



Detection of *mecA* Gene Associated with Methicillin Resistant *Staphylococcus aureus* and its Alternatives using Nanoparticles and Chia Seeds

M. Rajamani*, Jesteena Johny and R. Ragunathan

Centre for Bioscience and Nanoscience Research, Eachanari, Coimbatore, Tamil Nadu, India

*Corresponding e-mail: cbnrcindia@gmail.com

ABSTRACT

Staphylococcus aureus, a member of the family Micrococcaceae, is a Gram-positive coccus whose cells tend to occur either singly or if dividing cells do not separate, form pairs, tetrads and distinctive irregular "grape-like" structures, which is resistant to few antibiotics like Methicillin which is termed as Methicillin resistant *Staphylococcus aureus*. MRSA was isolated from the pus sample. Confirmation of MRSA was done by using Kirby Bauer disk method. Followed by sub culturing in Luria bertani broth and DNA isolation was performed by phenol chloroform method and confirmed by AGE (Agarose gel electrophoresis). The amplification of *mecA* gene thermocycler-PCR was done. Restriction fragmented linked polymorphism was done for knowing how much restriction sites are available for organism. To calculate the molecular weight of the protein SDS-PAGE (Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis) was performed. Followed by alternative measures was done, by using Silver nanoparticles at different concentrations and Chia seeds against MRSA.

Keywords: *Staphylococcus aureus*, MRSA, Isolation of DNA, PCR, RFLP, SDS-PAGE, Silver nanoparticles, Chia seeds

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) were first reported in 1961 and have since become a major nosocomial pathogen worldwide. An additional concern is the emergence of vancomycin-intermediate *S. aureus* (VISA) and more recently vancomycin-resistant *S. aureus* (VRSA). The reservoir of MRSA is infected and colonized patients, and the major mode of transmission from patient to patient is on the contaminated hands of healthcare workers [1]. Nearly all MRSA isolates produce an additional penicillin binding protein (PBP), named PBP2a. PBP2a binds β -lactams with a lower affinity than PBP2, the major physiological methicillin target. PBP2a is encoded by the *mecA* gene; a component of a larger DNA fragment designated the *mec* region.

The standard test used to identify MRSA is amplification of the *mecA* gene. The *mec* elements upstream and downstream of *mecA* are polymorphic. Nonetheless, two upstream genes, *mecR1* and *mecI*, are thought to regulate methicillin resistance [2].

Several PCR assays based on the DNA sequence information have been used for detection of MRSA strains. The PCR methods have high sensitivity and specificity and were independent of the physical and chemical conditions of culture. To carry out normal PCR, 24-hour time is needed. This time is required for DNA preparation, PCR performance and detection of PCR products into the gel [3]. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) studies of these genes were found to be quite useful in typing *S. aureus* strains and have proven absolute type ability, reproducibility and good discriminatory power [4].

Generally, nanoparticles are prepared by several methods such as physical and chemical, but these methods are not eco-friendly. In contrast biological methods urged as safe, cost effective, possible eco-friendly alternatives to physical and chemical methods [5].

Chia (*Salvia hispanica* L.) seeds are used widely because of its nutritional value, antioxidant, and antibacterial activity against various microorganisms but there are no studies that relate the antibacterial activity of Chia seeds against *Salvia hispanica* L. a biannually cultivated plant, is categorized under the mint family (Labiatae), super division of

Spermatophyta, and kingdom of Plantae, native to central and southern Mexico, Guatemala, and India. The available human and non-human studies showed possible effectiveness for allergies, athletic performance enhancement, cancer, coronary heart disease (CHD), endocrine disorders, hyperlipidaemia, hypertension, and stroke. Some evidence also suggests possible anticoagulant, antioxidant, and antiviral effects of *Salvia hispanica* [6].

MATERIALS AND METHODS

Collection of Sample

Pus sample was collected from Abirami hospital, Sundarapuram, Coimbatore, from the patients with infected skin. Skin lesions were swabbed, and pus samples were collected and maintained in saline. Totally 5 samples were collected.

