Int J Pharm Bio Sci 2013 July; 4(3): (P) 988 - 994

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

CHARACTERIZATION OF HEMOLYTIC ACTIVITY OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM PARANGIPETTAI COAST SOUTHEAST COAST OF INDIA

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ABSTRACT

The rising development of pollution has been extensive to the marine environment, although it has been one of natural places more stable for years, it is polluted marine aquaculture worldwide at present is being severely affected. It will be linked inter-relationships between human health and the oceans. The opportunistic pathogen is Staphylococci 21 strains isolated from parangipettai coastal waters. Further Identification of *S.aureus* was confirmed as Biochemical tests and PCR by using universal primers 27F and1492R. These strains were analyzed for the detection of α, β and δ hemolysin on sheep, bovine and horse blood used hemolytic activity. Out of 21 strains there is only one strain can detect β-hemolytic activity and hemolysins producing strain were confirmed *S.aureus*.

KEYWORDS: staphylococci, Hemolytic, PCR and Pollution

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INTRODUCTION

Pollution of coastal waters by residual effluents is a worldwide environmental problem[1]. Each year, an estimated 120 million cases of gastrointestinal illness and 50 million cases of respiratory ailments are contracted by bathers in wastewater polluted coastal waters[2]. The species of the Staphylococcus genus are ubiquitously disseminated in the environment with a number of species inhabiting specific ecological niches. They are found living naturally on the skin and mucous membranes of warm blooded animals and humans, which generally imply a commensal or symbiotic relationship with their host. *Staphylococci* are also isolated from a wide range of foodstuff such as meat, cheese and milk, and from environmental sources such as soil, sand, air and water[3, 4]. *Staphylococcus aureus* has been studied as a pollution indicator of direct contamination from bathers its detection is favored by its high survival capacity in marine environments, as it is more resistant to chlorine and salt. These bacteriological factors this also makes it a potential danger to the swimmers at the beaches[5]. *Staphylococcus aureus* in seawater has been shown to be positively correlated with skin, ear, and respiratory tract ailments[6]. The pathogenicity of the genus *Staphylococcus*, particularly for *Staphylococcus aureus*, is related to the production of a wide variety of exo toxins, including the alpha, beta, and delta hemolysins which contribute to its ability to cause diseases in many mammalian species. Staphylococcal alpha-hemolysin or alphatoxin is the most studied and characterized cytotoxin, and is considered a main pathogenicity factor because of its hemolytic, dermonecrotic and neurotoxic effects[7]. Additionally, beta-hemolysin is a sphingomyelinase that is highly active against sheep and bovine erythrocytes[8], while delta-hemolysin as well as alpha-hemolysin induce pore formation, perturbing the cell membrane permeability[9]. Hence in the present study was intended to verify the species of hemolytic activity and molecular identification using PCR to detection a *Staphylococcus aureus* isolated from Parangipettai coastal water the south east coast of India.

MATERIALS AND METHODS

COLLECTION AND ISOLATION OF STAPHYLOCOCCUS

The pathogenic strain was isolated from marine water collected from Parangipettai coastal water (Latitude11^{o}25'913''N and Longitude 79^{o}51'108''E) the south east coast of India. The surface water samples were collected in 100ml sterile screw caped bottles under aseptic conditions. All samples were brought to the laboratory in a portable ice box and bacteriological analyses were made immediately after collection. The marine water sample analysis to using membrane filtration technique was used to isolated bacteria from the water samples. Appropriate 10-fold dilutions of each sample prepared in 10ml sterile phosphate buffer saline. Each sample was filtered through a 0.45µm filter paper. Then the filter was placed on mannitol salt agar (Himedia-Mumbai). This is a medium specific isolation of staphylococcus species. The membrane filter paper was placed into petriplates were incubated at 37^{o}C for 24 hrs. The isolated colonies were screened and identified biochemical tests.

TAXONOMICAL AND BIOCHEMICAL IDENTIFICATION OF STAPHYLOCOCCUS AUREUS

The isolated colonies were identified using taxonomic, physiological and biochemical methods based on Bergey's manual of systematic bacteriology[10].

MOLECULAR IDENTIFICATION OF STAPHYLOCOCCUS ISOLATION OF GENOMIC DNA

Total genomic DNA was extracted from the brain heart infusion broth by phenol chloroform isoamyl alcohol method. This removes the protein and other cellular components from the nucleic acids to obtain the pure DNA. Log phase culture (2 to 4ml) was taken and centrifuged at 10,000rpm for 10minutes at 4^{o}C.

Centrifugation was repeated to wash the cells twice with 500µl of TE buffer. The pellet was resuspended 500µl of TE buffer and incubated for 10 minutes in boiling water bath and centrifuged. After centrifugation equal volume of phenol:chloroform:isoamyl alcohol was added to the supernatant and centrifuged. To the aqueous phase, 0.1 volume of 3M ammonium acetate pH (5.2) and 2.5 volume of ice-cold ethanol were added and incubated at -20^{o}C overnight. After incubation sample was centrifuged at 10,000rpm for 10 minutes at 4^{o}C and 70% ethanol was added to wash the pellet and air dried. After drying the DNA was resuspended in TE buffer pH 8.0 and stored at 4^{o}C. DNA sample (10µl) was mixed with 2µl of 6x loading dye and loaded in 1% Agarose gel. The separated DNA was visualized by UV-Transilluminator.

