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Research Article

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

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ABSTRACT

Pathogenic *Staphylococcus aureus was* isolated from the cases of subclinical mastitis in lactating cows. A total of 214 quarter milk samples were screened of which, 110 (51.40%) samples were found positive for subclinical mastitis (SCM) using California Mastitis test (CMT). Isolation of *Staph. aureus* was attempted in 68 samples, comprising 52 distinct and 16 strong CMT positive samples. All 68 samples yielded *Staph. aureus*. All the isolates were catalase positive while 54 (79.41%) and 56 (82.35%) isolates showed positive slide and tube coagulase test, respectively. On sugar fermentation all the isolates were positive for mannitol fermentation, while 59 (86.76%) and 53 (77.94%) isolates fermented glucose and lactose. On sheep blood agar, 48 (17.58%) isolates showed β-haemolysis and 59 (86.76%) samples were positive for nitrate reduction test. *Coa* gene was detected in all the *Staph. aureus* while 22 (32.35%) isolates revealed *Spa* (X region) gene. All the isolates were sensitive to Cefotaxime while highest number of isolates 63 (92.64%), were found resistant to ampicillin, followed by 20 (29.41%) for tetracycline, 17 (25.00%) for ofloxacin, 11 (16.17%) for lincomycin and 09 (13.23%) for ciprofloxacin, respectively.

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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 multietiological 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; Gianeechini et al., 2002; Wani et al., 2003). Rapid identification of such pathogenic bacteria can significantly reduce the time and cost of testing, resulting into 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 immunoglobulin 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 (Kaneene 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 37^0C 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

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for 5 min. The supernatant was directly used for the PCR assay. The coagulase (*Coa*) and Protein A (*Spa* X region) gene specific primers (Table -1), synthesized by Merck (India) were used. PCR conditions used for amplification of *Coa* 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).

Table-1: oligonucleotide primers used for PCR

Sequence name	Sequence (5' ⟶ 3')	Target	PCR amplicon	
			Size (bp)	Reference
Coa 1 (F)	CGAGACCAAGATTCAACAAG	Coagulase	710	Himabindu et al. (2009)
Coa-2 (R)	AAAGAAAACCACTCACATCA			
Spa-III (F)	CAA GCA CCA AAA GAG GAA	*Spa* (X region)	320	Frenay et al., (1996)
spa-IV	CAC CAG GTT TAA CGA CAT			

Antimicrobial sensitivity of the isolates was tested for Tetracycline, Offloxacin, Cephotaxime, Ciprofloxacin, Ampicilline, Lincomycin and Cephalexin 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 (95.59 %) 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.

Table 2: Multiple drug resistance pattern of *Staph. aureus* from mastitic milk

Organism	No. of isolates tested	Combination of resistance antibiotic		Name of antibiotic (number)
		No. of antibiotic	No. of isolates	
Staph. aureus	68	1	36 isolates (52.94%)	A (35) L (1)
		2	16 isolates (23.52%)	A + CF (7) T + L (4) OF + A (5)
		3	13 isolates (19.11%)	T +A + L (3) T + A+ CF (1) T + OF + A (9)
		4	2 isolates (2.94%)	T + OF+ A+ L (2)
		5	1 isolates (1.47%)	T + OF + A + L + CF (1)

T- Tetracycline; OF-Offloxacin; C-Cephotaxime; CF- Ciprofloxacin; A- Ampicilline; L- Lincomycin; CF- Cephalexin

The coagulase production is important phenonotypic determinant of *Staph. aureus* association with virulence (Loebe, 1903; 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 (Kaneene 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 offloxacin. Eleven (16.7%) isolates showed resistant for lincomycin and 9 (13.23%) were found to be resistant for ciprofloxacin. All the isolates were found to be sensitive to cefotaxime. 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

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five antibiotics. The most common group of multiple drug resistance was observed to be tetracycline + offloxacin + ampicillin recorded in 9 isolates, followed by ampicillin + ciprofloxacin (7 isolates), ampicillin + offloxacin (5 isolates). One isolate showed multiple resistance for 5 antibiotics viz. tetracycline + offloxacin + ampicillin + lincomycin + Ciprofloxacin. 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 betalactamase, 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 betalactamase (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 caogulase 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 (32.35%) 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., 20002).

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