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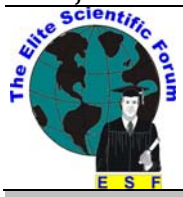

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RESEARCH ARTICLE

Molecular Characterization and Association of Local Isolates of *Staphylococcus aureus* on The Basis of 16S rRNA in Poultry and Human in Pakistan

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ABSTRACT

In human, food poisoning is a great problem and *Staphylococcus (S.) aureus* is frequently associated with it. *S. aureus* is ubiquitous in distribution and is among normal microflora of skin and mucous membranes of human and animals. It is also major cause of different systemic and skin infections in poultry and ultimately severe economic threat to the broiler industry of Pakistan. Poultry birds are frequently sold at retail with the skin intact, thus, the risk of *S. aureus* transmission to human population from whole raw poultry is even greater. Molecular biology is proving a helping hand in the accurate identification of microorganisms through sequence analysis of 16S rRNA gene. The objective of this study was to isolate and characterize *S. aureus* from poultry and poultry farm workers on the basis of 16S rRNA sequences and to investigate the relationship between these isolates. We amplified a region of 16S rRNA of *S. aureus* with subsequent sequencing. The BLAST analyses showed resemblance of sequences with that of previously submitted sequences to GenBank of *S. aureus* isolated from human and chicken. Phylogenetic analyses were performed by using MEGA *5.0* software which confirmed relationship between query sequences and that of human and chicken isolates. Thus, it may be concluded that *S. aureus* can be transferred from chicken to human and it has been proved by using advanced techniques of DNA sequencing and bioinformatics tools.

INTRODUCTION

Staphylococci, member of staphylococcaceae, are gram positive cocci ranging from 0.5 -1.5 µm in diameter and exist as irregular grape like clusters (Harris et al., 2002). Approximately 20 species of Staphylococci have been isolated till present and only *S. aureus* is of major veterinary importance. It is an important opportunistic pathogen that can cause superficial to life threatening diseases in a variety of animal species including poultry. The most common form of infection in poultry involves tenosynovitis and arthritis (Hill et al., 1989; Glisson and Smith, 1990). The most obvious route of infection is through a break in the skin; through the respiratory tract and the gut (Eric and Carolyn 2001). Poultry, meat and egg products are the common sources of *S. aureus*, posing a potential health risk. It is the predominant bacteria involved in food poisoning and is a leading cause of gastroenteritis resulting from ingestion of enterotoxins preformed in contaminated food (Ves et al., 2003).

Accurate and rapid identification of clinically important pathogens has been a challenge for microbiology laboratories. Conventional methods depend upon the comparison of morphological and phenotypic characteristics of the pathogens which are time consuming and are not reliable (Funke et al., 1997; Murray et al., 1999). Diagnostic laboratories have recently employed the molecular based tools to identify bacteria. The 16S rRNA gene sequencing is commonly used method for bacterial identification and classification (Fredricks and Relman, 1996). The 16S

rRNA sequence based identification is of great interest since ribosomal small subunit exists universally among bacteria and contains regions with species-specific variability that makes the identification of bacteria possible to the genus or species level by the comparison with databases in the public domain (Vandamme et al., 1996). This molecular approach has also been used for bacterial phylogeny (Clarridge, 2004; Raoult et al., 2004).

The 16S rRNA gene sequence of *S. aureus* is about 1,550 bp long containing both variable and conserved regions (Chen et al., 1989; Relman, 1999). Conserved regions are similar among bacterial genus and variable regions or signature sequences serve to differentiate at the species level. The sequence of the 16S rRNA gene acquires mutations slowly over time and is therefore considered a stable property (Woese, 1987). Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1,550 bp region) and the sequence of the variable region in between is used for the comparative taxonomy (Chen et al., 1989; Relman, 1999). The comparison of 16S rRNA sequence is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, archaebacteria and eukaryotic organisms.

In a similar type of study, pet animals of 49 Methicillin-resistant *Staphylococcus aureus* (MRSA) infected human patients were screened for MRSA carriage and their bacterial isolates were compared with those of infected human patients using Pulsed-Field Gel Electrophoresis (PFGE). It was found that pet animals of MRSA-infected patients were culture-positive for MRSA, indicating a potential source of infection for public health (Ferreira et al., 2011).

The goal of present study was the PCR amplification of 16S rRNA gene to characterize *S. aureus* isolated from birds and poultry farm personals and to investigate the relationship between these isolates to check the zoonoses. The study would be helpful to design control measures and to avoid the transmission of *S. aureus* between poultry birds and humans.

