PREVALENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN RETAIL BUFFALO MEAT IN CHENNAI, INDIA

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ABSTRACT

Staphylococcus aureus is a major pathogen, affecting both human and animals causing severe morbidity worldwide. In addition, several reports of isolation of *S. aureus*, especially methicillin-resistant *Staphylococcus aureus* (MRSA) from food animals and their meats have raised concern about its potential as food borne pathogen. The present study was carried out to evaluate the prevalence of *S. aureus* in buffalo meat marketed in retail outlets in Chennai and to detect the presence Methicillin resistance (*mecA*) gene in these isolates. A total of 40 buffalo meat samples were collected from retail outlets and isolation and identification. The results revealed that coagulase positive *Staphylococci* were isolated from 29 (72.5%) samples, coagulase negative *staphylococci* were isolated from 6 (22.5%) samples and 5 samples were negative for staphylococci species. Of the 29 coagulase positive isolates 25 (86.2%) isolates were presumptively confirmed as *S. aureus* by biochemical test with overall prevalence of 62.5% (25/40) in retail buffalo meat. Duplex PCR was performed for simultaneous detection of both *femA,* a marker specific for *S. aureus* and *mecA* gene, the gold standard for detection of methicillin resistance in *S. aureus*. All the 25 presumptive *S. aureus* isolates amplified *femA* gene (318 bp) and 13/25 (52%) of the isolates carried *mecA* gene (533 bp). The PCR product (*mecA*) was purified, sequenced and submitted to NCBI (Accession Number: KT885189). The higher prevalence of *S. aureus* indicates poor hygiene practices and presence of *mecA* gene in *S. aureus* isolated from retail buffalo meat indicates that buffalo meat sold in the retail outlets in Chennai could be a potential source for transmission of MRSA to consumers.

Keywords: *Bubalus bubalis*, buffaloes, prevalence, *S. aureus*, MRSA, buffalo meat, retail outlets

INTRODUCTION

Staphylococcus aureus is a gram positive, coagulase positive coccoi belonging to family Staphylococcaceae. It is a commensal organism present in the skin and mucous membranes of humans and animals and presence of these bacteria in meat and meat products is generally an indicator of poor sanitation. *S. aureus* contamination of meat is often attributed to unhygienic handling by persons involved in the slaughter and during

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processing meat (Rutala and William, 2006).

Staphylococcus aureus is one of the major foodborne pathogens, frequently causing diseases globally as a result of food ingestion contaminated with staphylococcal toxin. Although, humans and animals are well-known to be reservoirs and carriers of *Staphylococcus aureus* and are able to transmit the pathogen to food, the accurate prevalence of staphylococcal food poisoning is unknown because of inadequate and non existence of proper reporting system in developing countries like India.

Contamination of meat with bacteria resistant to antibiotics is a major threat to public health and prevalence of antimicrobial resistance among food-borne pathogens has increased during recent decades (Van *et al.*, 2007). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a type of bacteria that is resistant to methicillin and other β-lactam antimicrobials, such as oxacillin, penicillin and ampicillin (Price *et al.*, 2012). Resistance in MRSA is mediated by the presence of penicillin-binding protein (PBP)-2a, encoded by the chromosomal *mecA* gene. Disc diffusion and MIC methods for resistance against oxacillin have been the method used for detection of MRSA, but the results of these tests are affected by variation in test conditions and false resistant reports have been documented (Browm *et al*., 2005). Hence, the researchers have moved towards molecular methods like Polymerase Chain Reaction (PCR) that detect the *mecA* gene as the "gold standard" for defining classical methicillin resistance in *S. aureus* (Tiwari *et al*., 2009).The prevalence of MRSA has been reported in retail pork meat (Weese *et al.*, 2010), beef (Lim *et al.*, 2010) and in poultry meat (De Boer *et al.*, 2009) worldwide but few studies have been conducted to measure its prevalence and relevance in food products in India. With the above points in view, the present study was conducted to evaluate the prevalence of *S. aureus* in buffalo meat marketed in retail outlets in Chennai by conventional and molecular methods and to detect the presence Methicillin resistance (*mecA*) gene.

