results of catalase, coagulase, mannitol fermentation and biochemical tests were given in Table 1. Upon Gram's staining, the cocci forms with more uniformity in size and darkly stained were considered *S. aureus* and cocci found singly, in pairs and in bunches and lightly stained were thought as S. *epidermidis*.

Detection of methicillin-resistant *Staphylococci*

Nitrocefin disc test could detect 30.76% (16/52) microbial isolates as MRSA. While, percentage of methicillin resistant *S. epidermidis* detected with nitrocefin test was 25% (4/16). Referring to zone standards of CLSI, 23 (33.82%) out of 68 isolates of staphylococci were found methicillin-resistant through disc diffusion method using oxacillin 1µg and cefoxitin 10 µg discs. Disc diffusion test detected 32.69% (17/52) as MRSA and 37.5% (6/16) as coagulase negative *S. epidermidis* (Table 2).

Application of nitrocefin test directly on 25 mastitis milk samples detected 6 (24%) and oxacillin and cefoxitin discs detected 10 (40%) as MRS. Nitrocefin and disc diffusion tests findings of the present study showed that former test may be used with reliability for the detection of MRSA from isolated cultures and directly from mastitic milk samples.

Antibiotic sensitivity testing

Antibiotic sensitivity testing of methicillin-resistant *S. epidermidis* showed that enroflaxacin was most effective followed by norflaxacin and chloramphenicol. MRSA and *S. epidermidis* were resistant to amoxycillin (Table 4). MRSA were most sensitive to enrofloxacin (88.25%) followed by norflaxacin (76.47%) and chloramphenicol (58.82%). Amoxicillin were found resistant to all MRSA (Table 3). For coagulase negative *S. epidermidis*, the %age antimicrobial sensitivity was enrofloxacin (100%), norfloxacin (88.33%) and chloramphenicol (50%). While, amoxicillin was resistant for these microorganisms (Table 3).

DISCUSSION

Mastitis is recognized as the most costly disease in dairy cattle. The high losses are due to decreased milk production (10-26%), dumped milk after antibiotic usage, veterinary, labour, culling and death costs and low milk quality premiums due to increased somatic cell count and decreased milk fat.

The microorganisms which produced hemolytic zones of beta-haemolysis were *S. aureus* and those which did not were *S. epidermidis* and same was described by Freeman (1979). Upon Gram's staining of isolated microorganisms, the one which were occurring in grape like clusters and stained darkly by crystal violet were *S. aureus*. Contrary to this, the one which were occurring in single, pairs or in bunches and stained lightly were *S. epidermidis* and same was described by Leslie et al., 1998. The findings of present study for other biochemical tests including coagulase production, catalase and mannitol salt fermentation were according to previous works of Bisen and Verma (1998).

In present study, *S. aureus* was detected in 76% and coagulase negative *S. epidermidis* in 24% of clinical mastitis cases. Pyörälä and Taponen (2009) and Taponen et al. (2006) also found that the proportion of coagulase negative *S. epidermidis* is low as compared to *S. aureus* in clinical milk samples. Waller et al.

Table 1: Comparison of Biochemical characteristics of *S. aureus* and *S. epidermidis* isolated from bovine milk samples collected from Faisalabad District

Species	Catalase Test		Clumping Factor		Coagulase Test	
	Positive	Negative	Positive	Negative	Positive	Negative
S. aureus	52	Nil	48	4	52	Nil
S. epidermidis	16	Nil	Nil	16	Nil	16
Total	68	Nil	48	20	52	16

Table 2: Comparison of Nitrocefin with Oxacillin and Cefoxitin discs for the detection of coagulase positive MRSA and *S. epidermidis*

Antibiotic Disc	No of isolates Tested	Methicillin-resistant	Methicillin-sensitive	Percentage of MRSA
Coagulase positive MRSA				
Nitrocefin	52	16	36	30.76
Oxacillin 1µg & Cefoxitin 10µg	52	17	35	32.70
Coagulase positive *S. epidermidis*				
Nitrocefin	16	4	12	25
Oxacillin 1µg & Cefoxitin 10µg	16	6	10	37.5

Table 3: Pattern of antimicrobial susceptibility of coagulase positive MRSA and coagulase negative methicillin-resistant *S. epidermidis*

Antimicrobial Discs	Zone of Inhibition (mm)				Total Isolates	Resistant	Susceptible	%age Sensitivity
	1-10	11-20	21-30	≥30				
Coagulase positive MRSA								
ENR-5µg	1	1	10	5	17	2	15	88.23
NOR-10 µg	1	3	8	5	17	4	13	76.47
CHLOR-10µg	3	4	6	4	17	7	10	58.82
AMOX-25 µg	12	5	Nil	Nil	17	17	Nil	0.00
Coagulase negative methicillin-resistant *S. epidermidis*								
ENR-5µg	Nil	Nil	4	2	6	Nil	6	100
NOR-10 µg	1	Nil	3	2	6	1	5	88.33
CHLOR-10µg	1	2	3	Nil	17	3	3	50.00
AMOX-25 µg	5	1	Nil	Nil	6	6	Nil	0.00

(2011) recently showed that the most common strain isolated in subclinical mastitis cases was *S. epidermidis* and its prevalence was lower in clinical mastitis cases.

The detection of *mec A* gene by PCR is now a gold standard for the detection of methicillin resistance (Boubaker et al., 2004; Ganiere et al., 2001). Sixty eight isolates of Staphylococci from bovine mastitis were evaluated in the present study through disc diffusion method for the detection of methicillin resistance using oxacillin as well as cefoxitin disc. The use of both the discs for detection of MRS was also supported byBoubaker et al. (2004); Boyce et al. (1984), they compared two methods including oxacillin and cefoxitin disc diffusion test for detection of MRSA, with PCR for *mec A* as the reference method. Testing with both oxacillin and cefoxitin disc would give better sensitivity (100%) than cefoxitin disc alone, but at the expense of sensitivity (99.1%).

In the present study the enrofloaxacin was found most effective against MRSA and results obtained are similar to those obtained by (Ganiere et al., 2001; Kenar et al., 2012). All MRS were resistant to members of the penicillin family, such as ampicillin, oxacillin and penicillin.

Nitrocefin is a chromogenic cephalosporin developed by Glaxo research limited. (Coded 87/312: 3-2, 4 dinitrostryl)-(6r, 7r-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer). This compound exhibit a rapid distinctive color change from yellow (max at PH=7.0=390nm) to red (max at PH 7= 486nm). As the amide bond in the beta lactam ring is hydrolysed by a beta lastamase, it is sensitive to hydrolysis by all known lactamase produced by Gram positive and Gram negative bacteria (O'Callaghan et al., 1972). Nitrocefin discs were used for the detection for beta lastamase production by staphylococcus isolated from bovine IMI by (Gentilini et al., 2002; Watts and Salmon, 1997).

Moreover, there was higher incidence (34%) of methicillin resistant carrying pathogens as detected through the clinical nitrocefin test as compared to MRSA (16/52) and *S. epidermitidis* (4/16) combined involvement (29.4%) in the mastitis milk samples,

which may show some indications regarding the involvement of other pathogens than *Staphylococcus spp*. Present study would help in the early detection of methicillin resistant status of milk from the infected animals and antibiotic susceptibility findings would help the field veterinarians to treat the animals with even good antibiotics to reduce the losses. Further studies using PCR amplification of *mec A* gene for confirmation of MRS may be initiated as gold standard test.

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