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Genotyping *Staphylococcus aureus* Isolates with RS-PCR from Chicken Meat in Chennai City

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A B S T R A C T

The *Staphylococcus aureus* is a versatile opportunistic pathogen affecting both human and animals. The Genotyping of *S. aureus* isolates helps to understand the pathogen's dissemination and epidemiological clusters, Hence, the present study designed to study the genotyping *Staphylococcus aureus* with RS-PCR from Chicken meat in Chennai City. In the present study, ribosomal spacer PCR (RS-PCR) was found to be showing polymorphism with varying band size and on software based dendrogram analysis. The dendrogram obtained upon analysis found that 3 main clusters and named as Cluster A, Cluster B and Cluster C. The similarity co-efficient at 0.05 they were characterised as 23 ribotypes. The most frequent ribotype belong to Cluster C and ribotype no 23. Based on Cluster analysis in our study revealed that may be single evolutionary originated epidemiological similar *S. aureus* strain is circulating and its clustered with transmitted in chicken meat though out the city thou meat was not collected from the same farm or site. The PCR ribotyping is a rapid inexpensive technique that is highly reproducible and almost as discriminatory as other typing methods for *S. aureus* isolates and should be useful in the local investigation and control of outbreaks.

Introduction

Staphylococcus aureus is a versatile opportunistic pathogen (Sergelidis and Angelidis, 2017) example for amphixenosis which mainly involved in inter-transmission of diseases between humans and animals. The organism pathogenicity mainly depends on combination of genetic characteristics between different variants mediating virulence, immune evasion, invasive capacity, and antibiotic resistance (Chua *et al.*, 2014). They harbour variety of virulence factors which includes toxic shock syndrome toxin-1 (TSST-1), exfoliative toxin, enterotoxins, Panton-Valentine leukocidin and enterotoxin-like (Kot *et al.*, 2016; Wang and Muir, 2016). They are often being isolated from meat, raw milk, the processing surfaces and food handlers (Johler *et al.*, 2018). *S. aureus* strains major concern is antibiotic resistance evolving deadly Methicillin-resistant *S. aureus* (MRSA)

acquiring the mecA gene, encoding penicillin-binding protein 2a (PBP2a) and making treatment against them a tough battle (Fishovitz *et al.,* 2015). MRSA and other antimicrobial resistant *S. aureus* constitutes a serious public-health concern as they can affect humans, animals and presence in environment (Petinaki and Spiliopoulou, 2012).

The important routes of transmission of *S. aureus* are foods and food chains between food-producing animals and humans and vice versa (EFSA, 2009; Feingold *et al.,* 2012). Similarly, community-associated MRSA has been isolated from several animal-source foods such as poultry, pork and beef (Wang and Muir, 2016). Animals can act as source of zoonotic infections of *S. aureus*, most strains lack specific host tropism affecting many species (Luini *et al.,* 2015).

The Genotyping of *S. aureus* isolated from various sources and sites helps to understand the pathogen's dissemination and epidemiological clusters (Castelani *et al.,* 2013). The biochemical and molecular methods were earlier used for epidemiological investigations of human and bovine staphylococcal infections (Hartstein *et al.,* 1989; Kapur *et al.,* 1995). The introduction of PCR based genotyping methods are useful techniques because of sensitivity and speed of performance. RAPD-PCR based tool was used for molecular taxonomy and phylogenetic analysis among related individuals (Williams *et al.,* 1990). The repetitive element palindromic PCR (REP-PCR) and enterobacterial repetitive intergenic consensus sequence analysis (ERIC-PCR) were also used for discrimination of strains (Lessing *et al.,* 1995). The Pulsed-field gel electrophoresis (PFGE) has been recommended as a highly discriminatory method for typing MRSA but method is time-consuming and expensive typing method not well suited for screening large number of isolates (Tenover *et al.,* 1995).The similar PCR based amplification of the 16S-23S rRNA of intergenic spacer region (ribosomal spacer-PCR, RS-PCR) was found to be more valid in understanding evolutionary interest since there is sufficient conservation within this locus a universal organization of evolutionary relationships (Cedergren *et al.,* 1993). The 16S, 23S, and 5S rRNA genes are separated by spacer regions which exhibit significant sequence and length polymorphisms at the genus and species levels (Jensen *et al.,* 1993). The PCR based amplification of 16S-23S ribosomal spacer region can act as suitable method in generating database (Jensen *et al.,* 1993). The ribosomal spacer PCR technique has the advantage that it is less prone to variation because it is specific and is performed under high stringency conditions. Analysis of these sequences has been useful in differentiating closely related members of a number of genera. The present study designed to study the molecular based genotyping using RS-PCR of *S. aureus* isolates from chicken meat in Chennai city.

