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Multidrug Resistant *Staphylococcus aureus* Isolated from UTI Site and its Biofilm Inhibition Activity with Nanomaterials in Reference with Standard Antibiotics

Mohamed Mahroop Raja M.*, Reehana N. and Fasila Begum A.

PG and Research Department of Microbiology, Jamal Mohamed College (Autonomous), Affiliated to Bharathidasan University, Tiruchirappalli 620 020, Tamil Nadu, India

*Corresponding Author

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Abstract: Drug-resistant *Staphylococcus aureus* is a leading cause of nosocomial urinary tract infections and biofilm-associated illnesses worldwide. The present investigation intended to detect biofilm-forming *Staphylococcus aureus*, their antibiotic resistance, and plasmid profile. Thirty clinical urine samples were collected and tested for pathogen, drug-resistance pattern, plasmids profile, biofilm development, and molecular characterisation. Four strains of *Staphylococcus aureus* were discovered, according to Bergey's handbook. Kirby-Bauer technique was used to test antibiotic sensitivity and resistance. Four multidrug-resistant *Staphylococcus aureus* plasmids were examined by alkaline lysis. Among the four isolates, plasmid band was assigned, and additional investigations were employed for biofilm activity against four nanocomposite MoSe₂, Mn₃O₄, Nb₂O₅ and ZnO-SnO₂. Nanocomposite Nb₂O₅ inhibited biofilm at lower concentrations than cefotaxime. DNA was extracted and PCR was performed using the 16S rRNA gene for *Staphylococcus aureus*. Isolate phylogeny was analysed using Neighbor joining and uploaded to GenBank with accession number MG263510. *Staphylococcus aureus* multidrug resistance and biofilm formation are major medical challenges. Despite rising biofilm development rates, it is hard to compare, and hospitals have implemented tight infection control procedures. This study indicated that nanomaterials may help manage biofilm-related illnesses.

Keywords: Biofilm infections, UTI, Drug resistance, Nanomaterials, *Staphylococcus aureus*, Antibiotic

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Introduction

Biofilms are important for public health (Marti et al. 2011). Biofilm production involves polymeric molecules attached to live bacterium cells

(Anselme et al., 2010). Biofilm development adds to bacterial pathogenesis, which leads to chronic or long-lasting infections (Wozniak et al., 2003).

Antibiotics help control bacterial illnesses. Misuses of antibiotics have resulted to multidrug-resistant microorganisms in several species (Mohanasundaram *et al.*, 2010). *Staphylococcus aureus* is the second most prevalent cause of urinary tract and wound infections and the third cause of nosocomial bacteraemia (Akindele *et al.*, 2010). These germs are resistant to numerous prescribed medications and are termed multidrug resistant (MDR). They are frequent sources of disease mortality and morbidity (Jang, 2016).

Biofilm production is a technique for antibiotic-resistant plasmid transfer and an intercellular communication medium. Antimicrobial resistance in biofilm-producing organisms may be caused by delayed penetration of antimicrobial drugs through the biofilm matrix, changed growth rate of biofilm organisms, and other physiological changes. Thus, the capacity to produce biofilm might boost survival and persistence under stressful situations such as host invasion or antibiotic treatment (Gil-Perotin *et al.*, 2012; Narendiran *et al.*, 2014). Antimicrobial resistance among bacterial pathogens is on the rise, and controlling nosocomial infections has grown more difficult owing to the expansion of antibiotic-resistant *Staphylococcus aureus* (Mulugeta *et al.*, 2011). Antibiotic-resistant microorganisms are a clinical concern that nanotechnology might solve. Nanoparticles and nanomaterials generated by bacteria may replace antibiotics (Huh and Kwon, 2011).

Nanoparticles (NPs) are utilised in nanocrystalline silver dressings, lotions, and gels to minimise bacterial infections and treat chronic wound infections (Ip *et al.*, 2006; Magesh *et al.*, 2019). Nanomaterial offers antibacterial protection against microorganisms (Smriti *et al.*, 2012). NPs rupture the bacterial cell membrane, liberate metal ions, generate ROS, penetrate the bacterial cell membrane, and induce intracellular antibacterial effects including DNA and protein interactions (Wang *et al.*, 2017). *Staphylococcus aureus* biofilm production causes major contamination issues in medicine, dentistry, food

processing, water treatment, and other fields that impact human health and life. Nano-sized materials are the best way to destroy microorganisms since they act extracellularly and intracellularly (Roe *et al.* 2008; Hemalatha *et al.*, 2017). There is evidence that bacterial adhesion and subsequent biofilm development are greatly altered and identified as they arise; nanoparticles and nanomaterials display considerable bactericidal action against multidrug-resistant gram-positive and negative bacteria (Zeng *et al.*, 2007).

The current research examined biofilm development and its correlation with *Staphylococcus aureus* antibiotic resistance profiles from clinical impact urine specimens.

Materials and Methods

Identification of samples:

Thirty urine samples from hospital patients in Tiruchirappalli, Tamil Nadu, India, were collected. All samples were processed in 24 h. Gram staining, coagulase test, catalase test, and blood agar and mannitol salt agar were employed to identify clinical source urinary tract bacteria.