MATERIALS AND METHODS

Sample Collection and Culturing of Bacteria

Cotton swabs dipped in phosphate buffer saline (PBS) were used to collect the samples from nares and skin of 20 poultry birds and 20 poultry workers from three different poultry farms of Lahore district. The collected swabs were placed in nutrient broth and then allowed to grow on the nutrient agar plates for 24 hours. Using streak plate method, the organisms were isolated and purified on selective media including Staph110 medium and mannitol salt agar. Pure culture colonies isolated on agar plates were studied with gram staining technique and other biochemical tests i.e. coagulase, catalase, VP tests.

Isolation of Genomic DNA from Bacteria

After confirmation by culture characteristics and biochemical tests, genomic DNA was isolated from pure *S. aureus* colonies by Genomic DNA purification kit (MBI Fermentas, Graiciunau 8, Vilmius 2028, Lithuania).

Polymerase Chain Reaction amplification of 16S rRNA gene fragment

The specific primer set, targeting the 16s rRNA gene for *S. aureus*, was designed by using primer-3 software (Untergrasser et al., 2012). Subsequently, PCR was performed using specific primers with an anticipated product size of 756bp on the gel.

Primer	Sequence	Annealing temperature	Product size
FA	5´-AACTCTGTTATTAGGGAAGAA-3´	54°C	756 bp
RA	5´-CCACCTTCCTCCGGTTTGTCAC-3´		

These primers amplify 756 bp region of 16S rRNA gene fragment, which is highly conserved at species level. Total PCR reaction volume was 25 µl containing DNA (50 ng/µl), amonium persulphate buffer (10x), dNTPs (25 mM), $MgCl_2$ (2.5 mM), forward and reverse primers (10 pM, each), DNA Taq polymerase (0.5 U/primer) and double distilled water. The optimized PCR profile consisted of initial denaturation at 95°C for 4 minutes, followed by 30 cycles of template denaturation at 94°C for 1 minute, primer annealing at 54°C for 30 seconds and primer extension at 72°C for 30 seconds. Final extension was done at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis through a 1.2% agarose gel. The gel was stained with ethidium bromide and photographed.

Sequencing of the 16S rDNA fragment

The 16S rDNA (gene on the chromosomal DNA that encodes 16S rRNA) amplified PCR products were sequenced directly by ABI DNA Sequencer (Applied Biosystems Inc.).

Sequence analysis

Comparison of the amplified and sequenced PCR product (756 bp) of 16S rRNA genes of *S. aureus* isolates of present study against NCBI database reported sequences was done using Basic Local Alignment Search Tool (BLAST) hosted at website of NCBI. A phylogenetic tree was then drawn using the Neighbor joining method (Saitou and Nei, 1987). Phylogenetic and molecular evolutionary analyses were conducted at 1000 bootstrap value using Molecular Evolutionary Genetics analysis (MEGA) version 5.0 software (Center of Evolutionary Functional Genomics, Biodesign Institute, Arizona State University, USA) (Tamura et al., 2007).

RESULTS

The isolated bacteria grown well on Mannitol salt agar and showed positive results for catalase and coagulase tests. DNAs extracted from the purified colonies of *S. aureus* were subjected to PCR using the primer set targeting 16S rRNA gene. The gel electrophoresis showed a band of 756 bp on the agarose gel (Figure I). Amplified PCR products were sequenced directly. The generated sequences of the isolates were subjected to BLAST analysis at NCBI, which showed top most similarities with previously submitted sequences of *S. aureus* from different sources (Figure 2).

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and were in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons (Pair-wise deletion option). Phylogenetic analyses were conducted using MEGA version 5.0 software (Center of Evolutionary Functional Genomics, Biodesign Institute, Arizona State University, USA) and are shown in Figure 2, 3 and 4.

DISCUSSION

Currently world is under a heavy burden of continuous presence of infectious agents that are frequent cause of illnesses and deaths in both human and animals (Zeba and Altaf, 2008). Presently, emergence of bacterial strains has made it a bit difficult to identify infectious agents accurately, especially in case of slow growing and fastidious organisms. Traditional phenotypic identification is difficult and time consuming. Similarly phenotypic assessment can misidentify the organisms that have no sufficient unique metabolic reactivity. Universal presence of 16S rRNA gene in all bacteria has proved its importance in characterization of bacteria (Janda and Abbott, 2007). The sequence of 16S rRNA gene has been widely used as a molecular clock to estimate relationship among bacteria (phylogeny) (Sacchi et al., 2002).