Materials and Methods

Sample

A total of 259 chicken meat samples were collected aseptically using sterile Ziplocks from various retail vendors in the Chennai city. The study period from May 2017 to May 2019. The samples were transported to laboratory under refrigeration and stored at 4°C until further processing.

Isolation of *Staphylococcus aureus*

The chicken meat sample was enriched in brain heart infusion broth (HiMedia, India) containing 5-7% NaCl at 1:10 dilution and incubated at 35-37°C for 18-24 h. The enriched samples plated on Mannitol salt agar

(MSA) (HiMedia, India) incubated for 24 h at 35-37°C. The characteristic appearance of golden yellow colour colonies was considered to be presumptive *S. aureus.* The organism was demonstrated by Gram's stained smears which show Gram-positive cocci that occurred in grape like clusters. Further confirmation was done by positive reaction with catalase test (3% hydrogen peroxide), coagulase test using rabbit plasma and latex agglutination test (HiMedia, India).

DNA preparation

The 2-3 colony of presumptive isolates were transferred in 100 µL of sterile nuclease free water and washed thrice and the DNA was extracted by the boiling-snap chill method *viz.*, boiling at100°C for 5 min, followed by immediate chilling for 5 min at -20°C and then centrifuged at 10000 rpm for 5 min at 4°C, recovering the supernatant. The supernatant was used as DNA template for PCR reaction mixture.

PCR based ribotyping method for genotyping

The oligonucleotide primers G1-5' GAAGTCGTAACAAGG and L1- 5' CAAGGCATCCACCGT were used for amplification of 16S-23S ribosomal DNA inter spacer region *S. aureus* isolates as described by Jensen *et al.,* (1993). The polymerase chain reaction was performed in a reaction mixture of 25µL of final volume approximately containing 100 ng of template DNA, 12.5µL of Taq DNA polymerase 2X Master mix (Amplicon III, Denmark), 1µL of (20 pmol) each primer (Sigma Aldrich, India) and made up volume by nuclease free water. The PCR conditions in an A200 Gradient Thermal Cycler (LongGene®, Hangzhou, China) consisted of initial denaturation 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 55°C for 1min and 72°C for 1 min with a final extension of 72°C for 7 min. The amplified products were examined on agarose gel electrophoresis (1.5%) and 0.5% ethidium bromide staining. The gel was visualized using Imagecapture software in BioRad Gel documenter. The banding pattern was compared and scored as binary matrix with '0' & '1' which denotes that presence of a particular band as 1 and 0 for absence of that particular band. The similarity matrix constructed, based on Dice's correlation coefficient and by the method of the mean genetic distances UPGMA (UnweightedPair Group Method for Arithmetic Averages) using the Freetreeprogram (0.9.1.50 version). The dendograms were obtained to assess the genetic relationship among the *S. aureus* isolates with RS-PCR were and visualized using the online Phylogenetic tree (newick) viewer by *TreeDyn* by Phylogeny.fr: robust phylogenetic analysis (Chevenet *et al.,* 2006; Dereeper *et al.,* 2008)