Isolation and confirmation of *Staphylococcus aureus* using Biochemical tests and selective mediums

The pus sample was streaked onto nutrient agar plate and incubated for 37°C for 24 hours. Biochemical tests such as IMVIC, Catalase, Urease, Oxidase, Coagulase tests were performed. The culture was streaked onto Mannitol salt agar (selective medium) and kept for incubation at 37°C for 24 hours.

Confirmation of MRSA - Hi-Comb method

Staphylococcus aureus was isolated from the sample. Then, methicillin strip was used to confirm the MRSA by Disc Diffusion method - Susceptibility testing. The organism was swabbed on to the Muller Hinton agar plates and Hi-comb disc was placed using sterile forceps and the plates were incubated for 24 hours at 37°C.

Molecular characterisation - Genomic DNA isolation

Phenol-chloroform method was used to identify the DNA. The confirmed culture was re-inoculated into Luria bertani broth, after 24 hours of incubation the pellet was collected and added 500 µl of saline EDTA and Lysozyme. The tube was incubated for 37°C for 30 minutes. After incubation, 150 µl of 10% SDS was added; incubated for 65°C for 15 minutes. Added phenol, chloroform and isoamyl alcohol in the ratio of (25:24:1), and centrifuged at 10000 rpm for 10 minutes. Aqueous layer was collected, and 0.2 volume of sodium acetate and 5 volumes of isopropanol were added, to the pellet 500 µl of 70% ethanol was added and centrifuged. The confirmation of DNA was done by Agarose gel electrophoresis (1% Agarose) - 50 V for 90 min and viewed under the UV transilluminator.

PCR study - *mecA* gene amplification

The isolated DNA from the MRSA was taken for PCR study. To amplify the *mecA* gene by using the DNA after confirmation in AGE. About 2 µl of DNA was taken and 2 µl of forward and reverse primer was added.

Primer forward – 5' GGTTATGCCTTATATTCG

Reverse primer – 5'TTAGCGTTGCCAGTGCTC

About 8 µl of PCR Master Mix was added to it for efficient amplification. And then 6 µl of nuclease free water was added. The PCR cycling conditions were as follows:

Initial denaturation at 94°C for 1 min, denaturation for 30 secs at 94°C, followed by 20 cycles of 15 sec at 55°C for annealing process, extension for 72°C for 2 minutes with a final extension step at 72°C for 30 sec. About 5 µl of product were loaded onto 1.8% Agarose and viewed under the UV transilluminator.

Restriction Fragment Linked Polymorphism Studies

About 2 µL of PCR product and 1 µl Sau3A1 and EcoRI restriction enzyme was added. And 2 µl of 10x assay buffer was added, and then incubated at 37°C for 24 hours.

SDS-PAGE - Molecular confirmation of protein

Preparation of sample

The broth cultures were centrifuged at 10000 rpm for 15 min at 4°C. The cells were re-suspended with distilled water and washed by centrifugation at 3000 rpm for 15 min at 25°C. The pellets were finally, resuspended in an equivalent volume of sterile distilled water, placed in an ice bath. Protein concentrations were estimated by the method of Lowry's method. To identify the mg/g of protein content. Discontinuous gels were cast to allow for 10

mm of stacking gel. To give a final polyacrylamide content of 10%, the separation gel was prepared from a stock solution which contained 30% (w/v) acrylamide. The final concentrations of other components in the gel were 1.5 M Tris hydrochloride (pH 8.8) and 10% (w/v) SDS. Polymerization was achieved by addition of 10% (w/v) ammonium persulfate (10%) N,N,N',N'-tetramethyl ethylene diamine. The separation gel components, without SDS, were deaerated for 3 min before polymerization, which took approximately 15 min at 20°C.

The stacking gel was prepared from the same stock solution that was used for the separation gel, to give a polyacrylamide content of 30%. The remaining stacking gel components, including the stacking gel buffer, were added to give a final concentration of 0.125 M Tris hydrochloride (pH 6.8) and 0.1% (w/v) SDS. Polymerization was initiated by adding 0.05% (w/v) ammonium persulfate (10% solution) and 0.1% (v/v) N,N,N',N'-tetramethyl ethylene diamine. The stacking gel solution excluding SDS was deaerated for 3 minutes. Polymerization was evident after 6 to 8 min. The gel was run at a constant current of 30 mA, a period of 1-2 hours. Whole-cell polypeptide gels were stained with staining dye and destained in the same solvent.

