Production, Purification & Analysis of Enterotoxins from HA-MRSA Isolated from Clinical Samples

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Abstract
The problem of resistance is increasing for all types of bacterial isolates including Staphylococci. Methicillin resistant Staphylococcus aureus (MRSA) has emerged as an important hospital acquired and community acquired pathogen. Multi drug resistance among MRSA is a serious issue limiting the treatment options for the victims. MRSA strains that share the presence of staphylococcus cassette chromosome mec (SCC mec) in their genomes are frequently virulent and predominantly cause infections. Staphylococcal enterotoxins are a family of structurally related proteins that are produced by Staphylococcus aureus playing a major role in the pathogenicity. These microbial superantigens have profound effects on the immune system, which makes them useful tools for understanding its mechanism of action. The proposed study is mainly focused on collection of MRSA strains from clinical samples, isolation and Identification was done on the basis of microscopic examination and cultural characteristics, biochemical tests. The cultures were subjected for DNA extraction followed by AGE. All the samples were amplified targeting the gene Mec A and sent for sequencing. All the sequences were further analyzed and confirmed. The production and purification of Enterotoxins by MRSA was done and crude protein was collected. This was further purified by downstream processing. The sample was subjected for centrifugation followed by salt precipitation, Dialysis. The enterotoxins were produced form all the strains isolated and purified.

Keywords
MRSA, mec-A Gene, AGE, PCR, Enterotoxin

Introduction
Staphylococcus aureus is a critical human pathogen that can cause an assortment of illnesses, including skin and delicate tissue contaminations and orderly life undermine diseases. The proceeding with rise of methicillin resistant Staphylococcus aureus (MRSA), including hospital acquired (HA) - MRSA and community acquired (CA) - MRSA, is a noteworthy and expanding danger to general wellbeing. most of the nosocomial s. aureus infections are as a result of methicillin-resistant s. aureus (MRSA) lines and have turn out to be a broadly diagnosed motive of morbidity and mortality at some stage in the sector (ardic et al., 2006; pesavento et al., 2007; ho et al., 2008). In addition, MRSA lines proof against quinolones or multiresistant to other antibiotics have been emerging, leaving a restrained preference for their manipulate (mee-marquet et al., 2004; nejma et al., 2006; pesavento et al., 2007) The present study was the characterization of different staphylococcus aureus isolates collected from different clinical samples. Molecular level identification was done by DNA extraction followed AGE & then PCR based on 16s sequencing. This characterization was based on the ability of the isolates to produce and to express staphylococcal enterotoxins.

Materials & Methods
Collection of Samples: 5 no of samples were collected from various Research units, hospitals for the current research purpose.

Pure Culture Preparation: In order to obtain the pure cultures of the isolates streaking was performed on Muller Hinton agar medium which is specific for the selected bacteria.

Isolation & Identification of MRSA: The presumptive isolates were sub-cultured on MH medium for another 24hr. The suspected colonies were used for Identification of Staphylococcus aureus on the basis of Morphology and microscopic examination, cultural characteristics, and Bio chemical tests based on Bergey’s manual of systemic bacteriology. The final identification is however dependent on the sequencing of 16s R DNA of the isolates.

DNA Extraction: DNA was extracted using the standard method with some modifications.1.5ml of overnight culture was centrifuged at 6000rpm for 10 min, to the pellet 0.5ml of Lyses buffer was added and incubated at 60°C-65°C for 20 min. This was further centrifuged at 10,000 rpm for 10 min. To the supernatant equal volumes of Isopropyl alcohol was added and kept in ice cold conditions overnight. The sample was later centrifuged at 12,000rpm for 10 min and the pellet was collected to which 50µl of TE buffer was added and preserved until use.
Agarose Gel Electrophoresis: 0.32gm of Agarose was dissolved in 40 ml of TAE buffer by boiling. 3µl of Ethidium bromide was added to the solution which was then poured into the casting tray & kept for solidification. Gel was placed in the electrophoresis unit and the buffer tank was filled with TAE buffer. 5µl of dye with 10µl of Samples was load in the wells and the electrophoresis was run at 50 V for 40min. This protocol would confirm the quality of DNA obtained.

PCR Detection of S.aureus: The detection of *staphylococcus aureus* was performed by using 16S R RNA universal primers for amplification. The molecular level identification based on 16s sequencing confirms the identity of the samples.

Enterotoxin Production Media: Production medium was prepared by standard medium composition. Appropriate amount of glucose, carbon sources, in-organic salts, vitamins etc. were added in required concentrations and the media was subjected for sterilization.

Inoculation & Cultural Conditions: MRSA isolates were inoculated into the sterile production medium. All cultures were incubated in a water bath shaker at 37°C and 200 rpm for 4-5 days.

Centrifugation and Collection of Crude Enterotoxin: Cultured Broth was centrifuged at 5000X for 10 min & the supernatant containing crude enterotoxin was collected and stored in ice cold conditions.

