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PCR-RIBOTYPING OF BOVINE AND HUMAN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Abstract: The present study aimed at the assessment of genetic relatedness among the methicillin-resistant *Staphylococcus aureus* (MRSA) isolates of bovine and human origin. A set of twenty-four MRSA isolates obtained from cattle (11), buffaloes (10) and their handlers (3) were subjected to PCR-ribotyping using primers specific for 16S-23S ribosomal spacer region. Polymorphism was observed with 23 ribotypes identified among the 24 MRSA isolates. Phylogenetic analysis revealed wide genetic diversity with little host specificity among MRSA isolates. No genetic relatedness between isolates of cattle, buffalo and livestock handlers was observed. Further studies 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 MRSA strains from animals to animal handlers and vice versa.

Keywords: Bovine; human; MRSA; PCR-ribotyping.

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) have been a major public health issue for the last 50 years causing severe nosocomial infections worldwide (Verkade and Kluytmans, 2014). Of late it was noticed that MRSA was no longer restricted to humans and is emerging as an important zoonotic and veterinary pathogen (Cohn and Middleton, 2010). There are reports about MRSA in various animal species, foods of animal origin, personnel's working with livestock as well as pet owners in developed and other developing countries (Lee, 2003 and Abdul Aziz *et al*., 2006).

Polymerase Chain Reaction (PCR) based typing methods like PCR-Amplified Ribosomal DNA spacer Polymorphism (PCR-ribotyping or RS-PCR) have been used to study the population genetics, global epidemiology and outbreak investigation of MRSA (Kumari *et al.,* 1997). Amplification of 16S-23S ribosomal spacer region is a suitable process for generating database for use in a PCR based identification method (Jensen *et al.*, 1993). The

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objective of the present study was to assess the genetic relatedness among the MRSA strains obtained from cattle, buffaloes and their handlers by PCR- ribotyping.

Materials and methods

Reference strains: The reference strain MRSA (ATCC 25923) was purchased from Hi-Media Laboratories (Mumbai) and maintained in the Department of Veterinary Public Health and Epidemiology, N.T.R College of Veterinary Science, Gannavaram.

Source of MRSA isolates: A total of 24 MRSA isolates recovered from nasal swab samples of cattle (11), buffaloes (10) and their handlers (3) were used in this study. The identification of each isolate was carried out by cultural and biochemical tests viz., yellow colour colonies on mannitol salt agar, Gram staining (Gram positive cocci), catalase (positive), oxidase (negative), Voges-Proskauer (positive), haemolysis (positive) and coagualse activity (positive), growth on CHROM MeReSa agar with blue colour colonies, resistance to cefoxitin and oxacillin by disc diffusion method (Velasco *et al.,* 2005). Further, all the 24 isolates were confirmed as MRSA using the PCR targeting *mecA* and *blaZ* genes (Martineau *et al.,* 2000). DNA template was prepared by high salt method (Aravindakshan *et al.,* 1997). DNA concentration was determined spectrophotometrically (Nanodrop 200C, Thermo Scientific, USA) and diluted to a final concentration of 20ng/µl.

PCR-ribotyping of MRSA isolates: The primers used for amplification of 16S-23S ribosomal DNA spacer region of MRSA isolates were as described by Jensen *et al.* (1993). A 2.0 µl aliquot of MRSA genomic DNA was combined with 2.5µl of PCR reaction buffer with 15mM $MgCl_2$, 1.0 µl of a dNTP mixture [10mM], 1.25µl of each of two 15 base oligonucleotide primers (G1-GAA GTC GTA ACA AGG and L1- CAA GGC ATC CAC CGT) [20 pmol/µl] and 41.0 µl of nuclease free water. This mixture was heated to 94^{o}C for 5 min and 1.0 U of thermostable DNA polymerase was added. PCR assay was performed in Eppendorf (Germany) thermal cycler with heated lid. The cyclic conditions consisted of denaturation at 94^{o}C /1 min, annealing at 55^{o}C/1 min and extension at 72^{o}C/1 min for 34 cycles. The final elongation was done for 7 min at 72^{o}C. PCR products were subjected to 2% agarose gel electrophoresis (Sambrook and Russell, 2001).

Scoring of ribosomal spacer - PCR types: The resultant bands in analytical agarose gel electrophoresis were recorded by using image analysis system (Bio-Rad, USA) and were compared by visual inspection. The bands were scored as binary matrix with '0' & '1'. Presence of a particular band in a strain was scored as '1' and absence of that particular band in other strains was scored as '0'. The binary data was analysed using dollop programme of

phylip version 3.6 software and dendograms were constructed to assess the genetic relationship among the MRSA strains of bovine and human origin.

Results and discussion

A total of 24 MRSA isolates carrying *mecA* and *blaZ* genes were typed by PCR-ribotyping. The resultant amplicons ranged in size from 450 bp to 1000 bp; with 1 to 10 fragments resolved per isolate. The genetic diversity of MRSA from different sources and ribosomal spacer-PCR ribotypes detected in the present study was given in Table-1. Polymorphism was observed with 23 ribotypes identified among the 24 MRSA strains tested by PCR-ribotyping (Table 1). Two MRSA isolates of livestock handlers (H1 and H2) yielded the same banding pattern in PCR-ribotyping. To evaluate the strain diversity and genetic relatedness of MRSA isolates from different sources viz, cattle, buffaloes and livestock handlers; phylogenetic trees of PCR-ribotyping patterns were constructed (Fig. 1). Under cluster analysis, MRSA isolates from different sources of the study formed three clusters (Cluster I, Cluster II and Cluster III). There is a wide genetic diversity and little host specificity of MRSA among cattle, buffaloes and livestock handlers (Fig. 1).