MATERIALS AND METHODS

A total of 40 samples of raw buffalo meat were collected aseptically in separate containers from different retail outlets in Chennai, Tamil Nadu and the samples were transported in ice box to the Microbiology laboratory of Department of Livestock Products Technology (Meat Science for further processing.

Isolation and identification of *Staphylococcus aureus*

Isolation of *Staphylococcus aureus* was done as per the standard procedure (ISO standard 6888/1: 1999 and 6888/2: 1999). Twenty five grams of meat was homogenized with 225 ml of Brain heart infusion broth containing 10% NaCl for selective enrichment and was enriched at 37°C for 18 to 24 h. After 24 h, one loop full of inoculum from Brain heart infusion broth was streaked on the surface of sterile Baird parker Agar (BPA) base supplemented with 2% Egg yolk Tellurite emulsion (Hi-Media). The plates were incubated at 37°C for 24 to 48 h in an inverted position. Typical black colonies surrounded by white halo were considered to be presumptive *S. aureus* and those which produced black colonies without halo were *Staphylococci*. Both the colonies were subjected for the morphological characteristics by Gram's staining and Biochemical test *viz*., Catalase, Oxidase, Tube Coagulase and DNase for further identification as detailed below.

DNA extraction

For DNA extraction, the organisms were grown in Brain Heart Infusion broth (Himedia) and incubated at 37°C for 16 h and 1 ml of the bacterial suspension was centrifuged at 14,000 rpm for 1 minute. The pellets were washed thrice with Phosphate buffer saline to remove media components and the washed pellets were used for DNA extraction with Bacterial DNA extraction Kit (Quaigens) as per manufacturer's instruction. The DNA extracted was kept at -20°C until further use.

Oligonuleotide primers

The primers used in the present study targeted *femA* gene specific for *S. aureus* and *mecA* gene encoding PBP2a for detection of methicillin resistant *S. aureus*. The forward and reverse primers used for *femA* gene were 5'-CATGATGGCGAGATTACAGCT-3' and 5'-GTCATCACGATCAGCGAAAGC-3' yielding a PCR product of 318 bp (Al-Khafaji and Flayyih, 2015). The forward and reverse primers used for *mecA* gene were 5'-AAAATCGATGCTAAAGGTTGGC-3' and 5'-AGTTCTGCAGTACCGGATTTGC-3' amplified a 533 bp fragment (Merlino *et al*., 2002). The reference strain for Methicillin resistant *Staphylococcus aureus* N-315 was obtained from Department of Veterinary Microbiology, RIVER, Puducherry and was used as positive control.

Duplex PCR

PCR amplification of both the genes was carried out in 25 µl reaction consisting of 12.5 µl of master mix (Amplicon Red Dye mix), 1.0 µl of each forward and reverse primers (10 pmol/µl), 3.0 µl of DNA template and 7.5 µl of nuclease free water. Negative control contained all material except template DNA, so instead that distilled water was added. The reaction mixture was placed in thermal cycler (Eppendorf) and the conditions of amplification of *femA and mecA* gene are as follows: Initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 63°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 5 minutes. The PCR products along with 100 bp DNA ladder were separated by electrophoresis on a 2.0% Agarose gel in TAE buffer (40 mol/l Tris-acetate, pH 7.6 and 1 mmol/l Na_2EDTA) at 80 V for 90 minutes. Gels were stained with Ethidium bromide and were viewed under UV transilluminator in gel documentation system (BioRad, USA).

