The Bioscan 6(4) : 543-546, 2011
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

16S rDNA BASED PHYLOGENY OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES FROM THE PUS SPECIMENS

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KEY WORDS
Antibiotic resistance
mecA gene
Pathogens
Pus
Phylogenetic tree

Received on :
01.07.2011

Accepted on :
17.10.2011

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ABSTRACT
The genomic comparison of the MRSA isolates from pus sample has been done with that of the standard strains from GenBank. The rapid identification of bacteria in clinical samples is very important for patient management and antimicrobial therapies. Methicillin Resistant *Staphylococcus aureus* (MRSA) isolated from pus samples of clinical patients. Isolated MRSA named as SMKV-1 and SMKV-2 grown properly using with Oxoid CM3 Nutrient agar medium. Genomic DNA from above strains was isolated and 16S rDNA were amplified using suitable primers. The amplified 16S rDNA sequences have been deposited in the GenBank (DQ 306890 and DQ 306891) and selected 16S rDNA sequences of the genus *Staphylococcus* from the database were used to study the phylogenetic relationship with the present MRSA isolates. *mecA* gene appeared on 310 bp region on agarose gel electrophoresis. In the present study we found that the MRSA isolates were 100% similar to GenBank strains.

INTRODUCTION

Staphylococcus aureus is an important human pathogen capable of causing diseases in the hospital and community settings. It can produce enterotoxin in humans and animals. *S. aureus* is also known to be the common cause of nosocomial community-acquired infections and surgical wound infections (Kennedy *et al*., 2008). Numerous states have reported an increased incidence of community-acquired infections caused by multidrug-resistant organisms where reportedly no exposure to antibiotics or health care in the 3 months before symptom onset occurred (Kenneley, 2010**;** Nicolau and Stein, 2010). CA-MRSA soft tissue infections may clinically present as cellulitis, folliculitis, furuncles, carbuncles, and abscesses (Cohen and Kurzrock, 2004). The infection often begins as a mild superficial infection of the skin, which may look harmless at onset but rapidly develop into large abscesses within 24 to 48 hr. The lesions are often mistaken for spider bites (File, 2007). Although skin and soft tissue infections are more common with CA-MRSA, osteomyelitis, otitis media, respiratory tract, and blood sepsis can occur (Fridkin *et al*., 2005). CA-MRSA infections have become epidemic in many parts of the world and are responsible for significant morbidity and mortality (Macario, 2010). Several risk factors, such as recent hospitalization or exposure to a health care setting, residence in long term- care facilities, invasive or surgical procedures, and injection drug use, predispose a patient to MRSA acquisition (Salim *et al.,* 2005). Panton-Valentine leukocidin (PVL) is a synergohymenotropic toxin (SHT) (Horts *et al.,* 2010). It is two-component cytolytic toxin epidemiologically linked to CA-MRSA infections, including serious invasive infections caused by the epidemic clone referred to as strain USA300 (Olsen *et al*., 2010).

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique16S rRNA gene sequence analysis lacks widespread use beyond the large and reference laboratories because of technical and cost considerations (Clarridge, 2004). The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level (Sacchi *et al*., 2002). The present study has been carried out to understand the phylogenetic relationship of the MRSA isolates (SMKV-1 and SMKV-2) from the pus samples using PCR technique.

MATERIALS AND METHODS

Two strains of Methicillin Resistant *Staphylococcus aureus* (MRSA) named as SMKV-1 and SMKV-2 were isolated from

the pus samples of the patients using Oxoid CM3 Nutrient agar medium supplemented with 0.04% (wt/vol) KH_2PO_4 and 0.24% (wt/vol) $Na_2 HPO_4.12 H_2O$ (pH 6.8). The pellets from actively grown cultures (1.5mL) were obtained by centrifugation at 13,000 rpm for five minutes and washed with TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). The cell pellets were then dissolved in 200 μL of TE. Cell lysis was obtained at 37ºC after treatment with lysozyme (2mg/mL; final concentration) for 30 min and using SDS (1%). The lysate was extracted twice with phenol/chloroform followed by two extractions with chloroform/isoamyl alcohol (24:1). After adding 1/10 volume of sodium acetate (3 M, pH 5.2) and 0.5 volume of isopropanol, the supernatant was incubated at -20ºC for 30 min. The DNA was then sedimented by centrifugation at 13000 rpm for 20 min and the resulting pellet was washed with 70% ethanol and dried under vacuum. The DNA pellets were then dissolved in 100μL TE and used as template for PCR amplification of 16S rDNA sequence (Pitcher *et al*., 1989).

