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Prevalence of Panton Valentine Leukocidin (*pvl*) Gene in Methicillin Resistant *Staphylococcus aureus* Isolated from Market Samples of Chicken Meat

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A B S T R A C T

The present study was conducted to evaluate the prevalence of Panton Valentine Leukocidin (*pvl*) in methicillin resistant *S. aureus* isolated from Chicken meat marketed in retail outlets in Chennai city, Tamil Nadu. A total of 120 meat samples were collected from different retail outlets and it was observed that 66.67 (80/120) per cent of the samples were positive for the presence of *S. aureus*. The isolates were screened for methicillin resistance phenotypically by methicillin, oxacillin and cefoxitin disc diffusion assay and for presence of *mecA* gene by PCR. The results revealed that 54 isolates were positive for presence of *mecA* gene by PCR indicating that the prevalence of methicillin resistant *S. aureus* (MRSA) was 67.5 per cent. Comparison of different disc diffusion assays with *mecA* PCR revealed that cefoxitin disc diffusion assay has sensitivity, specificity, Positive predictive value (PPV), Negative predicative value (NPV) and accuracy of100, 91, 100, 89 and 95 per cent respectively as compared to oxacillin and methicillin disc diffusion assay.Both MRSA and Methicillin susceptible *S. aureus* (MSSA) were screened by PCR for the presence of *pvl* gene and it was observed that 38 isolates carried *pvl* gene of which 28 isolates were MRSA and 10 isolates were MSSA indicating an overall prevalence of 47.5 per cent (38/80). The results of the present study indicates that MRSA isolated from retail chicken meat carries pvl geneclearly indicating the presence of Community associated-MRSA involving human contamination and hence proper hygiene is essential to prevent possible ill effects to the consumers.

Introduction

S. aureus is a gram positive commensal pathogen commonly found in skin and nasal cavity of both human and animals. These organisms have evolved over decades as one of the pathogen to gain resistance to commonly used antibiotics in human and animal treatments. *S. aureus* is extraordinarily adaptable pathogen with a proven ability to

develop resistance to antibiotics with majority of the genes encoding resistance being mediated through plasmids (Chambers and DeLeo, 2009). Methicillin resistance in *Staphylococcus aureus* (MRSA) in humans as well as in foods of animal origin and its prevalence has been increasing worldwide. This resistance is due to the acquisition of genes encoding a unique penicillin-binding protein (PBP2'or PBP2a) (Chen *et al.*, 2009). Studies in different countries have strongly suggested that consumption of under cooked MRSA contaminated meat could be responsible for the prevalence of MRSA in the community (Ogata *et al.*, 2102). Hence, there is an urgent need to document the prevalence of methicillin resistant *S. aureus* in meats marketed in retail outlets.

In addition, *S. aureus* especially MRSA often harbor gene encoding for Panton–Valentine leukocidin (PVL), and this is an exotoxin encoding gene and has been associated with most CA-MRSA (Community Associated MRSA) strains which causes severe skin infections and necrotizing pneumonia in human (Deurenberg *et al.*, 2007). Since, *S. aureus* and MRSA have been found in human, food-producing animals and retail meat, the concern about the exposure for humans through the food chain is increasing day by day and hence the present study was aimed at evaluation of retail chicken meat samples for presence of *mecA* (associated with methicillin resistance) and PVL (virulence factor) genes.

Materials and Methods

The protocol and methodology used in the present study for isolation and characterization of *S. aureus* from Chicken meat was carried out with approval from Institutional Biosafety Committee of Tamil Nadu Animal and Veterinary Sciences University, Chennai.

Study area and source of material

A total of 120 chicken meat samples collected in sterile containers from different retail outlets in Chennai city (South, Central and North Zone) were used in his study.