Table 1: Genetic diversity of MRSA strains of bovine and human origin

Source	No. of MRSA isolates	No. of PCR-ribotypes
Cattle	11	11
Buffaloes	10	10
Livestock handlers	3	2
TOTAL	**24**	**23**

Cluster I comprised of MRSA isolates of cattle (C1, C3, C5, C7), buffaloes (B5, B6, B10) and livestock Handler (H3). In cluster II, MRSA isolates from cattle (C2, C4, C6) and buffaloes (B2, B4, B7, B9) were the major source. Livestock handlers MRSA were absent in cluster II. In cluster III, MRSA isolates of cattle (C8, C9, C10, C11), buffaloes (B1, B3, B8), and livestock handlers (H1, H2) were present (Fig. 1). In cluster I and III, livestock handlers MRSA were also grouped along with cattle and buffaloes MRSA isolates. However those samples were not collected from the same farm, ruling out the possibility of zoonotic transmission. These findings are in agreement with the reports of other researchers (Kumari *et al.,* 1997 and Pereira *et al.,* 2002).

The 16S-23S intergenic sequence has been shown to be highly conserved and is more stable and more direct indicator of the evolutionary divergence of MRSA strains (Gurtler and

Barrie, 1995). Accurate epidemiological typing is of primary importance for the identification of MRSA clones found in animals, animal handlers and hospitalized patients for enabling sources and routes of transmission to be identified (Mulligan and Arbeit, 1991). In the present study, though livestock MRSA strains clustered with animal MRSA strains, they were not collected from the same farm. Livestock MRSA strains are not spreading to human population under the Indian farm conditions and is not alarming when compared to Europe countries. Pereira and Siqueira-Junior (1995) showed that *S. aureus* isolated from animals exhibit antibiotic resistance patterns very different from those of human origin.

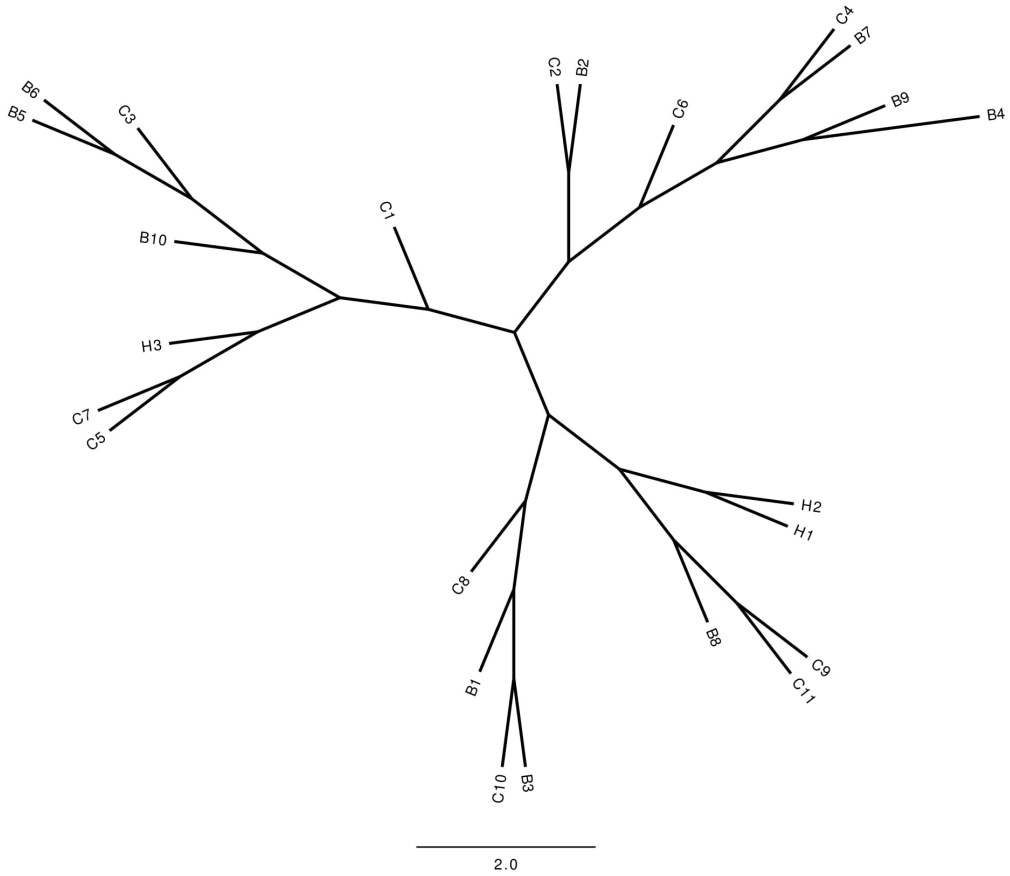

Fig. 1: An unrooted phylogenetic tree of PCR-ribotyping patterns of MRSA isolates generated using dollop programme of phylip version 3.6.

In the view of the considerable genetic heterogeneity in natural population of *S. aureus,* appropriate typing systems are needed 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 MRSA isolates and should be useful in the local investigation of MRSA outbreaks

(Kumari *et al*., 1997). The ribosomal spacer PCR technique has the advantage that it is less prone to variation due to its specificity and is performed under high stringency conditions.

Conclusion

In this study, MRSA isolates have wide genetic diversity with limited host specificity. PCR-ribotyping can be used for the detection of polymorphism and to assess the genetic relatedness of MRSA strains from animals and livestock handlers. 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.

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