For the amplification of 16SrDNA, 100μL reaction mixture contained 200 μM dNTP (Perkin-Elmer Product, France), 0.1 μM concentration of forward and reverse primers (27f: 5'-GAGTTTGATCCTGGCTCAG-3' and 1525r: 5'-AGAAAGGAGGTGATCCAGCC-3') (Shrestha *et al*., 2002), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of *Taq polymerase* (Qiagen) and 10 μL of template DNA. An initial denaturing step of 95ºC for 10 min was followed by 30 cycles of amplification (1 min at 94ºC, 1 min at 55ºC and 2 min at 72ºC) and a final extension step at 72ºC for 10 min. DNA was amplified with RT- PCR and amplified DNA was checked by electrophoresis of 5 μL of PCR product in a 1% agarose gel in Tris-borate-EDTA (TBE) buffer (pH 8.3) and by staining with Ethidium Bromide. The PCR product was purified by using Nucleotrap PCR extraction kit (Macherey Nagel Product) and sequenced (ABI Prism 377 DNA sequencer, Applied Biosystems) using the primers, 27 f (5'-GAGTTTGATCCTGGCTCAG-3'), 343r (5'-CTGCTGCC TCCCGTA-3'), 357f (5'- TACGGGAGGCAGCAG-3'), 519r [5'-G(T/A) ATTACCGCGGC (T/G) GCTG-3'], 536f [5'-AGC(C/A) GCCGCGGTAAT (T/A) C-3'], 803f (5'-ATTAGATACCCTG GTA G-3'), 907r (5'-CCGTCAATTCATTTGAGTTT-3'), 1114f (5'-GCAACGAGCGCAA CCC-3'), 1385r[5'-CGGTGTGT(A/G) CAAGGCCC-3') and 1525r (5'-GAAAGGAGG TGA TCCAGCC-3') (Ludwig *et al*., 1992). For the phylogenetic analysis, related 16S rRNA gene sequences from the Genbank within the genus *Staphylococcus* were compared with the two isolates (SMKV-1 and SMKV-2). Using MEGALIGN program of DNASTAR aligned the 16S rDNA sequences. An evolutionary distance matrix was generated as described by Jukes and Cantor (1969). An evolutionary tree for the datasets was prepared from the neighbor-joining method of Saitou and Nei (1987) and also using the neighbor-joining program of MEGA version 2.1. Performing bootstrap analysis of the neighbor-joining data based on 1000 bp resembling was used to assess the stability of relationships.

RESULTS AND DISCUSSION

The first taxonomic description of the genus *Staphylococcus* is classified based on colony pigmentation, a key factor used to differentiate the opportunistic pathogen *S. aureus*, which causes high morbidity and mortality, and other staphylococci generally, considered being harmless commensals or saprophytic bacteria. Using a variety of morphological criteria and physiological and biochemical tests, it soon became obvious that more staphylococcal species are associated with humans and animal species than had been recognized so far (Kloos and Schleifer, 1975). In the present study, the 16S rDNA sequences of the MRSA isolates SMKV-1 and SMKV-2 were deposited in the GenBank (NCBI, USA) with the accession numbers DQ306890 and DQ306891 respectively. The results showed 100% sequence similarity with *S. aureus* strain MSSA476 (BX571857) and *Staphylococcus* sub sp. *aureus* MRSA252 (BX571857) followed by 99.86% similarity with *S. aureus* (L36472), 99.79% similarity with *S. aureus* (X68417), 99.65% with *S. aureus* (X70648), 98.59 with *Staphylococcus* sp. H780 (AB 177644) (Fig. 1). The 16S rDNA sequence results were comparable with the findings of Saitou and Nei (1987) study coincides with the present study. They reported that the *S. aureus* genomes were composed of a complex mixture of genes, many of which seem to have been acquired by lateral gene transfer. Gurtler and Barrie (1995) reported that the sequence conservation of the *rrn* operons argues for the use of the 16S-23S spacer region as a stable and direct indicator of the evolutionary divergence of *S. aureus* strains. As per the report of Garrity and Holt (2001), the recent results of molecular phylogenetic classification, it was proposed to reclassify the genus *Staphylococcus* into a family *"Staphylococcaceae"*. Differentiation up to the species level may also have substantial consequences for the management of patients (Jones *et al*., 2002). While conventional differentiation schemes based on physiological and biochemical tests are relatively cumbersome and time-consuming and require various approaches. The commercial "rapid" identification systems share the problems of failure to identify commonly encountered bacteria, uselessness in identifying uncommon isolates, and lack of adequate strains in the accompanying databases (Renneberg *et al*., 1995; Spanu *et al*., 2003). Furthermore, commercial systems may provide ambiguous results, presenting two or more suggestions for identification with a comparable safety level. The techniques based on amplification of 16S rRNA genes for comparing bacterial communities are now widely used in microbial ecology, but calibration of these techniques with traditional tools, such as cultivation, has been conspicuously absent (Dunbar *et al*., 1999). The presence of *mecA* gene allows a bacterium to be resistant to antibiotics such as Methicillin, Penicillin, and other penicillin-like antibiotics. The *mecA* gene does not allow the ring like structure of penicillin-like antibiotics to attack the enzymes in the cell wall of the bacterium, thus the penicillin cannot enter the bacterium and destroy it (Sendi *et al*., 2005). A study carried out by Banerjee *et al*., (2010) reported the genome resequencing of MRSA strain, CRB, revealed that it differs from its parent by five single-nucleotide polymorphisms in three genes. More specifically, the genes which are encoding PBP4, a low-molecular-weight penicillin-binding protein, GdpP, a predicted signaling protein, and AcrB, a cation multidrug efflux transporter. CRB displayed resistance to a variety of β-lactams but was hyper susceptible to cefoxitin. In this present study, two MRSA isolates SMKV-1 and SMKV-2 are positive for *mec*