Sequencing of purified PCR product

The purified PCR product (532 bp amplicon of mecA gene) was sequenced by automated sequencing procedure by MWG Biotech Pvt. Ltd., Bangalore. The cycle sequencing reaction was performed using BigDye Terminator V3.1 cycle sequencing kit containing AmpliTac DNA polymerase. The sequence obtained were analyzed using Edit Seq of Laser gene (DNA STAR Inc.) software. The comparison of sequence was done by clustral method using Megalign™ software package with BLAST (Basic Local Allignment Search Tool). The *mecA* sequence of *S. aureus* isolated from buffalo meat was compared with reference sequences (www.ncbi.nlm.nih.gov) to observe for level of homology (identity). The sequence was submitted to Genbank for accession number.

RESULTS AND DISCUSSION

In the present study, out of the 40 buffalo meat samples used for detection of *S. aureus*, 35

samples were positive for presence of *Staphylococci* sp. characterized by typical black colonies with halo around them in Baird Parker Agar supplemented with Egg Yolk Tellurite emulsion, which is selective and differential medium used for the isolation and identification of *Staphylococcu*s species from foods (O'Brien *et al*., 2009). The colonies were examined microscopically by gram staining and all the isolates were gram positive cocci arranged in grape-like irregular clusters which are characteristics to *Staphylococcus* spp. (Dubey and Maheshwari, 2009).

Biochemical characterization of the 35 isolates revealed that all the isolates were negative for oxidase test, which is used to differentiate *Staphylococcus* from *Micrococcus* genus (Vos *et al*., 2009) and positive for the Catalase test that differentiates *Staphylococcus* from the genus *Streptococcus*. To determine coagulase positive and coagulase negative isolates all the 35 isolates were subjected to tube coagulase test and results revealed that 29 isolates (82.85%) showed the ability to produce coagulase enzyme (coagulase positive) and 6 isolates (17.15%) were coagulase negative.All the coagulase positive isolates (29) were streaked on mannitol salt agar and it was observed that all isolates had the ability to grow on mannitol salt agar and but only 25 (86.21%) isolates were capable of fermenting mannitol and turned the color of the medium from pink to yellow. DNase production is characteristic of most of pathogenic isolates of *S. aureus*, which produce DNase enzyme which destructs the host DNA and increases the invasiveness and pathogenecity of staphylococci. The results revealed that only 25/29 isolates (86.21%) were DNase producers (Brooks *et al*., 2007).

In the present study of the 40 meat samples screened for the prevalence of *S. aureus*, 25 samples were positive, with an overall prevalence rate of 62.5%, which clearly indicates poor hygiene practices followed in retail outlets resulting in contamination. The results of the present study are in concurrence with the findings of Jackson *et al*. (2013); Ahmad *et al* (2013) who reported prevalence of *S. aureus* from retail beef as 63% and 58% respectively. Similarly Gundogan *et al.* (2005) reported 53% of *S. aureus* contamination of meat and chicken samples and Atanassova *et al.* (2001) found 51.1% *S. aureus* contamination in raw pork meat. However contrary to the findings of the present study lower prevalence of 20% to 37% *S. aureus* in retail beef products have been reported by Hanson *et al*. (2011); Waters *et al* (2011); Hanning *et al*. (2012), whereas higher prevalence (80% to 100%) of *S. aureus* in retail meats have been reported by Ruban *et al*. (2012); Badhe *et al* (2013).

Cho *et al*. (2007) opined that *femA* appears to be present in all *S. aureus* strains, but not in other Staphylococcus species strains. All the 25 presumptive isolates were subjected to duplex PCR for amplification of *femA* and *mecA* gene (Al-Khafaji, 2013). All the isolates amplified PCR product of 318 bp (Figure 1) specific for femA gene indicating that all the isolates were *S. aureus.* It has been observed that coagulase negative staphylococci did not carry *femA* gene, since it is species specific marker for *S. aureus* (Sabet *et al*., 2007).