PCR AMPLIFICATION

The PCR amplification was performed in a thermal cycler (Model GENE) by using a recombinant Taq DNA polymerase (AmpliTaq, Perkin-Elmer Cetus Corp., Norwalk, Conn). The 16S rRNA coding gene was amplified using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The reaction mixture consisted of 50µl consisted of lysate, 10 µl of l0x PCR amplification buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.5 mM MgCl2, 0.2 mM of each dNTP of Tritton X-100 and 200 µM primer stock, 1.5 Unit Taq DNA polymerse and template DNA (50ng) in a 1 µl volume were added. PCR was performed using 37- 40 cycles of amplification following conditions: DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 0.5 min, and DNA extension at 72°C for 1.5 min. After the final cycle, the reaction was terminated by keeping it at 72°C for 3.5 min. The PCR products were stored in the cycler at 4°C until they were collected. The amplified PCR products were visualized simultaneously by standard gel electrophoresis in a 4% agarose gel stained with etidium bromide. The gels were photographed under ultra violet light using the BioProfile system (Mitsubishi, Tokio, Japan). Molecular size markers (1.5-bp and 1.5-kb DNA ladder; Genei India) were included in each agarose gel[11].

16S rDNA SEQUENCING

A sequence of 935 bp was sequenced and amplification using universal primers was performed. The amplicons were sequenced directly with the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit using a model 373 A sequencer in (Applied Biosystem). The results were processed into sequence data with sequence analysis chromas software (version2.33;TechnelysiumPvt.Ltd;http://www.technelysium.com.au/chromas.html) and the Partial sequences were combined into a single consensus sequence by finding multiple matching sub segments in both forward and reverse sequences with the aid of William Pearson's lalign program (http://www.ch.embnet.org/software/lalign_form.html). A single sequence analysis data obtained was blasted in NCBI database.

PHYLOGENETIC ANALYSIS

The sequences were edited using Vecscreen and a BLAST search of the National Centre of Biotechnology Information (NCBI) was performed to identify the closest neighbours to the cloned sequences. Taxonomic assignments were performed using the Ribosomal Database Project (RDP) classifier program (http://rdp.cme.msu.edu/). Phylogenetic tree was inferred using the neighbor joining. Tree building algorithm and analyses were performed with MEGA 4.0 software[12].

ASSAY OF HEMOLYTIC ACTIVITY

The hemolytic activity was evaluated by plating method. The Staphylococci strain on triplicate plates of blood agar base supplemented with 5% sheep, ovine and horse blood for alpha, beta and delta-hemolysin assays respectively[13]. To remove any possible anti hemolysin compounds present in the serum, the red blood cells were washed with sterile saline and resuspended in saline to the original volume of the blood. Strain were streaked on the surface

of the blood agar plates and incubated at 37 °C for 24 to 48 hrs. The criteria for hemolysin identification were: complete lytic zone transparent with blurred edges for α-hemolysin on ovine and incomplete (non- transparent) lytic zone, which became complete with sharp edges after overnight incubation at 4 °C on bovine blood agar, for β-hemolysin. The δ-hemolysin production was determined as complete hemolytic zones on horse blood agar[13, 14].

RESULTS

Nucleotide sequence accession number

The nucleotide sequence corresponding to the 16 s rRNA gene of *Staphylococcus aureus* CASMTK1 have been assigned the respective accession number is JX435813 in the gene bank database.

Table.1

Isolation and Identification of Staphylococcus aureus from parangipettai coastal water to identify Biochemical Tests.

Tests	***Staphylococcus aureus***
Morphology	Cocci in clusters
Gram's reaction	Positive
Motility	Non-motile
Pigmentation	Yellow
Nacl.Conc	5-10%
pH range of growth	7-10
Indole	Negative
Methyl red	Positive
Voges proskauer	Positive
Catalsae	Positive
Coagulase	Positive
Dnase	Positive
Oxidase	Negative
Mannitol	Positive
Citrate	Negative
Fructose	Negative
Xylose	Negative
Arabinose	Negative
Arginine	Negative
Maltose	Negative
Lactose	Negative
Sucrose	Negative
Xylitol	Negative
Cellobiose	Negative
Urease	Negative
Phosphotase	Negative
Novobiocin	Negative
Rhamnose	Negative
Dextrose	Negative