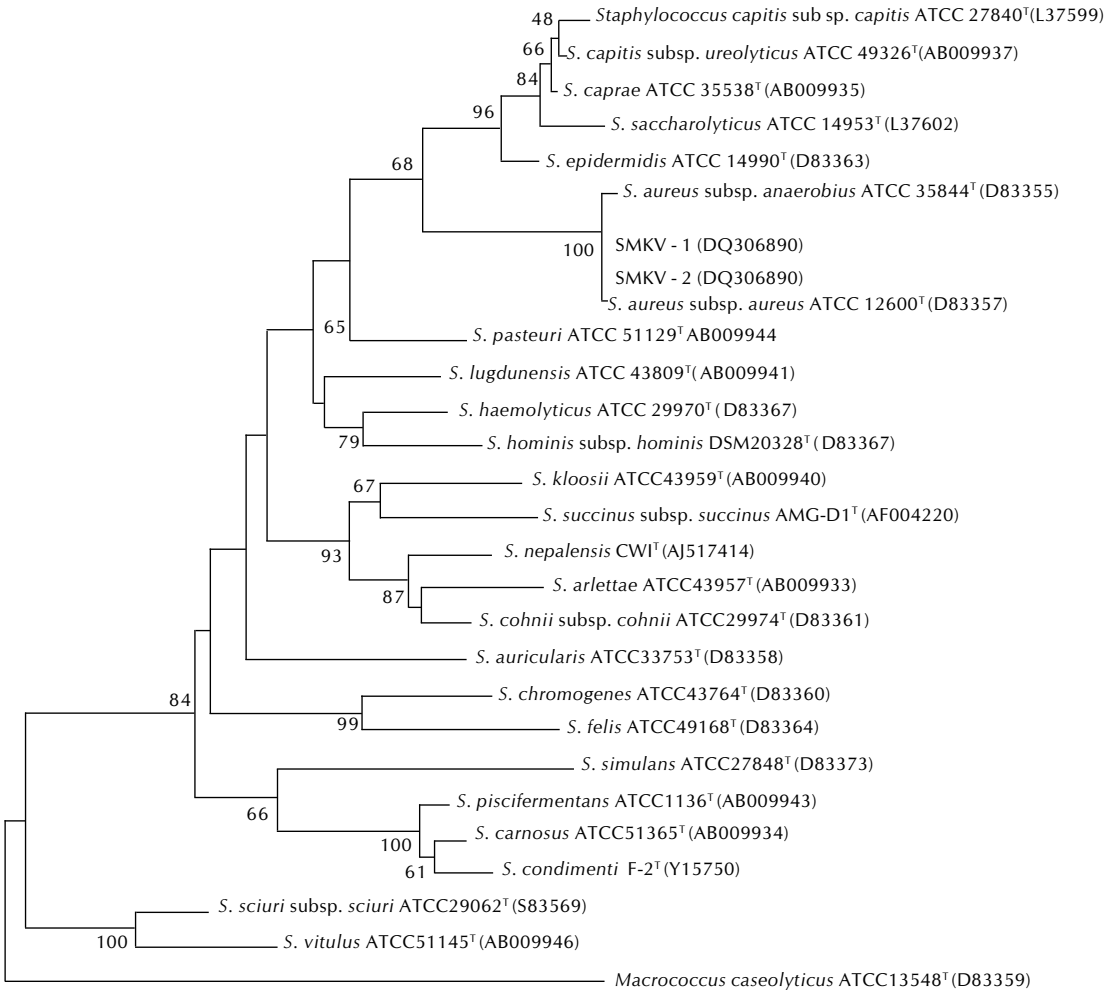

Figure 1: Phylogenetic tree derived from 16S rRNA gene sequences showing the position of strains SMKV 1 and SMKV 2 within the genus *Staphylococcus*. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1390 nt . Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50 %. Bar, 0.01 substitutions per nucleotide position