In present study, 16S rRNA gene was amplified using PCR primers. Several researchers characterized methicillin resistant *S. aureus* (MRSA) through 16S rRNA gene amplification by PCR (Moussa and Shible, 2008; Ibed and Hamim, 2014).

The sequences of amplified PCR products from isolates (present study) of *S. aureus* were compared with

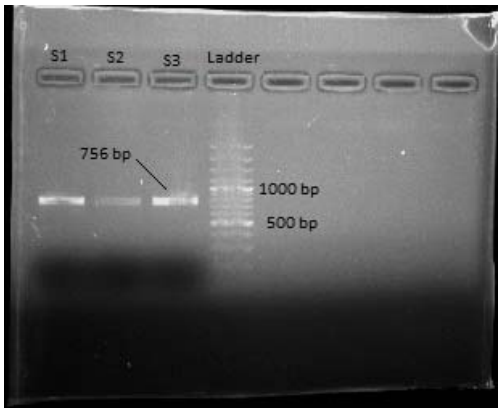

Fig. 1: Amplification of 756bp fragment of 16S rRNA from *S. aureus* isolates

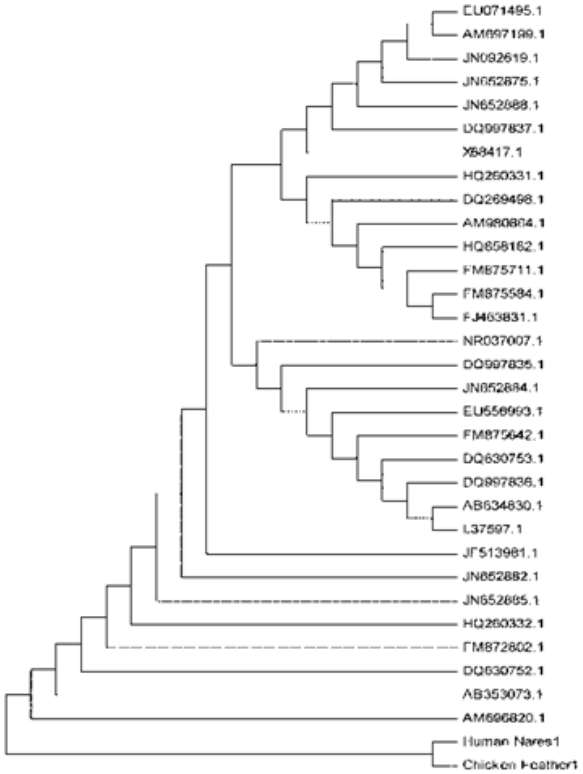

Fig. 2: Phylogenetic tree including local isolates and NCBI reported *S. aureus* isolates

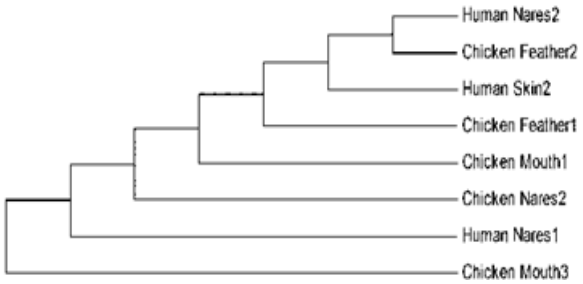

Fig. 3: Phylogenetic analysis showing relatedness of study isolates of *S. aureus*

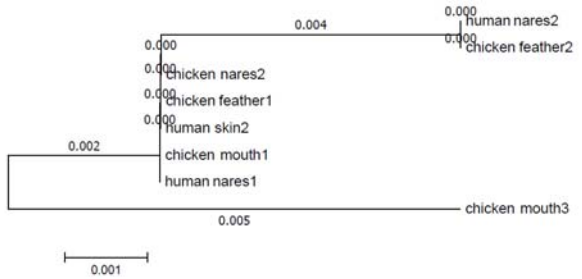

Fig. 4: Phylogenetic analysis with branch lengths showing relatedness of study isolates of *S. aureus*

already reported sequences at NCBI by BLAST tool of bioinformatics with the help of blast. The results revealed 98-99% similarity of the local isolates (*S. aureus*) with the previously submitted sequences from different regions of the world. The similarity index could help in designing the control strategies for the prevention of *S. aureus* infection.