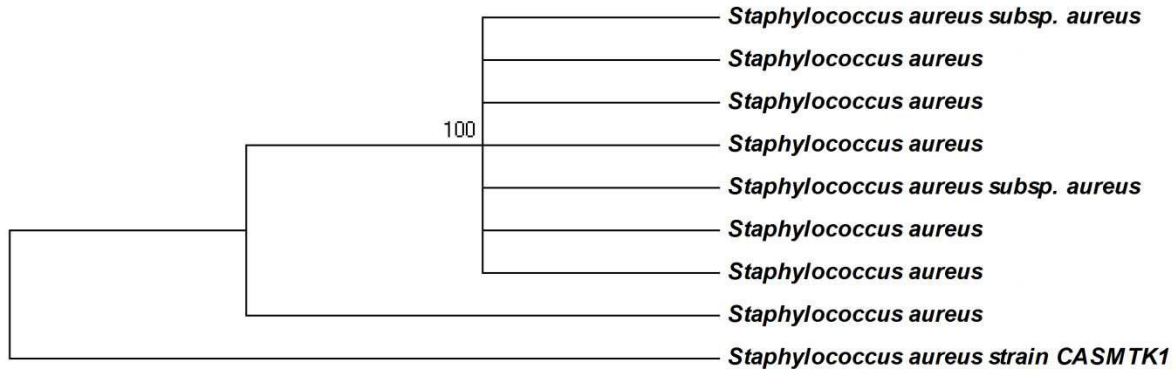

Figure 1

The phylogram showing the position of strain CAS MTK1 and related strains based on 16S rRNA gene sequence. Phylogenetic tree based on neighbor joining analysis and only bootstrap values expressed as percentage of 1000 replications are shown at the branch points.

Hemolytic Activity

The marine *Staphylococcus aureus* CASMTK1 strain were tested for haemolytic acitivity used sheep blood agar plates. *S. aureus* CASMTK1 strain found β-haemolytic activity colonies on the blood agar plates. These strain developing transparent zones around colonies are considered has β-haemolytic activities. The β-haemolytic zone of inhibition assessment is 1.0cm.

DISCUSSION

Staphylococcus is a species rich genus with importance from both on human health and economic perspective. Relevant to the first goal of this study, our results provide strong evidence that the current groupings of Staphylococcus species require revision, and provide a clear consensus across analyses on how this could bend one to reflect inferred evolutionary relationships among species groups. Hence in the present study isolated and identified for Staphylococcus species. Bergey's manual of systematic bacteriology was used for the examination of biochemical properties to identify this marine *Staphylococcus* species. Biochemical tests were able to identify *S. aureus* in comparison with the 16S rRNA sequence analysis. In the present study the isolated *S. aureus* tested blood agar plates it shows β-haemolytic activity. Whereas the similar study of β-haemolytic activity carried out[15] Moreover 82.8% of the isolates were beta haemolytic, 13.8% isolates were alpha haemolytic and 3.4% of the isolates were non haemolytic on blood agar. Nearly similar results were obtained by[16]. In the early days of molecular phylogenetics, a gene tree was usually equated with the species tree. This view was typified using ribosomal RNA (rRNA) sequences as the principal molecular phylogenetic marker. Phylogenetic analysis of rRNA transformed our understanding of the history of life resulting in the discovery of a previously unrecognized domain of life the Archaea and in a tree topology that has been aptly called the standard model of evolution[17, 18, and 19]. This model involves the early descent of the bacterial clade from the last universal common ancestor and a subsequent separation of archaea and eukaryotes. In addition to the formulation of the standard model, phylogenetic analysis of rRNAs brought 'the winds of evolutionary change' onto taxonomy by revealing supporting or correcting many major clades among bacteria, archaea and eukaryotes[20]. So in the present study we used 16S rDNA sequence to construct the phylogenetic tree using MEGA 4 software. Multiple alignments of 16S rRNA gene data

sequences of Staphylococcus marine water isolates were obtained using ClustalW program. Sequence similarities of 16S rRNA gene among 9 possess *Staphylococcus* species range level is 100%. The relationships among species of the genus *Staphylococcuss* were confirmed by phylogenetic analysis based on the 16S rRNA gene sequencing, and the topology of the tree was evaluated by bootstrap values. The phylogenetic tree (Figure 1) showed that *Staphylococcus* species were divided in distinct subgroups. The group consisting of *S. aureus subsp. aureus* ST228, *S.aureus subsp. aureus* FUA2080, *S. aureus* RA 20, *S. aureus* S11, *S. aureus* GSA79, *S. aureus* FFL 34, *S. aureus sc-sa*. This grouping formed a monophyletic clade in the phylogenetic tree with 100% of the bootstrap value. Better results have been obtained by comparing sequences of other housekeeping genes such as *hsp60*, *sodA*, *rpoB*, *tuf*, and *gap*[21]. The availability of such a comprehensive estimate of the evolutionary origins of and relationships among staphylococci provides an important context for understanding patterns of gain and loss of genetic and physiological attributes and the potential role of lateral gene transfer in both pathologically relevant phenotypes and in estimation of phylogenetic relationships among species.

CONCLUSION

Oceans directly and indirectly impact the extent to which humans are exposed to disease causing organisms. Recent research has greatly enhanced our understanding of the relationships between pathogens, coastal and marine environments, and human health. In the present findings shows that the β-haemolytic activity of *Staphylococcus aureus*, And the biochemical tests were used to identification of *Staphylococcus aureus* and final confirmation method is the PCR technique has been used shown to be accurate, fast and reliable tool for identification of *Staphylococcus aureus*. These opportunistic bacteria' habitats are acting as pollution indicator of ecosystems and also help to warn of threats from emerging pathogens.

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