Remedial measures by using silver nanoparticles and chia seeds

Synthesis of Silver nanoparticles

The cell free filtrate was obtained by centrifugation of *Bacillus subtilis* at 10000 rpm for 10 minutes and temperature maintained at 4°C. For synthesis of silver nanoparticles 10ml of the cell free filtrate was brought in contact with 1 mM of silver nitrate concentration in 100 ml Erlen Meyer Flask and agitated at 37°C in dark conditions under neutral pH 7. Simultaneously, control without Silver nitrate solution was incubated under same conditions.

Preparation of Chia seeds extract

Total 100 grams of the seeds powder was weighed and macerated in 600 ml of distilled water in a sterile glass container for aqueous extract and similarly 100 grams of seeds powder were weighed and macerated in 600 ml of ethanol in a sterile glass container for ethanolic extract respectively. Cold maceration was performed for two days by shaking at regular intervals. It was subjected to filtration using Whatman filter paper to obtain a clear filtrate. This was kept on a water bath set at 60°C to obtain crude extract of Chia seeds.

Antimicrobial Susceptibility Testing - Well diffusion (Kirby Bauer) method was performed

To the prepared muller hinton agar 50 µl of *S. aureus* was added and spreaded, after spreading well were made and 20 µl of Nanoparticles and chia seeds were added respectively and the plate were incubated at 37°C for 24 hours. Zone of inhibition were observed after 24 hours of incubation.

RESULTS AND DISCUSSION

Sample collection

After the collection and subculturing of culture this were further characterised by following methods

The isolated pathogen was further subcultured on 1% nutrient agar medium and stored at 4°C.

Biochemical test

Subcultured samples were used for the confirmation of the organisms, IMVIC, catalase, coagulase, oxidase, and urease tests. In this IMVIC, coagulase and oxidase showing negative results and for catalase and urease showing positive results (Table 1).

Table 1 Biochemical tests for the confirmation of *S. aureus*

S. No	Techniques	Results
1	IMVIC test	Negative
2	Catalase Test	Positive
3	Coagulase Test	Negative
4	Oxidase Test	Negative
5	Urease	Positive

For the confirmation of *S. aureus* in specific medium of mannitol salt agar it gives yellow colour after the incubation time, so it confirms the *S. aureus* organism (Figure 1).



Figure 1 Confirmation of *Staphylococcus aureus* in mannitol salt agar

Confirmation of MRSA

MRSA *S. aureus* were confirmed by using Hi-Comb method, after placing the comb and incubation time no zone of inhibition was observed, which is confirming the organism is fully resistant to the methicillin antibiotic, this were showing in the Figure 2.

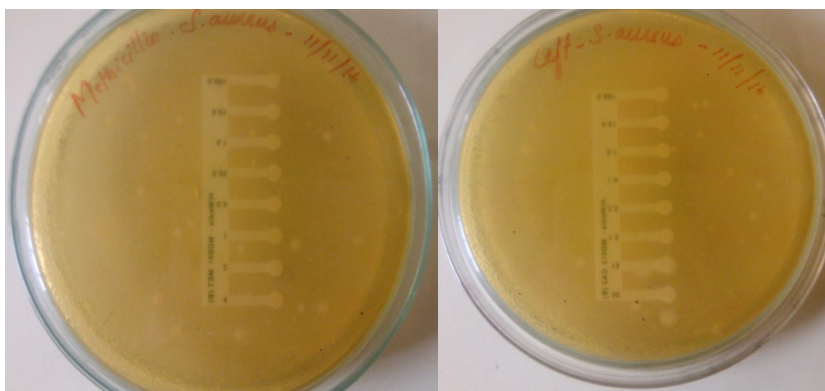


Figure 2 The culture is completely resistant to the methicillin disc

Staphylococcus aureus (MRSA) is one of the most important causes of hospital infections worldwide. High-level resistance to methicillin is caused by the *mecA* gene, which encodes an alternative penicillin-binding protein, PBP 2a [7]. MRSA is also emerging in the community, particularly in the United States, where 28% of community-acquired *S. aureus* strains may be resistant to methicillin [8].