Isolation of *Staphylococcus aureus*

Isolation of *Staphylococcus aureus* was done as per the standard procedure (ISO standard 6888/1:1999 and 6888/2: 1999). In brief, ten grams of each sample was added to 90 ml of sterile Brain Heart Infusion broth supplemented with 10 % NaCl and enriched for 8-10 hours at 37^{o}C. The enriched samples were streaked onto mannitol salt agar plates (Himedia, India) and were incubated for 24 to 48 h at 37°C. The presumptive suspected colonies were identified by Gram staining, catalase test, mannitol fermentation, coagulase and thermonuclease test as per standard protocol.

Reference strains

The reference strains of *S. aureus* (MTCC 87) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh and the reference strains of methicillin resistant *S. aureus* N- 315 (Juntendo University, Tokyo, Japan)were provided by Department of Veterinary Microbiology, Rajiv Gandhi Institute of Veterinary Education and Research (RIVER), Pondicherry were used in this study for standardization of PCR protocols.

Disc diffusion assay

Antibiotic susceptibility testing for detection of methicillin resistance among the isolates was performed by Kirby-Bauer disc diffusion method using methicillin (5 µg), oxacillin (1 µg) and cefoxitin (30 µg) disc. A 0.5 McFarland standard suspension of the isolate

was made and lawn culture done on Mueller Hinton agar plate and were incubated at 37 ^{0} C for 18 h and zone diameters were measured. The sensitivity, specificity, positive predictive value and negative predictive value of the cefoxitin, oxacillin, and methicillin disk diffusion test in detecting phenotypic methicillin resistance in the *S. aureus* isolates using the presence of the *mecA* gene as "gold standard" as per the procedure outlined by Olowe *et al.,* (2013).

Polymerase chain reaction

The genomic DNA was extracted by using DNA extraction kit (Qiagen) and the primers were custom synthesized. The sequences of the primers used for gene amplification are presented in Table 1. All oligonucleotide primers were custom synthesized by M/s. Eurofins, Bangalore. Polymerase chain reaction (PCR) for the detection of *mecA* and *pvl* genes was performed according to the methods described by Merlino *et al.,* (2002) and Lina *et al.,* (1999). Briefly, amplification reactions were performed in a 25 µL mixture containing 12.5 µL of 2X PCR master mix (Amplicon, Denmark), 10pmol of each primers and 2 µL of DNA template and the final volume was adjusted to 25 µL by adding nuclease free water. Amplification reactions were performed using a DNA thermal cycler (Master Cycler Gradient, Eppendorf, Germany) with the following program: for *mecA* gene- denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation for 1 minute at 95°C, annealing for 30 seconds at 59°C and extension for one minute at 72°C and final extension for 5 minutes at 72°C and for *pvl* gene- denaturation for 10 minutes at 95°C, followed by 30 cycles of denaturation for 3 seconds at 94°C, annealing for 30 seconds at 55°C and extension for one minute at 72°C and final extension for 10 minutes at 72°C. The PCR products were stained with 1% solution of ethidium bromide and visualized under UV light after gel electrophoresis on 2.0% agarose gel. Nuclease free water was used as the negative control.

Results and Discussion

The results of the present study revealed that 66.67 (80/120) per cent of the samples were positive for the presence of *S. aureus*. The results of the disc diffusion assay for detection of methicillin resistanceusing methicillin, oxacillin and cefoxitin are presented in table 2 and the sensitivity, specificity, Positive predictive value (PPV), Negative predicative value (NPV) and accuracy of this assay in comparison with *mecA* PCR are presented in table 3.

The result of the disc diffusion assay and comparison with *mecA* gold standard PCR clearly indicated that cefoxitin disc diffusion assay was superior compared to methicillin and oxacillin disc diffusion assay for detection of methicillin resistant *S. aureus.* Similarly, several workers have reported that the cefoxitin disc method has better sensitivity than the oxacillin disc method for MRSA detection (Pramodhini *et al.,* 2011; Kali *et al.,* 2014; Vyas *et al.,* 2015). The higher sensitivity to cefoxitin can be explained by the increased expression of the *mecA*-encoded protein PBP2a, as cefoxitin being an inducer of the *mecA* gene (Datta *et al.,* 2012). In addition, Clinical and Laboratories Standards Institute (CLSI) (2010) recommends usage of cefoxitin 30 µg disc for disc diffusion method for identification of MRSA.