Results and Discussion

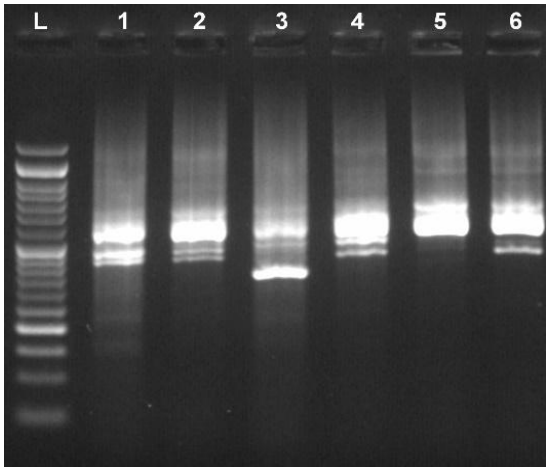
A total of 259 chicken meat samples when subjected for isolation of *S. aureus* using conventional methods, found that 41.31 percent (107/259) presumptive positive isolates. The presumptive *Staphylococcus aureus* isolates were further confirmed with latex agglutination test and coagulase test. The positive isolates were further subjected for DNA extraction and used for genotypic based RS-PCR typing using oligonucleotide primers G1 and L1 for the amplification of 16S-23S ribosomal DNA inter spacer region conserved fragments (Jensen *et al.,* 1993). The total of 107 positive *S. aureus* isolates on typing with RS-PCR found that amplicon bands varying size from 350 bp to 900bp; with 3 to 8 fragments resolved per isolate (Figure 1). Similar kind of result was found with Reshma *et al.,* (2017) who reported band size between 450 bp to 1000 bp and 1 to 10 fragments resolved in *S. aureus* isolates whereas Rao and

Surendran, (2010) reported bands were narrow band spectrum between 530 and 870 bp in *Vibrio choleare.* The presence of more amplified products might be due to the fact that *Staphylococcus aureus* has 9 operons and the weaker band may represent heteroduplex molecules resulting from cross hybridization of amplification products from different kinds of operons.

The gel images were further subjected for similarity matrix construction, based on Dice's correlation coefficient, by the method of the mean genetic distances UPGMA (Unweighted Pair Group Method for Arithmetic Averages) using the Freetree program for phylogenetic tree construction. In the present study, ribosomal spacer PCR (RS-PCR) was found to be showing polymorphism with varying band size and on software based dendrogram analysis. The 16S-23S intergenic sequence was showed as highly conserved, stable and appropriate indicator to study the evolutionary divergence of *S. aureus* strains (Gurtler and Barrie, 1995).