Methicillin-resistant *Staphylococcus aureus* (MRSA) were first reported in 1961 and have since become a major nosocomial pathogen worldwide. An additional concern is the emergence of vancomycin-intermediate *S. aureus* (VISA) and more recently vancomycin-resistant *S. aureus* (VRSA). The reservoir of MRSA is infected and colonized patients, and the major mode of transmission from patient to patient is on the contaminated hands of healthcare workers. It is axiomatic that the sooner an MRSA infection is diagnosed, and the susceptibility to antimicrobial agents established, the sooner appropriate therapy and control measures can be initiated [1].

In confirmation of MRSA, Methicillin strip method was used, and the organism was to be resistant to both Methicillin and Ceftaroline. Though, Ceftaroline is a fifth generation antibiotic and also MRSA is said to be multidrug resistance was stated by Doğan et al. [9].

Genomic DNA isolation

Phenol chloroform method were used to isolate the DNA from *S. aureus*, the presence of DNA is confirmed by running in AGE at 1% agarose in buffer. After running this were visualised in UV-Transilluminator, reddish orange colour band were observed which is given in Figure 3.



Figure 3 Electrophoretic analysis of extracted DNA from MRSA on 1% Agarose gel
Lines 1; 1 kb DNA ladder, line 2 and 3; sample

DNA isolation shows confirmation of DNA of MRSA (Methicillin resistant *Staphylococcus aureus*) while comparing with 1 kb ladder. DNA extraction is suitable for all PCR approaches where *Staphylococci* are subject of the DNA analysis. Nevertheless, for some sensitive molecular methods such as restriction fragments length polymorphisms (RFLP) or DNA sequencing, at least one step phenol/chloroform extraction plus ethanol perception is needed to get rid of DNase and contaminated proteins [3].

PCR study-*mecA* gene amplification

The isolated DNA were further used for the *mecA* gene amplification, which is shown in Figure 4. To correlate the resistance profiles of MRSA strains with their current dissemination, the susceptibilities of the recent European *S. aureus* isolates were compared. The *mecA* gene was present in all isolates resistant to four or more antibiotics. Moreover, this multiresistance was displayed by the most prevalent and geographically widespread MRSA types, which together represented 99% of the *mecA*+ population in Europe [10].

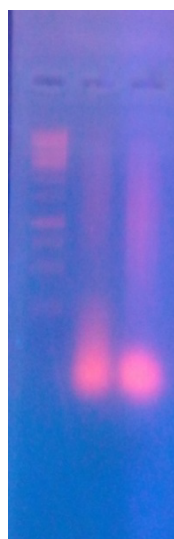


Figure 4 Electrophoretic analysis of amplified gene from extracted DNA of MRSA on 1.8% Agarose gel: Lines 1 -3; 1 kb DNA ladder marker: PCR products respectively

mecA gene was amplified through PCR analysis which confirmed with the 1Kb ladder on the 1.8% Agarose gel electrophoresis. PCR is a sensitive, specific, and rapid method for the detection of *mecA* positive strains. In the study

of Aziz Japoni, et al., find that the *Staphylococci* cell walls are surrounded by the thick peptidoglycan. To weaken *Staphylococcal* cell wall in normal DNA extraction, usually achromopeptidase or lysostaphin is recommended. On the other hand, for *Staphylococci* with thick peptidoglycan (20 nm - 80 nm diameters), it would be difficult to penetrate into the cell wall except by specific enzymes (achromopeptidase or lysostaphin) adding to the lysis buffer. Using vancomycin discs (cell wall inhibitor antibiotic) instead of expensive enzymes, because almost all *Staphylococci* are sensitive to this antibiotic, and can weaken a thick bacterial cell wall. As a consequence, boiling of suspension containing several colonies of bacteria from the edge of inhibition zone will readily open the disintegrated cell wall.

Restriction Fragment Length Polymorphism (RFLP)

RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. For the RFLP study different enzymes were used and after that running in 1.2% agarose different cutting cites were observed and which is confirming the sample have specific restriction endonucleases. The restriction digestion of MRSA by using enzymes Sau3A1 and ECORI, clearly acted on restriction sites of MRSA and bands were observed under UV transilluminator (Figure 5).