In the present study, it was observed that 54 out of the 80 *S. aureus* isolates screened by PCR amplified 533 bp product (Fig 1) specific for *mecA* gene as described by Merlino *et al.,* (2002). PCR based on *mecA* gene is considered the gold standard method for detection of MRSA (Shahraz *et al.,* 2012; Ahmed *et al.,* 2014). Based on the results it

was evident that the prevalence of MRSA in retail chicken meat marketed in Chennai was 67.5 per cent (54/80). Similar prevalence of MRSA have been reported by Fesler *et al.*, (2011) in chicken (37.2 %), Karmi (2013) in chicken (24-52 %) and AgwuUluNnachi *et al.*, (2014) in beef and goat meat (85.7 & 63.2 %). However, in India no reports are presently available on the prevalence of MRSA in retail meats, however higher MRSA (80 %) have been reported from human in hospital settings (Verma *et al.*, 2000). Contrary to our findings lower prevalence of MRSA were recorded in various retail meats by Boost *et al.*, (2013) in Hong Kong (4.4 to 21.9 %), Wang *et al.*, (2013) in China (1.7%) and Eldaly *et al.*, (2014) in Egypt (5 to 15 %). The literature clearly suggested that there is a considerable variation in the prevalence of MRSA in different countries and the variation may be attributed to factors like sample size, sampling and culture methods, regulation in use of antibiotics in farm animals, monitoring systems in place for use of antibiotics as growth promoters, unhygienic slaughter/ processing as well as regular screening of retail samples to evaluate the present status of MRSA.

Table.1 Primers used in this study

Target gene	Sequence	Product size	Reference
mecA (Methicillin Resistance)	F - AAAATCGATGGTAAAGGTTGGC R- AGTTCTGCAGTACCGGATTTGC	533 bp	Merlino*et al.*, (2002)
pvl (Panton- Valentine Leukocidin)	F-ATCATTAGGTAAAATGTCTGGACATGATCCA R- GCATCAACTGTATTGGATAGCAAAAGC	433 bp	Lina*et al.*, (1999)

Table.2 Results of disc diffusion assay of *S. aureus* isolated from retail chicken meat

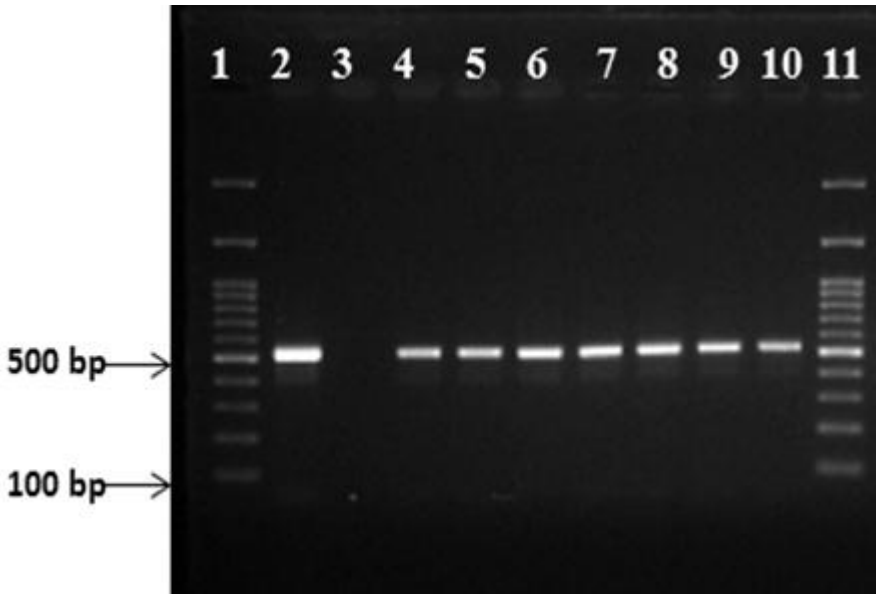
Antibiotics	Chicken isolates (n=80)	
	Sensitive	Resistant
Methicillin	44 (55.00)	36 (45.00)
Oxacillin	28 (35.00)	52 (65.00)
Cefoxitin	26 (32.50)	54 (67.50)