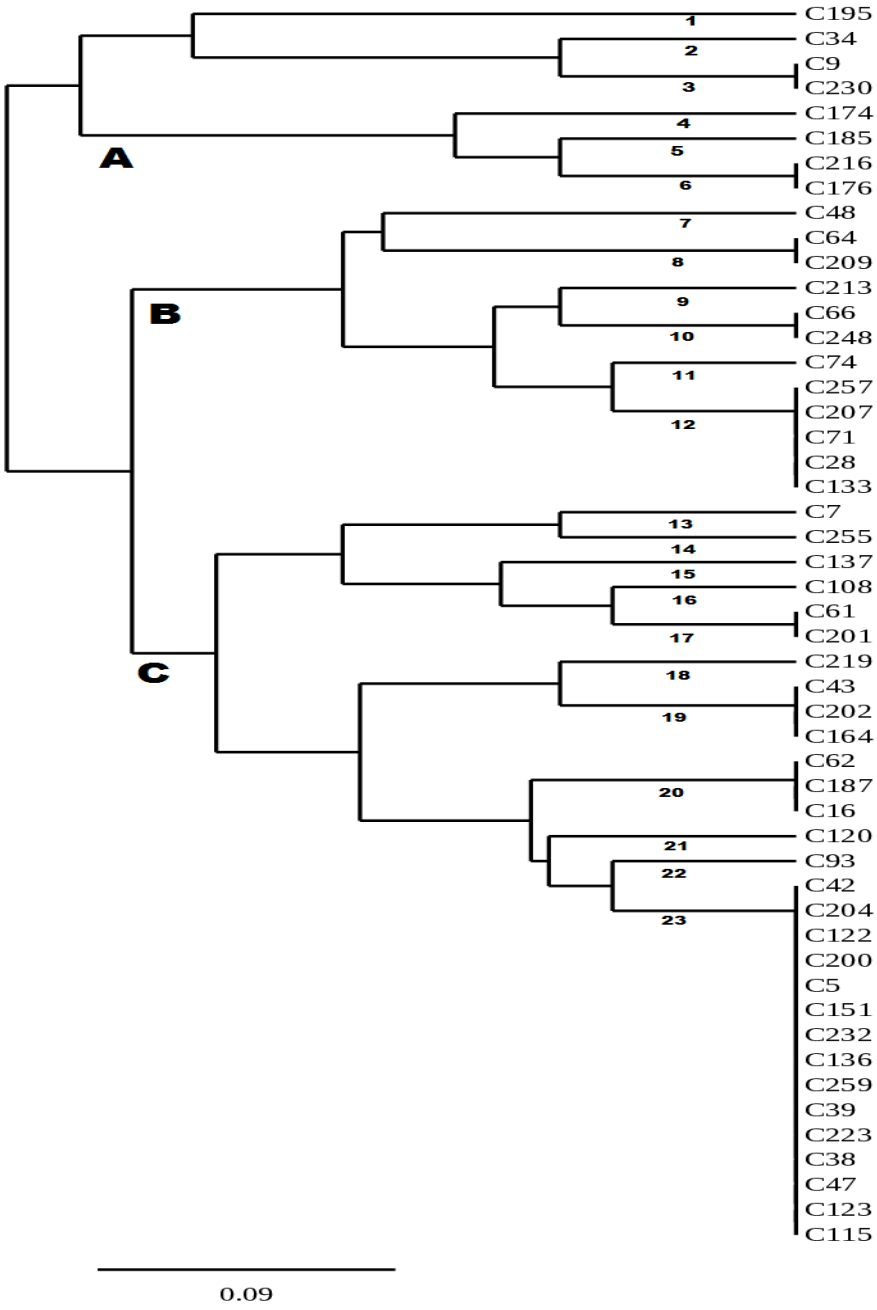
The dendrogram obtained upon analysis found that 3 main clusters and named as Cluster A, Cluster B and Cluster C (Figure 2). The similarity co-efficient at 0.05 they were characterised as 23 ribotypes. The Cluster A and B comprised of 6 ribotypes each and Cluster C comprised of 11 ribotypes, respectively. The most frequent ribotypebelong to Cluster C and ribotype no 23. The remaining isolates were evenly distributed in 22 other ribotypes groups of smaller number per ribotype. Based on Cluster analysis in our study revealed that may be single evolutionary originated epidemiological similar *S. aureus* strain is circulating and its clustered with transmitted in chicken meat though out the city thou meat was not collected from the same farm or site. The spread of antimicrobial resistance gene and other virulence factors among the isolates since they have genetic relatedness would we major contributing factor. The results conclude that RS-PCR can be used for detection of polymorphism to know the genetic relatedness of *S. aureus* isolated from different sites. Though PCR based ribotyping can be used for the detection of polymorphism and to assess the genetic relatedness of *S. aureus*.

Figure.1 The gel image of *S. aureus* isolates on typing with RS-PCR



(L: DNA ladder (50bp); 1,2,3,4,5,6 – RS-PCR ribotypes showing polymorphism with band bands between 350bp-900bp)

Figure.2 The dendrogram showing clusters and ribotypes based on RS-PCR gel image analysis on Free tree software



Other molecular techniques like Pulsed Field Gel Electrophoresis (PFGE), Multilocus sequence typing (MLST), *Staphylococcal* protein A (*Spa*) typing and whole genome sequencing are required to establish zoonotic transmission of these strains from animals to animal handlers and vice versa.

In conclusion, the present study provides on input that ribosomal spacer PCR (RS-PCR) was found to be showing polymorphism with varying band size and on software based dendrogram analysis. There was considerable genetic heterogeneity in population of *S. aureus* isolated with this appropriate typing

system to determine the genetic structures of the isolates, enabling a rational and effective strategy for epidemiological control. PCR-ribotyping is a rapid inexpensive technique that is highly reproducible and almost as discriminatory as PFGE for typing *S. aureus* isolates and should be useful in the local investigation and control of outbreaks.

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