Furtherance, our isolates from poultry and human origin were in close association with each other. One of the isolate from chicken feather and other from human nares showed very close association between them suggesting that *S. aureus* can be transmitted from poultry to human. It has been reported that poultry and poultry products are vehicles for *S. aureus* transmission to human beings (Ves et al., 2003; Lee et al., 2003). But we confirmed this fact with the help of molecular biological techniques.

REFERENCES

Chen K, H Neimark, P Rumore and CR Steinman, 1989. Broad-range DNA probes for detecting and amplifying eubacterial nucleic acids. FEMS Microbiology Letters, 57: 19-24.

Clarridge JE, 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clinical Microbiology Reviews, 17: 840-862.

Eric LJ and LM Carolyn, 2001. Staphylococcus infections in broiler breeders. AviaTech, 1: 1-4.

Ferreira JP, KL Anderson, MT Correa, R Lyman, F Ruffin, L Barth, Reller and VG Fowler, 2011. Transmission of MRSA between companion animals and infected human patients presenting to outpatient medical care facilities. PLoS ONE, 6: 11.

Fredricks D and DA Relman, 1996. Sequence based identification of microbial pathogens: a reconsideration of Koch's postulates. Clinical Microbiology Reviews, 9: 18-33.

Funke G, AV Graevenitz, JE Clarridge and K Bernard, 1997. Clinical microbiology of coryneform organisms. Clinical Microbiology Reviews, 10: 125-159.

Glisson JR and JA Smith, 1990. Staphylococcal tenosynovitis in broiler breeders. In Proceedings of the avian skeletal disease symposium. AAAP/AVMA, San Antonio, TX, USA, pp: 83-85.

Harris LG, SJ Foster and RG Richards, 2002. An introduction to *Staphylococcus aureus* and techniques for identifying and quantifying *S. aureus* adhesins in relation to adhesion to biomaterials. European Cells and Materials, 4: 39-60.

Hill JE, GN Rowland, JR Glisson and P Villegas, 1989. Comparative microscopic lesions in reoviral and staphylococcal tenosynovitis. Avian Diseases, 33: 401-410.

Ibed AN and SS Hamim, 2014. Molecular detection of methicillin resistant *staphylococcus aureus* isolated from burns infection in Al-nasiriyah city. World Journal of Pharmaceutical Sciences, 2: 950-954.

Janda JM and SL Abbott, 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal of Clinical Microbiology, 45: 2761-2764.

Lee JH, 2003. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Applied Environmental Microbiology, 69: 6489-6494.

Moussa I, AM Shible, 2008. Molecular characterization of methicillin-resistant *S. aureus* recovered from outpatient clinics in Riyadh, Saudi Arabia. Saudi Medical Journal, 30: 611-617.

Murray PR, EJ Baron, MA Pfaller, FC Tenover and RH Yolken RH, editors, 1999. Manual of clinical microbiology, 7th ed. ASM Press, Washington DC, USA, pp: 264-282.

Raoult D, S Audic, C Robert, C Abergel, P Renesto, H Ogata, B La Scola, M Suzan and JM Claverie, 2004. The 1.2-megabase genome sequence of Mimivirus. Science, 306: 1344-1350.

Relman DA, 1999. The search for unrecognized pathogens. Science, 284: 1308-1310.

Sacchi CT, AM Whitney, MW Reeves, LW Mayer and T Popovic, 2002. Sequence diversity of Neisseria meningitidis 16S rRNA genes and use of 16S rRNA gene sequencing as a molecular subtyping tool. Journal of Clinical Microbiology, 40: 4520-4527.

Saitou N and M Nei, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

Tamura K, Dudley J, Nei M and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

Untergrasser A, I Cutcutache, T Koressaar, J Ye, BC Faircloth, M Remm and SG Rozen, 2012. Primer3 - new capabilities and interfaces. Nucleic Acids Research, 40: 115

Vandamme P, B Pot, M Gillis, P de Vos , K Kersters and J Swings, 1996. Polyphasic taxonomy, a consensus approach to bacterial systematic. Microbiological Reviews, 60: 407-438.

Ves Y, B Florence and G Michel, 2003. *S. aureus* and food poisoning. Genetics and Molecular Research, 2: 63-76.

Woese CR, 1987. Bacterial evolution. Microbiological Reviews, 51: 221-271.

Zeba PI and A Altaf, 2008. Molecular approaches for epidemiological analysis of infectious diseases. Infectious Diseases Journal, 17: 98-104.