The isolates (25) confirmed as *S. aureus* were subjected to PCR for amplification of *mecA* gene, which encodes production of an altered PBP (PBP2a or PBP2) a modified transpeptidase that has a low affinity for all β-lactam antimicrobials like penicillins, cephalosporins and carbapenems (Scott and Van Duijkeren, 2010). In the present study, 13 out of the 25 (52%) *S. aureus* isolates amplified

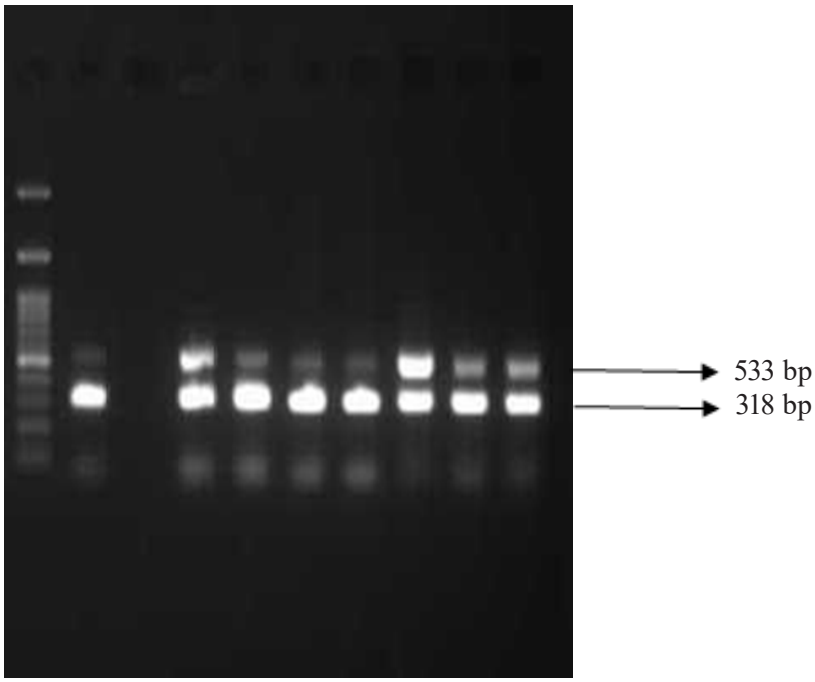

Figure 1. Agarose gel Electrophoresis of PCR products for detection of femA and mecA gene (M- Molecular Marker (100bp), P-Positive control (*Staphylococcus aureus* N- 315), N- Negative Control, Lane 1-7: Isolates positive for both femA and mecA gene.

533 bp specific for *mecA* gene (Figure 1). MRSA has been identified in retail meat worldwide, and pork had the highest contamination rate in the US and Canada (Pu *et al*., 2009; Weese *et al*., 2010), beef in South Korea (Lim *et al*., 2010) and poultry in the Netherlands (De Boer *et al*., 2009).

The results of the present study were in concurrence with the findings of Shareef *et al*. (2009), who reported 52.04% MRSA in poultry in Iraq, Suk-kyung *et al*. (2010) who reported 43.3% in retail poultry meat in Korea, Costa *et al*. (2014), who have reported a prevalence of 23.3% of MRSA in retail beef in Brazil and Contreras *et al*. (2015), who reported prevalence of 32% MRSA in Raw Hamburgers in Brazil.

The *mecA* PCR product was sequenced and BLAST analysis results reveled 100% identity with several reference sequences of Methicillin resistant *S. aureus*. The sequence was submitted to NCBI (Accession Number: KT885189).

CONCLUSION

The results of this study clearly indicate a high incidence of *S. aureus* and methicillin resistance in retail buffalo meat. Consumption of such meat with *S. aureus* might be of low risk to consumers as these organisms are killed by high temperature employed during cooking. However, there is high risk of transmission from raw meat to persons handling meat and hence, meat handlers should take appropriate measures to prevent the spread of MRSA by contaminated raw foods and to prevent the occurrence, growth and survival of MRSA in prepared food. In addition, the

duplex PCR developed can be effectively used for simultaneously identifying both the species as well as methicillin resistance.

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