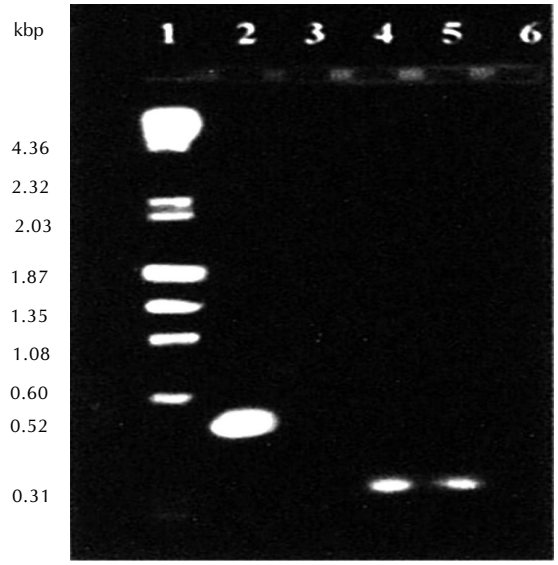

Figure 2: *mec-A* gene amplification of MRSA isolates
1 – Marker, 2 – MTCC 87, 3, 6 - MSSA, 4, 5 – MRSA strains.

A gene (Fig. 2) which was confirmed by PCR analysis. Anvari and Safarimotlagh (2010) recently reported the rapid detection of methicillin-resistant staphylococci by multiplex PCR. The development of molecular typing methods has enabled the tracking of different strains of *S. aureus*. This may lead to better control of outbreak strains. A greater understanding of how the staphylococci evolve, especially due to the acquisition of mobile genetic elements encoding resistance and virulence genes helps to identify new outbreak strains and may even prevent their emergence (Lindsay *et al.,* 2006).

ACKNOWLEDGMENTS

The authors are acknowledged to their family members for their constant encouragement and support.They are thankful to the research scholars of Microbiology department, Bharathidasan University for their technical support.

REFERENCES

Anvari, M. and Safarimotlagh, M. R. 2010. Rapid detection of methicillin-resistant staphylococci by multiplex PCR. *Afr. J. Biotechnol*.

9: 7629-7631.

Banerjee, R., Gretes, M., Harlem, C., Basuino, L. and Chambers, H. F. 2010. A *mecA*-negative strain of methicillin-resistant *Staphylococcus aureus* with high-level β-lactam resistance contains mutations in three genes. *Antimicrob. Agents. Chemother.* **54:** 4900-4902.

Clarridge, J. E. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin. Microbiol. Rev*. **17(4):** 840-862.

Cohen, P. and Kurzrock, R. 2004. Community acquired methicillin-resistant *Staphylococcus aureus* skin infection: an emerging clinical problem. *J. Am. Acad. Dermatol*. **50:** 277–280.

Dunbar, J., Takala, S., Barns, S. M., Davis, J. A. and Kuske, C. R. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Appl. Environ. Microbiol*. **65(4):** 1662-1669.

File, T. M. 2007. Impact of community acquired methicillin-resistant *Staphylococcus aureus* in the hospital setting. *Cleve. Clin. J. Med.* **74(4):** 6 -11.

Fridkin, S. K., Hageman, J. C. and Morrison, M. 2005. Methicillin-resistant *Staphylococcus aureus* disease in three communities. *N. Engl. J. Med.* **352:** 1436–1444.

Garrity, G. M. and Holt, J. G. 2001. The Road Map to the Manual. *In* Bergey's Manual of Systematic Bacteriology, Boone, D.R., Castenholz, R. W. and Garrity, G. M. (Ed.). 2nd Edn., Springer-Verlag, New York. pp. 119-166.

Gurtler, V. and Barrie, H. D. 1995. Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S-23S rDNA spacer regions: characterization of spacer sequences. *Microbiol.* **141:** 1255-1265.

Horts, T. B., Dumitrescu, O., Badiou, C., Thomas, D. and Benito, Y. 2010. A histidine-to-arginine substitution in panton-valentine leukocidin from USA300 community-acquired methicillin-resistant *Staphylococcus aureus* does not impair its leukotoxicity. *Infect. Immunol.* **78(1):** 260-264.

