
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 from 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 turned 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^oC-65^o 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 loaded 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 conditions 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

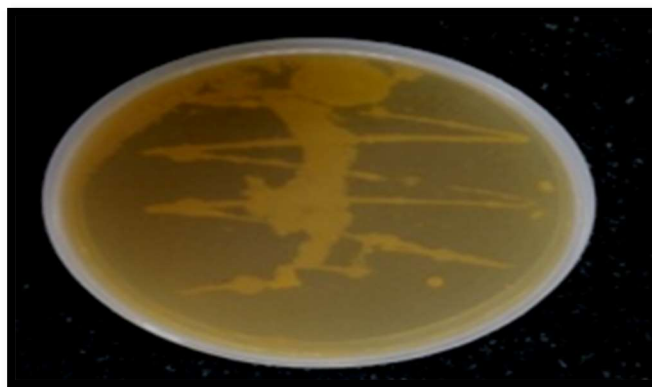


Fig 1: Shows the Pure Culture Obtained by Streak Plate Method

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 for 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.

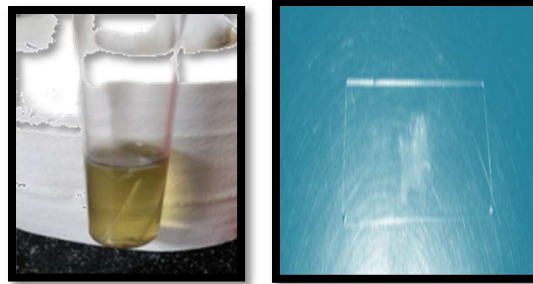


Fig 2 & 3 indicate the Results of Mannitol Fermentio Test and Atalase Test respectively. The Bacteria is Positive for Both the Tests.

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 baught from another laboratory. All the amplified samples were further purified and sent foe sequencing so as to conform their identity at the molecular level.

Sequencing Results and BLAST Analysis for Identification

Sample 1:

CTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG AACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGG

Staphylococcus sp. strain IOM2617228 16S ribosomal RNA gene, partial sequence					
Sequence ID: MK038968.1 Length: 1145 Number of Matches: 1					
Range 1: 885 to 1012 GenBank Graphics					
Score	Expect	Identities	Gaps	Strand	
237 bits(128)	6e-59	128/128(100%)	0/128(0%)	Plus/Plus	
Query 1		CTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA		60	
Sbjct 885		CTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA		944	
Query 61		CGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCC		120	
Sbjct 945		CGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCC		1004	
Query 121		TTCGGGGG 128			
Sbjct 1005		TTCGGGGG 1012			

Inference: The bacteria has been identified as Staphylococcus sp. Strain: [MK038968.1](#)

Sample 2:

GTGAGTTAAGCAATTTCAAGCAAACCTTACAACAATTCAATGCTCAGGATAAAGTGTGATGATTTTTG
GTCCTGAGGGTGGTTTGTGAGAAAATGAAATTTCAATATTTAGTAATACTAGTACAGTTGTTGGTCTTG
GGCCGAGA

Staphylococcus aureus strain 82 chromosome, complete genome					
Sequence ID: CP031661.1 Length: 2823570 Number of Matches: 1					
Range 1: 1604374 to 1604519 GenBank Graphics					
Score	Expect	Identities	Gaps	Strand	
270 bits(146)	7e-69	146/146(100%)	0/146(0%)	Plus/Minus	
Query 1		GTGAGTTAAGCAATTTCAAGCAAACCTTACAACAATTCAATGCTCAGGATAAAGTGTG		60	
Sbjct 1604519		GTGAGTTAAGCAATTTCAAGCAAACCTTACAACAATTCAATGCTCAGGATAAAGTGTG		1604460	
Query 61		TGATTTTTGGTCTTGAGGGTGGTTTGTGAGAAAATGAAATTTCAATATTTAGTAATACTA		120	
Sbjct 1604459		TGATTTTTGGTCTTGAGGGTGGTTTGTGAGAAAATGAAATTTCAATATTTAGTAATACTA		1604400	
Query 121		GTACAGTTGTTGGTCTTGGGCCGAGA 146			
Sbjct 1604399		GTACAGTTGTTGGTCTTGGGCCGAGA 1604374			

Inference: The given bacteria has been identified as Staphylococcus aureus strain: [CP031661.1](#)

Sample 3:

GAGTGCTAAGTGTAGGGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG
GAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGA

Staphylococcus stepanovicii strain 4G159 16S ribosomal RNA gene, partial sequence
Sequence ID: [MK026833.1](#) Length: 1451 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
268 bits(145)	3e-68	145/145(100%)	0/145(0%)	Plus/Plus
Query 1	GAAGTCTTAAGTGTATTGTTACAGCTCGTGCAGTAGCGAGATTGACTGTCTTAAGTGAGTTGTGCTTACC			60
Sbjct 772	GAAGTCTTAAGTGTATTGTTACAGCTCGTGCAGTAGCGAGATTGACTGTCTTAAGTGAGTTGTGCTTACC			831
Query 61	CGCTTGGGAGTACGACCGAAGGTTGAAATTCGAAAGGAAATGACGGGGACCCGCACAAG			120
Sbjct 832	CGCTTGGGAGTACGACCGAAGGTTGAAATTCGAAAGGAAATGACGGGGACCCGCACAAG			891
Query 121	CGGTGGAGCATGTGGTTTAAATTCGA			145
Sbjct 892	CGGTGGAGCATGTGGTTTAAATTCGA			916

Inference: The bacteria has been identified as Staphylococcus stepanovicii strain : [MK026833.1](#)

Sample4:

GGAGTCTTATGATATTGTTACAGCTCGTGCAGTAGCGAGATTGACTGTCTTAAGTGAGTTGTGCTTACC
TTTAGTAAATAAAGGTGGACAGTTCCTTGCACCTAAATCATCAAAAGGTAAGA

Staphylococcus epidermidis strain SEI, complete genome
Sequence ID: [CP009046.1](#) Length: 2500626 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
228 bits(123)	4e-56	123/123(100%)	0/123(0%)	Plus/Plus
Query 1	GGAGTCTTATGATATTGTTACAGCTCGTGCAGTAGCGAGATTGACTGTCTTAAGTGAGTT			60
Sbjct 1487795	GGAGTCTTATGATATTGTTACAGCTCGTGCAGTAGCGAGATTGACTGTCTTAAGTGAGTT			1487854
Query 61	GTGCTTACCTTTAGTAAATAAAGGTGGACAGTTCCTTGCACCTAAATCATCAAAAGGTA			120
Sbjct 1487855	GTGCTTACCTTTAGTAAATAAAGGTGGACAGTTCCTTGCACCTAAATCATCAAAAGGTA			1487914
Query 121	AGA 123			
Sbjct 1487915	AGA 1487917			

Inference: The bacteria has been identified as Staphylococcus hominis strain: [MH423313.1](#)

Sample 5:

TTTTTAATGGCAGAAGTTGATATTTCTATATTAAGTATGGTTCCAAGAGAATATTTTCATCCTAACCT
AAAGTGAATAGCTCACTTATCAGATTAATAGAAAAAATCAAGAATATCACACAAAGATAAACAGA
AGTATAATTATTCGTTATGAAATGGGTTAACAAAGAATACAAGAAAATATTTACAAAAAATCAATTT
AAC

Staphylococcus hominis strain Sho-115Lar plasmid pSho-115Lar, complete sequence
Sequence ID: [MH423313.1](#) Length: 2473 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
383 bits(207)	1e-102	207/207(100%)	0/207(0%)	Plus/Minus
Query 1	TTTTTAATGGCAGAAGTTGATATTTCTATATTAAGTATGGTTCCAAGAGAATATTTTCAT			60
Sbjct 1699	TTTTTAATGGCAGAAGTTGATATTTCTATATTAAGTATGGTTCCAAGAGAATATTTTCAT			1640
Query 61	CCTAAACCTAAAGTGAATAGCTCACTTATCAGATTAATAGAAAAAATCAAGAAATATCA			120
Sbjct 1639	CCTAAACCTAAAGTGAATAGCTCACTTATCAGATTAATAGAAAAAATCAAGAAATATCA			1580
Query 121	CACAAAGATAACAGAAGTATAATTTTCGTTATGAAATGGGTTAACAAAGAATACAAG			180
Sbjct 1579	CACAAAGATAACAGAAGTATAATTTTCGTTATGAAATGGGTTAACAAAGAATACAAG			1520

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.



Fig 4 & 5: Production Medium & Crude Protein Collection



Fig 6: 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 for 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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