Figure 5 Electrophoretic analysis of restriction fragments of MRSA on 1.2% Agarose gel: Lines 1 -3; 1 kb DNA ladder marker: Restriction fragments respectively

SDS-PAGE

In the present the identification of molecular weight of the sample is 56 kDa which is showing in Figure 6, which confirms of MRSA.

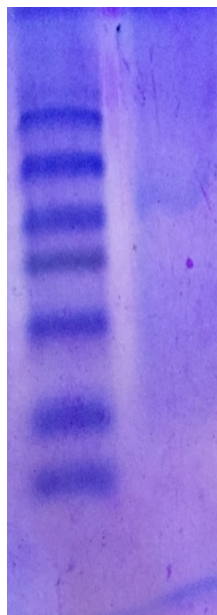


Figure 6 SDS-PAGE results; Lane 1 Marker Lane 2 sample - 56kDa

In India, the prevalence of nosocomial infections caused by MRSA varies between 20% and 40% methicillin resistance in *S. aureus* is associated with production of an altered penicillin-binding protein, a 78 kDa protein termed PBP2a, which has a low affinity for β -lactam antibiotics. The production of PBP2a is mediated by the *mecA* gene present in MRSA [11].

Threat of MRSA is serious. Usually molecular weight of MRSA ranges between 20 KDa - 205 KDa [12].

Remedial measures for MRSA by using synthesized silver nanoparticles and chia seeds

The present study deals with the biological process for the formation of silver nanoparticles using *Bacillus subtilis* and the synthesis of the nanoparticles which is obtained within hours of contact with the cell free filtrate. A colour change from colourless to brown took place within a few minutes indicating the formation of silver nanoparticles.

Majorly the colour change of the medium is indicated as formation of silver nanoparticles and its characterisation of silver nanoparticles can be done by UV-visible spectroscopy. The absorbance intensity of the brown colour increased steadily as a function of reaction time. The absorption maximum between 400 nm and 450 nm clearly indicates the formation of silver nanoparticles [5]. In the highest peak value observed at 300 nm - 360 nm of absorbance and confirmed as silver nanoparticles. And also, the colour changes indicate the synthesis of silver nanoparticles by using *Bacillus subtilis* [13] (Figure 7 and Table 2).



Figure 7 Zone of inhibition observed in MHA plates against *S. aureus*

Table 2 Zone of inhibition in mm

S. No	Treatment	Zone of inhibition (mm)
1	Silver nanoparticles	12
2	Chia seeds	14

But in case of silver nanoparticles it showed up to 12 mm. If the concentration of silver nanoparticles was increased, surely there might be a chance of treating MRSA by using silver nanoparticles. And in case of natural medicine, chia seeds have been used, which showed nearly 14 mm zone of inhibition. Concentration of chia seeds to increase for a better activity towards MRSA. Because the aqueous chia seeds were found to show as stated by Divyapriya et al. [6]. The antibacterial activity showed of the silver nanoparticles can be enhanced by increasing the concentration of the nanoparticles and chia seeds against MRSA. In the current study, comparative analysis was done to determine the effectiveness of the nanoparticles, chia seeds and conventional antibiotics.

SUMMARY AND CONCLUSION

Pus sample were collected from infected patients. The pus samples were streaked onto basal media - nutrient agar. Basic identification and biochemical tests were performed. Gram positive cocci were observed and also coagulase negative *Staphylococci* was observed, which is followed by the confirmation of MRSA was done by using methicillin strips. DNA was extracted by using phenol chloroform method and also compared with 1 Kb ladder. And also, *mecA* gene was amplified by using thermocycler. And control was compared with 1 Kb ladder. Restriction fragments of MRSA were studied using ECORI and SAU3AI. The result of restriction fragments confirms the restriction sites of MRSA. The protein components of MRSA were identified by using SDS-PAGE. The molecular weight of about 56 KDa, where the MRSA molecular weight will be of 20 KDa - 205 KDa. The silver nanoparticles were synthesized by using *Bacillus subtilis*. Colour change of medium, confirms from the synthesized silver nanoparticles, compared with the control. And the absorbance readings of silver nanoparticles were taken. The highest peak value ranged between 300 nm - 360 nm. Then, chia seeds were extracted by using ethanol. Then well diffusion technique was performed for treating MRSA. Silver nanoparticles and chia seeds have shown better bacterial and medicinal activity against (methicillin resistant *Staphylococcus aureus*). Further studies will be carried out for drug formation for *Staphylococci* infection