Jones, R. M., Jackson, M. A., Ong, C. and Lofland, G. K. 2002. Endocarditis caused by *Staphylococcus lugdunensis. Pediatr. Infect. Dis. J.* **21:** 265-268.

Jukes, T. H. and Cantor, C. R. 1969. Evolution of Protein molecules. Munro, I. H.N. (ed.), mammalian Protein Metabolism. Academic press, New York. pp. 21-123.

Kennedy, A. D., Otto, M., Braughton, K. R., Whitney, A. R. and Chen, L. 2008. From the cover: Epidemic community-associated methicillin-resistant *Staphylococcus aureus:* Recent clonal expansion and diversification. *Proc. Natl. Acad. Sci. USA*. **105:** 1327-1332.

Kenneley, I. L. 2010. *Infection control and the home care environment.* Home Health Care Manage. Practice. ***22(3):*** *195-201.*

Kloos, W. E. and Schleifer, K. H. 1975. Simplified scheme for routine identification of human *Staphylococcus* species. *J. Clin. Microbiol.* **1b:** 82-88.

Lindsay, J. A., Moore, C. E., Day, N. P., Peacock, S. J., Witney, A. A., Stabler, R. A., Hussain, S. E., Butcher, P. D. and Hinds, J. 2006. Microarrays reveal each of the ten dominant lineages of *Staphylococcus aureus* have unique combinations of surface associated and regulatory genes. *J. Bacteriol.* **188:** 699-676.

Ludwig, M., Kirchof, G., Klugbauer, N., Weizenegger, M. and Betzl, D. 1992. Complex 23s ribosomal RNA sequences of Gram positive bacteria with a low DNA G + C content. *J. Syst. Appl. Microbiol.* **15:** 487-501.

Macario, E. 2010. Using cognitive interviews to refine a household contacts survey on the epidemiology of community-associated methicillin resistant *Staphylococcus aureus. J. Infect. Prevent.* **11(2):** 44-48.

Nicolau, P. D. and Stein, G. E. 2010. Therapeutic options for diabetic foot infections*:* A review with an emphasis on tissue penetration characteristics. *J. Ameri. Pedia. Medical. Asso.* 100 (1): 52-63 .

Olsen, R. J., Kobayasi, S. D., Ayeras, A. A., Ashraf, M. and Graves, S. F. 2010. Lack of a major role of *Staphylococcus aureus* panton – Valenine leukocidin in lower respiratory tract infection in non human primates. *Am. J. Pathol.* **176:** 1346 -1354.

Pitcher, D. G., Saunders, N. A. and Owen, R. J. 1989. *Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Letter. Appl. Microbiol.* ***8(4****): 151- 156.*

Renneberg, J., Rieneck, K. and Gutschik, E. 1995. Evaluation of Staph ID 32 system and Staph-Zym system for identification of coagulase-negative staphylococci. *J. Clin. Microbiol.* **33:** 1150-1153.

Sacchi, C. T., Whitney, A. M., Mayer, L. W., Morey, R. and Steigerwalt, A. 2002. Sequencing of 16S rRNA Gene: A Rapid Tool for Identification of *Bacillus anthracis. Emerg. Infect. Dis*. **8(10):** 1117–1123.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Bio. Evol.* **4(4):** 406-425.

Salim, B. S., Mathema, B., Braughton, K., Davis, S. and Sinsimer, D. 2005. Differential distribution and expression of panton-valentine leucocidin among community-acquired methicillin-resistant *Staphylococcus aureus* Strains. *J.Clnic. Microbiol.* **43(7):** 3373–3379.

Sendi, P., Graber, P. and Zimmerl, W. 2005. Loss of ***mec****A* ***gene*** in *Staphylococcus epidermidis* after prolonged therapy with vancomycin. *J. Antimicrobial. Chemother.* **56(4):** 794-795.

Shrestha, N. K., Tuohy, M. J., Hall, G. S., Isada, C. M. and Procop, G . W. 2002. Rapid identification of *Staphylococcus aureus* and the *mecA* gene from BacT/ALERT blood culture bottles by using the lightcycler system. *J. Clinical. Microbiol.* **40(7):** 2659-2661.

Spanu, T., Sanguinetti, M., Ciccaglione, D., Dinzeo, T., Romano, L., Leone, F. and Fadda, G. 2003. Use of the VITEK 2 system for rapid identification of clinical isolates of Staphylococci from bloodstream infections. *J. Clin. Microbiol.* **41:** 4259-4263.