Research Article

Characterization and Antimicrobial Sensitivity of Staphylococcus aureus Isolates from Subclinical Bovine Mastitis

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INTRODUCTION
Mastitis is single largest problem in dairy animals causing economic losses in the tune of Millions of rupees annually in India. Although, Mastitis is a multifactorial disease, Staphylococcus aureus is the primary and probably the most lethal agent that causes chronic and deep infection in the mammary glands which is extremely difficult to be cured (Saravanan et al., 2000; Gianecheini et al., 2002; Wani et al., 2003). Rapid identification of such pathogenic bacteria can significantly reduce the time and cost of testing, resulting in timely cure, low cost of treatment and can significantly reduce production losses. The traditional method of bacterial culture is labor intensive and time consuming. Therefore, rapid PCR based methods have been developed to identify real pathogens. Although, very few strains of Staph aureus do not produce detectable amount of coagulase, all strains seem to possess a coagulase gene (Coa) (Vandenesch et al., 1994). Similarly protein A, which is the major surface proteins of staphylococci (Spa), binds immunoglobin G and impairs opsonisation by serum complement and phagocytosis by polymorphonuclear leukocytes (Gao and Stewart, 2004). Development of PCR based methods for detection of such genes enabled rapid identification of virulent Staph aureus (Riffon et al., 2001; Strommenger et al., 2006). The single most common use of antimicrobial agents in dairy cattle is for prevention and treatment of bovine mastitis (Kanencne and Miller, 1992; Moore and Heider, 1984). The resistance of Staph aureus to antimicrobial agents has been extensively documented and it contributed significantly to the treatment failure (Sumathi et al., 2008; Sudhakar et al., 2009; Kumar et al., 2010). Present investigation reports the isolation and identification of pathogenic Staphylococcus aureus in the subclinically mastitic milk samples and their antibiogram.

MATERIALS AND METHODS
A total of 214 quarter milk samples were collected from lactating cows as per the recommendations of National Mastitis Council (NMC, 1990), transported on ice to Microbiology laboratory for further processing. The samples were subjected to California mastitis test (CMT) according to the procedure described by Quinn et al. (1994) and were graded as negative, trace(+1), weak(+2), distinct (+3), or strongly positive (+4). A total of 68 milk samples, graded as distinct and strongly positive, were selected for isolation of Staph aureus using Mannitol salt agar as selective media. The isolates were characterized by Gram's staining, haemolysis on 7% sheep blood agar and biochemical tests viz. Catalase test, Coagulase test (bound as well as free coagulase), nitrate test and fermentation of glucose, mannitol and maltose sugars (NMC, 1990, Quinn et al., 1994).

The isolates were tested for the presence of coagulase (Coa) and Protein A (Spa x region) genes specific for virulent Staph. aureus using PCR. The heating technique described by Franco et al. (2008) was used for preparation of DNA template. The isolates were grown overnight at 37°C in brain heart infusion broth and 1 ml of the culture was centrifuged to collect the pellet. The pellet was suspended in 100 μl of distilled water into eppendorf tubes. The cellular suspension was brought to a boil for 10 min, and immediately was centrifuged at 14,000 RPM.
for 5 min. The supernatant was directly used for the PCR assay. The coagulase (Cox) and Protein A (Spa X region) gene specific primers (Table 1), synthesized by Merck (India) were used. PCR conditions used for amplification of Cox gene were: 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 58°C for 45 sec and 72°C for 1 min, followed by a final extension of 72°C for 10 min. and for amplification of Spa gene were: 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 10 min. The amplified PCR products were detected by electrophoresis in 1.5% agarose gel prepared in 0.5X TBE buffer as per the method of Sambrook et al. (1989) and analyzed using Gel documentation system (Uvi Tech).

Antimicrobial sensitivity of the isolates was tested for Tetracycline, Ofloxacin, Cephalexin, Ciprofloxacin, Ampicilline, Lincomycin and Cepahlexin using the modified disc diffusion method of Bauer et al. (1966).

RESULTS AND DISCUSSION

In present study, out of 214 quarter milk samples collected from lactating cows, 110 (51.40%) samples were found positive in California Mastitis Test (CMT). Of these 18 (8.41%), 24 (11.21%), 52 (24.30%) and 16 (7.48%) samples exhibited trace, weak positive, distinct and strong positive CMT reactions respectively. CMT has been recognized as a highly sensitive test to detect bovine SCM (Dangore et al., 2000; Madut et al., 2009).