Table.3 Comparison of methicillin, oxacillin and cefoxitin disc diffusion assay with *mecA* gene PCR for detection of methicillin resistant *S. aureus*

	Specificity (%)	Sensitivity (%)	PPV (%)	NPV (%)	Accuracy (%)
Methicillin	67	53	70	48	58
Oxacillin	84	89	89	84	87
Cefoxitin	100	91	100	89	95

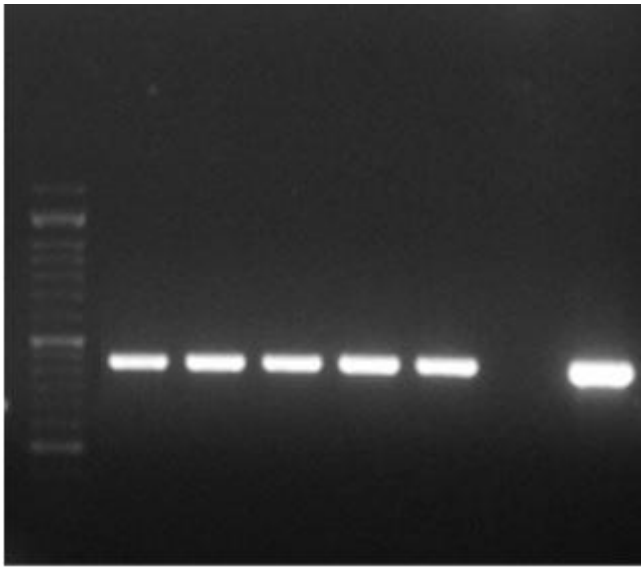
PPV: Positive Predictive Value; NPV: Negative Predictive Value

Fig.1 PCR amplification of *mecA* (533 bp) gene in *S. aureus* isolated from chicken marketed in Chennai city



Lane 1& 11: 100 bp DNA ladder, Lane 2: *S. aureus*standard isolate, Lane 3: Negative Control, Lane 4-10: Isolates from Chicken meat positive for *mecA*gene

Fig.2 PCR amplification of *pvl* (433 bp) gene in *S. aureus* isolated from chicken marketed in Chennai city



Lane M: 100 bp DNA ladder, Lane 1-6: *S. aureus* isolates from Chicken and Beef positive for *pvl* gene; Lane 6: Negative Control and Lane 7: *pvl* reference strain (MVCMSTC27)

All the 80 isolates (54 MRSA and 26 MSSA) were screened for the presence of Panton–Valentine leukocidin (*pvl*) gene, a marker for Community Associated MRSA (CA-MRSA) based on PCR and it was observed that that 38 isolates amplified 433 bp product specific for

pvl gene as described by Lina *et al.,* (1999), of which 28 isolates were MRSA and 10 isolates were MSSA indicating an overall prevalence of 47.5 per cent (38/80).The results of the present study were in accordance with Bhutia*et al.,* (2012) and Kaur *et al.,* (2012) in India who suggested that MRSA is an important reservoir of *pvl*gene and are now being slowly acquired by MSSA strains. Similarly, Abdalrahman *et al.,* (2015) observed that 66.7 per cent MRSA isolates obtained from chicken meat carried *pvl* gene.

In conclusion, the results of the present study clearly indicates that the retail chicken marketed in Chennai is highly contaminated with Methicillin resistant *S. aureus (MRSA) and majority of these isolates also harbor pvl*gene, which encodes exotoxin responsible for virulence of these strains and with ability to causes severe skin infections in human and person in contact with such contaminated meat. In addition, PVL being a marker of CA-MRSA, this study clearly indicates that the major source of contamination of meat is human handlers. However, further molecular characterization and validation of these isolates will provide better insights of the origin as well as source of contamination.

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