Salt Precipitation: Ammonium per sulphate (0.8mg/ml) was added pinch by pinch to the suspension under ice cold conditions on magnetic stirrer for 1hr. Later the solution was stored in ice cold concisions for overnight for complete saturation. The precipitated proteins were recovered by centrifugation at 10,000X for 10 min & dissolved in fresh Tris- HCl buffer for subsequent stage of purification.

Dialysis: Dialysis membrane was added into 100 ml of boiling water and boiled for 10 minutes. The membrane was transferred to a beaker containing 2% Sodium bi Carbonate and boiled for 10 minutes. The membrane was re transferred to 100 ml boiling water & boiled for 10 minutes. Later it has to be taken out and dried and sealed on one end. The Protein to be purified is poured into the membrane and was again sealed on the other end. The whole sample is suspended in a beaker with water on a magnetic stirrer and subjected for constant shaking for overnight.

Results & Discussion

Pure Culture Preparation of the Selected Bacteria

The source sample was inoculated onto the MH medium. The colonies produced all are resistant to methicillin antibiotic. One colony was selected and used for pure culture preparation.

![Fig 1: Shows the Pure Culture Obtained by Streak Plate Method](image)

Gram’s Staining to Identify the Culture Based on their Cell Wall Composition

As per the microscopic view after Gram’s staining the bacteria were found to be Gram positive Cocci in groups. These cultures were further screened for Biochemical test to identify the species.

Biochemical Identification of Bacteria

All the isolates were tested or the Catalase and mannitol fermentation activity. All the samples showed a positive reaction towards catalase and mannitol tests indicating the samples to be *staphylococcus aureus* species.
Fig 2 & 3 indicate the Results of Mannitol Fermentation Test and Atalase Test respectively. The Bacteria is Positive for Both the Tests.

DNA Extraction, PCR Amplification
All the samples were subjected for DNA extraction using basic lysis SDS method and the extracted DNA was subjected for amplification using PCR and universal primers. The primers were bought from another laboratory. All the amplified samples were further purified and sent for sequencing so as to conform their identity at the molecular level.

Sequencing Results and BLAST Analysis for Identification
Sample 1:
CTCAAAGGAATTTGACGGGGACCCGCACAAGCGGAGCATGTTATTGGAAGACAGCAACCGGAAGTTCAATGACAATCTTCACCAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGG

Inference: The bacteria has been identified as Staphylococcus sp. Strain: MK038968.1

Sample 2:
GTGAGTTAAGCAATTTCAAGCAAAACTTTCCAACATTCAATGCTCAGGATAAAAGTGTGATGATTTTTG
GTCTTGAGGGGTGGTTGTCAGAAAATGAAATTTCATTATTTAGTAATACGTACGTATGTTGTTGGCTTGG
GGCCGAGA

Inference: The given bacteria has been identified as Staphylococcus aureus strain: CP031661.1

Sample 3:
GAGTGCTAAGTGTATAGGGGTTCTCCCTCCCCTTATGCTGACGCTAAGCATTAAAGCAGCTCAGCCTGGG
GAGTACGACCCGAAGGTTTGAACACTCAAAGGAATTGACCGGGACCCCGCACAAGCGGTTGAGCATGTTG
TTTAATTCGA

Inference: The bacteria has been identified as Staphylococcus aureus strain: CP031661.1
Inference: The bacteria has been identified as Staphylococcus stepanovicii strain: MK026833.1

Sample 4:
GGAGTCTTATGATATTGTTACAGCTCGTGACGTAGCGAGATTGACTGTCTTAAGTGAGTTGTGCTTTACC TTGTAATAAAAGGTGGACAGTTCCTTGCACTTAAATCATCAAAAAAGGTAAAGA

Inference: The bacteria has been identified as Staphylococcus hominis strain: MH423313.1

Sample 5:
TTTTTAATGGCAGAAGTTGATATTTCTATATTAAGTATGTTCCAAGAGAAATATTTCATCCTAAACCT AAGTGAATAGCTCACTTATCAGATTAAATAGAAAAAAATCAAGAATATCACACAAAGATAAACAGA AGTATAATTATTGCCATTAGAAATGGGTAAACAAAGAATACACAGAAAATATTTCACAAAAATCAATT AAC

Inference: The bacteria has been identified as Staphylococcus hominis strain: MH423313.1

After performing PCR detection of *S. aureus*, the bacterial cells were cultured in enterotoxin production medium. After incubation crude protein were subjected to centrifugation and dialysis.
Conclusion
The above work concludes that all the bacteria isolated from the clinical samples was identified to be Staphylococcal sub species and are known fro the presence of Methicillin resistance gene. Also the work included the extraction and purification of enterotoxin that can have a therapeutic role in the further investigations.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this paper

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