Staph. aureus was isolated from all 68 samples that showed distinct (52) and strong positive reaction (16) in CMT. The Staph. aureus has been reported as the major pathogen involved in bovine mastitis (Singh and Baxi, 1982; Hameed et al., 2006; Sudhakar et al., 2009; Ranjan et al., 2011) and its association with subclinical mastitis as a major pathogen has been reported from various parts of the country (Ranjan et al., 2011). Out of 68 isolates tested, 65 (93.59 %) isolates showed catalase positive reaction. In Coagulase test all the isolates showed Coagulase activity. Of these, 54 (79.41%) and 56 (82.35 %) samples were found positive in slide and tube Coagulase test respectively. Further, 12 (17.65%) samples were positive for tube Coagulase test only and 14 (20.59) samples were positive in slide test, while 42 (61.76 %) samples exhibited positive reaction both in slide as well as tube Coagulase tests. In sugar fermentation reaction the mannitol was fermented by all the 68 isolates with production of acid and gas, while glucose was fermented with production of acid and gas by 59 (86.74 %) and 48 (70.58 %) isolates and lactose was fermented by 53 (77.94 %) and 52 (76.47 %) isolates respectively. On blood agar, 48 (70.58 %) isolates showed β haemolysis and 59 (86.76 %) isolates were positive for nitrate reduction tests.

The coagulase production is important phenotypic determinant of Staph. aureus association with virulence (Loebe, 1993; Jeljaszewicz et al., 1983). Coagulase positive Staphylococci were reported to be mostly positive to mannitol fermentation (Higgins and Chartier, 1984; Erasmus, 1985). β haemolysis is another trait used extensively for characterization of pathogenic Staph. aureus (Arshad et al., 2006; Franco et al., 2008.). Bovine mastitis is the single most common ailment for the use of antibiotics in dairy cattle (Kanecne and Miller, 1992; Moore and Heider, 1984). Out of 68 isolates tested highest number of isolates (63, 92.64 %) were resistant against ampicillin followed by 20 (29.41 %) for tetracycline and 17 (25%) for ofloxacin. Eleven (16.7%) isolates showed resistant to lincomycin and 9 (13.23%) were found to be resistant for ciprofloxacin. All the isolates were found to be sensitive to cephalotaxine. Out of 68 isolates tested, 36 isolates were resistant to single antibiotic (Table-2). Of these, 36 (52.94 %) isolates were resistant to ampicillin and one for lincomycin. Remaining 32 isolates exhibited multiple drug resistance involving two and more antibiotics. Of these, 16 (23.52 %) isolates were resistant to two antibiotics, 13 (19.11 %) to three antibiotics, 2 (2.94 %) to four antibiotics and one (1.47 %) isolate exhibited resistance to

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates tested</th>
<th>Combination of resistance antibiotic</th>
<th>Name of antibiotic (number)</th>
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<tbody>
<tr>
<td>Staph. aureus</td>
<td>68</td>
<td>T + OF + A + L + CF (1)</td>
<td>Tetracycline; OF-Ofloxacin; C-Cephalexine; CF-Ciprofloxacin; A-Ampicilline; L-Lincomycin; CF-Cepahlexin</td>
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five antibiotics. The most common group of multiple drug resistance was observed to be tetracycline + oxolinic + ampicillin + ciprofloxacins in 9 isolates, followed by ampicillin + ciprofloxacins (7 isolates), ampicillin + oxolinic (5 isolates). One isolate showed multiple resistance for 5 antibiotics viz. tetracycline + oxolinic + ampicillin + lincomycin + Ciprofloxacins. The resistance of Staph. aureus to antimicrobial agents has been extensively documented and it contributed significantly to the treatment failure (Sumathi et al., 2008; Sudhakar et al., 2009; Kumar et al., 2010). The high resistance of Staph. aureus to ampicillin may be attributed to the production of beta lactamase, an enzyme that inactivates penicillin and closely related antibiotics (Abera et al., 2010). It is believed that around 50% of mastitis causing Staph. aureus strains produce beta lactamase (Green and Bradely, 2004).

In PCR, the Coa gene could be amplified in all the 68 isolates with a uniform amplicon size of 710 bp. The presence of Coa gene in all the Staph. aureus isolates is due to the fact that all the isolates included in the study, are coagulase producers as evident in biochemical tests. In the last few years, the Coa gene analysis has been extensively used for typing Staph. aureus isolates identified from bovine mastitis cases (Aarestrup et al., 1995; Lange et al., 1999; Schlegelova et al., 2003). In present study, 22 (32.3%) isolates yielded a uniform SpA (X region) gene product of 320bp. Staphylococcal protein A (Spa) is an important virulence factor of Staph. aureus. The Spa gene of Staph. aureus encodes protein A and is used for typing of Staph. aureus (Harmsen et al., 2003). The isolates showing Spa gene were also positive for coagulase, catalase and nitrate tests and all fermented glucose, lactose and mannitol sugars on biochemical characterization. Single-locus DNA sequencing of repeat regions of the Coa and the Spa gene could be used for reliable and accurate typing of Staph. aureus strains (Frenay et al., 1996; Shopsin et al., 2000; Tang et al., 2000).

REFERENCES
Green M and Bradely A (2004). Clinical forum Staph aureus mastitis in cattle UK Vet. 9:

