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Virulence, enterotoxigenicity and antibiotic profile of *Staphylococcus aureus* from buffalo clinical mastitis

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Staphylococcus aureus is responsible for 40–70% of all intra-mammary infections in bovine (Wilson *et al*. 1997). Their ready transference from human and animal body to a variety of food stuffs, accounts for their role as an important cause of food poisoning as well as zoonotic threat. It leads to great economic losses in the dairy industry, attributed mainly to mastitis.

Widespread use of antimicrobial drugs for treatment of mastitis has favoured the selection of resistant *S. aureus* strains. Because of its intracellular nature the bacteriological cure rate of this organism in infection is relatively low (Bramley 1992). Resistance to antimicrobial agents in *S. aureus* associated mastitis has 2 relevant aspects. First, is a reduction in cure rates after treatment of clinical mastitis cases (Sol *et al*. 2000) and; the second is the potential impact of transmission of resistant bacteria to humans via raw milk and their milk products. Progressive emergence and rapid dissemination of antimicrobial resistance in *S. aureus* constituting a major public health concern globally (Chambers 1997, Martynez and Baquero 2000) with the identity of their virulence characters that principally relate the pathogenicity. However, drug resistance alludes to link the public health potential of this organism by categorizing whether the virulence associated genes are more frequent in drug resistant *S. aureus* than their drug susceptible population.

S. aureus from persistent intra-mammary infection (IMI) produces a spectrum of extracellular protein toxins, viz. haemolysins, leukoudins, exfoliative toxins, enterotoxins, toxic schock syndrome toxin (tst), antibiotic resistant genes, pyrogenic toxin superantigen (PTSAg) etc. Among these, staphylococcal enterotoxins (SE), are the major exotoxins responsible for food intoxications and have the most widespread potential significance for human health as well as dairy products safety (Srinivasan *et al.* 2006).

Phenotypic expression of virulence characters (e.g. coagulase, thermonuclease and enterotoxin production) of *S. aureus* usually depends on culture conditions and thus characterization is time taking effort; however, molecular techniques, particularly polymerase chain reaction (PCR) asserted the presence of specific gene sequences by amplification of signature DNA could provide a more sensitive and quick estimate of the potential for *S. aureus* mastitis isolates (Haveri *et al*. 2007). In Indian context, no synchronized study has been reported on the status of virulence and enterotoxigenic genes among antibiotic resistant *S. aureus* in comparison to their antibiotic sensitive population. Keeping the above points in consideration, the present study was designed to determine the virulence and entertoxigenicity profile of antibiotic resistant and sensitive isolates of *S. aureus* in buffalo mastitis and also to understand the association of these factors in context to public health.

Composite quarter milk samples from buffaloes (75) with history of clinical mastitis of different periurban herds. The milk samples were collected aseptically and transported to the laboratory on ice within 1 h of collection. Each sample was given a pre-enrichment in brain heart infusion (BHI) broth for overnight at 37°C. Subsequently seeded on to the Baird-parker agar with egg-yolk emulsion and the plates were incubated at 37°C for 24 to 72 h. *S. aureus* suspected colonies (distinct black in color, with or without opalescence around colonies) were subjected to biochemical tests for catalase, oxidase and anaerobic glucose and mannitol fermentation (Cowan and Steel 1993) and thermostable nuclease activity (Lachica *et al*. 1972). Antimicrobial susceptibility test was performed on Mueller hinton agar by disc diffusion method (NCCLS, 2002) using penicillin G (10units), cephotaxime (5µg), amoxyclav (30µg), cloxacillin (10 µg), ciprofloxacin (30µg) and methicillin (30µg) antibiotics discs.

PCR assays were performed using published primers and protocols directed against coagulase (*coa*) (Akineden *et al.* 2001), thermonuclease (*nuc*) (Brakstad *et al.* 1992) and

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enterotoxin *se*A, *se*B and *se*C genes (*ent*A, *ent*B and *ent*C) (Sharma *et al.* 2000) to evaluate pathogenic potential of *S. aureus* isolates.

Template DNA was prepared by heating and chilling method and such DNA diluted in 1: 10 was used as template**.** *S. aureus* (Cowan-I) and *E. coli* K_{12} strain were used as positive and negative control, respectively in all PCR assays. PCR amplified product was electrophoresed in 1.5% agarose gel, stained with ethidium bromide, visualized and documented using gel documentation system.

PCR amplicon for *ent*A and *ent*B was purified from gel using the gel extraction kit. Sequencing of purified DNA of *ent*A and *ent*B was done through the kind courtesy of National Institute of Cholera and Enteric Diseases, Kolkata, and nucleotide sequences were compared to the published sequence data using NCBI BLAST search in Pub Med to obtain the sequence homology for *ent*A and *ent*B.

A total of 70 isolates of *S. aureus* were recovered from clinical mastitic buffaloes (75). All isolates were (+) ve for coagulase production followed by 92.85% (65 of 70 isolates) for thermostable nuclease production. PCR results for *coa* gene showed that all isolates were positive *coa* and had distinguished 3 types of PCR amplicon of approximately 840 bp, 600 bp and 440 bp exhibiting polymorphism of this gene. The 440 bp PCR product was observed in 33 (47.82%) isolates, whereas 600 bp and 840 bp products were found in 22 (31.88%) and 15 (20.28%) isolates, respectively. Further, 278 bp amplicon for *nuc* gene was identified in 65 (92.85%) of 70 isolates of *S. aureus.* Phenotypic observation co-related well with PCR findings and revealed all in all expression of *coa* and in 92.85% isolates for *nuc* gene

Antimicrobial susceptibility and resistance result showed that 59 (84.28%) isolates were resistant to penicillin-G and 55 (78.57%) to cephotaxime; 41(58.57%) to cloxacillin; 23 (32.85%) to amoxyclav. On the other hand, 85.71% (60 of 70) and 92.85% (65 of 70) isolates were sensitive to ciprofloxacin and methicillin, respectively. It was observed (Table 1) that all the isolates that were resistant to the 6 antibiotics were also positive for *coa* (100%) and *nuc* (100%) gene by PCR (data not shown). All the 59 penicillin-G resistant isolates were carrying *coa* and *nuc* gene. Similarly all the isolates that were resistant to cephotaxime (55), cloxacillin (41), amoxyclav (23), ciprofloxacin (10) and methicillin (5) were harboring *coa* gene as well as *nuc* gene.

Six (8.57%) isolates were positive for *ent*A gene and 11 (15.71%) for *ent*B gene; nevertheless, none of the 70 isolates was found positive for *ent*C gene. Interestingly, no isolate carried more than 1 enterotoxin gene. On the analogy, all the 6 *ent*A gene positive isolates were resistant to all the antibiotics, except 1 isolate that was susceptible to methicillin. Likewise, the *ent*B gene positive isolates (11) were resistant to all the used antibiotics except 2 and 6 isolates that were sensitive to ciprofloxacillin and methicillin, respectively.

On the other hand, 11 15, 60, 29, 47 and 65 isolates that were susceptible to penicillin-G, cephotaxime, ciprofloxacin, cloxacillin, amoxyclav and methicillin, respectively, were harbouring *coa* gene. Like wise, 6, 11, 55, 25, 43 and 60 isolates that were sensitive to penicillin-G, cephotaxime, ciprofloxacin, cloxacillin, amoxyclav and methicillin, respectively were harboring *nuc* gene. Only 1 methicillin susceptible isolate carried *ent*A gene; however, 2 and 6 isolates susceptible to ciprofloxacin and methicillin, respectively, were also possessing *ent*B gene.

Enterotoxin (SE) production status of these *S. aureus* isolates could not be judged by antigen-antibody reaction using the specific antisera to SE because the corresponding SE antisera could not be arranged. However, determination of SE producing genes was attempted in this study by targeting the amplification of SEA, SEB and SEC genes with the view that the finding could assess the potential of these isolates regarding enterotoxigenecity as well as their public health importance.

Table 1. PCR assays for virulence and enterotoxin genes in antibiotic resistant and sensitive *Staphylococcus aureus* strains

Virulence gene		Virulence and enterotoxin producing gene(s) in											
		Antibiotic resistance isolates						Antibiotic sensitive isolates					
Name	No. of isolate	P (n=59)	Ce (n=55)	Cf (n=10)	Cx (n=41)	Ac (n=23)	Me (n=5)	P (n=11)	Ce (n=15)	Cf (n=60)	Cx (n=29)	Ac (n=47)	Me (n=65)
coa	70	59 (100)	55 (100)	10 (100)	41 (100)	23 (100)	05 (100)	11 (100)	15 (100)	60 (100)	29 (100)	47 (100)	65 (100)
nuc	65	59 (100)	54 (98.18)	10 (100)	40 (97.56)	22 (95.65)	05 (100)	06 (54.55)	11 (73.33)	55 (91.66)	25 (83.33)	43 (89.58)	60 (92.3)
entA	06	6 (10.16)	06 (10.9)	06 (60)	06 (14.63)	06 (26.08)	05 (100)	0	0	0	0	0	01 (1.53)
entB	11	11 (18.64)	11 (20)	09 (90)	11 (26.83)	11 (47.82)	05 (100)	0	0	02 (3.33)	0	0	06 (9.23)
entC	0	0	0	0	0	0	0	0	0	0	0	0	0

P, Penicillin G (10 units); Ce, cephotaxime (5µg); Cf, ciprofloxacin (30 µg); Cx, cloxacillin (10µg); Ac, amoxyclav (30µg); Me, methicillin (30 µg); n, indicates number of isolates of *S. aureus*; value in parenthesis indicates percentage.

S. aureus was the main subject of studies on antimicrobial resistance because of its importance for mastitis in bovine. Constant and irrational use of antimicrobial agents in mastitis treatment fetches it essential to monitor the susceptibilities of *S. aureus* to antimicrobial agents as it emerged as one of the troublesome pathogens being resistant to many antimicrobial agents (Kim *et al*. 2004).

In this study, a multidrug resistance phenomenon was observed among *S. aureus* isolates where resistance to penicillin-G (84.28%), cephotaxime (78.57%) and cloxacillin (58.57%) was high. Ciprofloxacin and methicillin were found to be the most effective antimicrobial agents. About 32.85% isolates were resistant to amoxycillin/clavunate antibiotic, a ß- lactamase inhibitor combination.

PCR assay for virulence genes as well as enterotoxin producing genes showed that the frequency of these genes in *S. aureus* was comparatively high in antibiotic resistant isolates than their susceptible isolates. *coa* gene was constant in all the antibiotic resistant isolates. Interestingly, the *nuc*$^+$ isolates showed a high degree of resistance to all antibiotics except methicillin and ciprofloxacin where a paltry population carried *nuc* gene.

The isolates carrying enterotoxin genes (*ent*A and *ent*B) were resistant to all the antibiotics used in this study, suggesting their probable role in perpetuation and propagation of antibiotic resistance property in considerable population of *S. aureus* isolates from mastitic milk. On the other hand, a meager population of methicillin sensitive isolate (1.53%) carried *ent*A gene and 2 (3.33%) ciprofloxacin sensitive isolates and 6 (9.23%) methicillin-sensitive isolates also possessed *ent*B gene. The findings suggest that barring a few, almost all of the *S. aureus* isolates that carry the *ent*A and *ent*B were resistant to recent generation antibiotics adopted in clinical practice which indicates public health burden.

The findings revealed that all the enterotoxigenic isolates were carrying virulence genes (*coa*, *nuc*) and except a few all of them were resistant to the used antibiotics. Present results support to suggest that milk or milk products of buffalo mastitis source that is refractory to antibiotic treatment pose a potential public hazard in terms of staphylococcal food intoxication.

SUMMARY

Antibiotic resistant *Staphylococcus aureus* are frequent in mastitis cases and food intoxication is common in consumption of suspicious milk and milk products where virulent and enterotoxigenic isolates of *S. aureus* are implicated. The study was aimed to determine the presence of virulence genes and enterotoxigenicity status in antibiotic resistant and sensitive isolates of *S. aureus* from buffalo mastitis cases. Isolates (70) of *S. aureus* were recovered from buffaloes (75) with clinical-mastitis of different periurban herds and were tested for antimicrobial susceptibility against penicillin G (10units), cephotaxime (5µg), ciprofloxacin (30µg), cloxacillin (10µg), amoxyclav (30µg) and methicillin (30µg) by disc diffusion method and further characterized for biochemical characteristics and virulence (*coa* and *nuc*) and enterotoxigenic genes (e*ntA, entB and entC*). All the strains were positive for coagulase and 65 (92.85%) were for thermonuclease production. PCR results showed that except a few, the isolates harbouring the coagulase (*coa*) and thermonucelase (*nuc*) genes were resistant to used antibiotics. Six isolates were positive for enterotoxin gene A (*entA*) and all were antibiotic resistant except 1 isolate which is sensitive to methicillin. Similarly, 11 isolates were carrying enterotoxin gene B (*entB*) and were resistant to penicillin-G, cephotaxime, cloxacillin and amoxyclav and 9 and 5 were resistant to ciprofloxacin and methicillin; however, 6 and 2 *entB*$^+$ isolates were sensitive to methicillin and ciprofloxacin, respectively. Nevertheless, *ent*C gene was not detected in any of the 70 isolates. All the enterotoxigenic isolates were carrying virulence genes (*coa*, *nuc*) and except few all of them were resistant to the used antibiotics. It suggested that in majority, milk or milk products of buffalo mastitis source that is refractory to antibiotic treatment harbour the virulent and enterotoxigenic *S. aureus* and pose an alarm to public health in terms of staphylococcal food intoxication.

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