REFERENCES

- [1] Brown, Derek FJ, et al. "Guidelines for the laboratory diagnosis and susceptibility testing of methicillin-resistant *Staphylococcus aureus* (MRSA)." *Journal of Antimicrobial Chemotherapy* Vol. 56, No. 6, 2005, pp. 1000-18.
- [2] Felten, Annie, et al. "Evaluation of three techniques for detection of low-level methicillin-resistant *Staphylococcus aureus* (MRSA): a disk diffusion method with cefoxitin and moxalactam, the Vitek 2 system, and the MRSA-screen latex agglutination test." *Journal of Clinical Microbiology* Vol. 40, No. 8, 2002, pp. 2766-71.
- [3] Japoni, Aziz, et al. "Modified DNA extraction for rapid PCR detection of methicillin-resistant *Staphylococci*." *Iranian Biomedical Journal* Vol. 8, No. 3, 2004, pp. 161-65.
- [4] Mehndiratta, P.L., et al. "Molecular typing of methicillin-resistant *Staphylococcus aureus* strains by PCR-RFLP of SPA gene: a reference laboratory perspective." *Indian Journal of Medical Microbiology* Vol. 27, No. 2, 2009, p. 116.
- [5] Manikprabhu, Deene, and K. Lingappa. "Antibacterial activity of silver nanoparticles against methicillin-resistant *Staphylococcus aureus* synthesized using model *Streptomyces* sp. pigment by photo-irradiation method." *Journal of Pharmacy Research* Vol. 6, No. 2, 2013, pp. 255-60.
- [6] Divyapriya, G.K., D.J. Veeresh, and Puja C. Yavagal. "Evaluation of antibacterial efficacy of chia (*salvia hispanica*) seeds extract against *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter Actinomycetes comitans*-An *in-vitro* study." *International Journal of Ayurveda and Pharma Research* Vol. 4, No. 4, 2016.
- [7] Beck, Wolfgang D., B. Berger-Bächi, and Fritz H. Kayser. "Additional DNA in methicillin-resistant *Staphylococcus aureus* and molecular cloning of *mec*-specific DNA." *Journal of Bacteriology* Vol. 165, No. 2, 1986, pp. 373-78.
- [8] Herold, Betsy C., et al. "Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk." *Jama* Vol. 279, No. 8, 1998, pp. 593-98.

- [9] Doğan, Göksel, et al. "Antibiotic susceptibilities and SDS-PAGE protein profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) strains obtained from Denizli Hospital." *Current Progress in Biological Research*. InTech, 2013.
- [10] Wienders, C.L.C., et al. "mecA gene is widely disseminated in *Staphylococcus aureus* population." *Journal of Clinical Microbiology* Vol. 40, No. 11, 2002, pp. 3970-75.
- [11] Datta, Priya, et al. "Evaluation of various methods for the detection of Methicillin-resistant *Staphylococcus aureus* strains and susceptibility patterns". *Journal of Medical Microbiology*. Vol. 60, 2011, pp. 1613-16.
- [12] Saçılık, Sefa CAN, et al. "Analysis of Methicillin Resistant *Staphylococcus aureus* Isolates by Polyacrylamide Gel Electrophoresis in an Intensive Care Unit of Übni-Sina Hospital." *Turkish Journal of Medical Sciences* Vol. 30, No. 4, 2000, pp. 367-72.
- [13] Parry, Christopher M., et al. "Clinically and microbiologically derived azithromycin susceptibility breakpoints for *Salmonella enterica* serovars Typhi and Paratyphi A." *Antimicrobial Agents and Chemotherapy* Vol. 59, No. 5, 2015, pp. 2756-64.