Multiple antibiotics resistance (MAR) Index and DDP:

The disc diffusion test was used to determine the antimicrobial drug resistance pattern of clinical isolates against various antibiotics. Growth plates were incubated for 24 h at 37°C. Clinical and Laboratory Standard Institute recommendations judged the isolate drug-resistant after incubation. Multiple antibiotics resistance index formula:

$$\text{MAR Index} = \frac{\text{Number of resistant antibiotics}}{\text{Tested total antibiotics}}$$

Tested antibiotics:

Plasmid isolation:

All four *Staphylococcus aureus* pure isolates were sub-cultured in Luria-Bertani broth at 37°C for 12-16 h. Alkaline-SDS lysis isolated resistant plasmid DNA. Purified DNA was kept at -20°C after phenol and ethanol extraction. Horizontal gel electrophoresis was done at 100 volts for 60 min

using TAE buffer. Plasmids were run on 0.8% agarose gel and stained with ethidium bromide. Electrophoresed samples included a DNA molecular weight marker. Gel documentation system was used to observe plasmid bands in dyed gels after electrophoresis.

MIC (Minimum Inhibitory Concentration) screening:

By dilution, Mueller-Hinton broth's minimum inhibitory concentration was determined. MIC testing includes MoSe₂, Mn₃O₄, Nb₂O₅, and ZnO-SnO₂. 1000 g, 500 g, 250 g, 125 g, 62.5 g, 31.2 g, 15.6 g, and 7.6 g of diluted nano composites were evaluated on test organisms. After 24 h at 37°C, findings were collected and analysed using CLSI criteria.

Agar diffusion test:

Mueller's secondary actinomycetes strain screens *Klebsiella pneumoniae* and *Staphylococcus* on Hinton agar via disc diffusion. Multidrug-resistant bacteria were swabbed onto agar. 100 µl of pure fractions were put on a sterile disc and agar plate. Standard Positive control was 10 mg cefotaxime. Plates were heated to 37°C. ZI was measured after incubation.

The test organism was swabbed onto Mueller-Hinton agar. After inoculation, 1000 µg, 500 µg, 250 µg, 125 µg, 62.5 µg, 31.2 µg, 15.6 µg, and 7.6 µg nano composite were put on agar wells. Positive control used was 10 mg cefotaxime. 24 h 37°C incubation was done. After incubation, ZI was measured.

Biofilms Assay:

For biofilm production, 13 mm x 0.2 mm TMX cover slips were inserted in Falcon 24 well polystyrene plates. 24 h SA cultures in 0.2 OD600 wells. Negative controls were broth-only wells. 5 day covered incubation at 27°C was done. TMX coverslips were taken from each well of the 24 well plates (static biofilm development), washed with double distilled water, and inserted in the corresponding wells of a fresh 24 well plate containing 1 ml of 4.0% crystal violet solution (wt/vol) incubated at room temperature for 30

min. After incubation, the crystal violet solution was removed and the cover slips were cleaned and stored in ethanol. The plates were incubated at room temperature for 1 h, and the extracted crystal violet was measured at 640 absorbance.

Assay anti-biofilm:

Crude ethyl acetate extract was tested against MRSA biofilm. Strong biofilm-forming MRSA was injected overnight into 0.2 OD 600 wells. Inoculated 24 well plate with 100 µl (100 µg) crude extract. Negative controls were ethyl acetate and empty broth. Antibiotic control was cefotaxime (10 mg). 5 day incubation at 27°C was performed. Crystal violet test measured biofilm development after incubation.

Extracting DNA:

Staphylococcus aureus DNA was extracted using SDS (Goldenberger *et al.*, 1995). The bacterial cell suspension was treated with SDS, Tris HCl, and EDA (EDTA). Cell detritus and contaminants were removed successively by centrifugation. Chilled ethyl alcohol precipitated genomic DNA. Centrifuged DNA was pelletized. Pellet was dissolved in TE buffer and kept at 40°C.

rRNA amplification:

Forward (16S rRNA F) and reverse (16S rRNA R) primers were developed for amplification (forward primer and reverse primer, purchased from Sigma-Aldrich, Hyderabad). 94°C for 1 min; 94°C, 63°C, and 72°C for 1 min for 35 cycles; 72°C for 10 min. The PCR results were identified by electrophoresis using 1.5% agarose, and the bands were stained with 7 µl/100 ml of ethidium bromide (Nyx Technic, Inc. USA).

Phylogeny:

Software created phylogenetic trees demonstrating sequences' ancestry. Contigs were searched for nucleotide similarity using BLAST (Benson *et al.*, 2014). The sequences were aligned pairwise using Clustal W, and the phylogenetic tree was built using MEGA5.05 software (Kumar *et al.*, 2016; Sivakumar *et al.*, 2022). BLAST was used to identify genera and species.

Table 1: Frequency of clinical urine positive samples

Age factor	Number of urine samples collected	No of positive urine samples
20-30	5	0
30-40	7	3
40-50	10	4
50-60	8	6
Total	30	13

Table 2: Biochemical identification of *Staphylococcus aureus*

S. No.	Microbiological Examinations	Growth observations
1.	Cell wall nature	Gram positive cell wall
2.	Shape	Cocci
3	Mannitol salt agar	Yellow colour growth
4.	Blood agar	Beta- haemolysis
5.	Coagulase test	Positive
6.	Catalase	Positive

Results and Discussion

In this investigation, multidrug-resistant bacteria were isolated from clinical urine specimens. Infectious patients ages 20-30, 30-40, 40-50, and 50-60 gave urine samples. 17 of 30 samples were negative, while 13 were positive. 50-60-year-olds had the most infections (Table 1). All positive samples were microbiologically analysed. Four of 13 samples had *Staphylococcus aureus*. Bergey's handbook of systematic bacteriology mention that *Staphylococcus aureus* has yellow colonies on Mannitol salt agar and - hemolytic activity on blood agar. Positive coagulase and catalase assays were prepared for *Staphylococcus aureus* (Table 2). *Staphylococcus aureus* causes nosocomial respiratory, urinary, bloodstream, and post-surgical infections (Choo and Chambers, 2016; Rajakumari *et al.*, 2017).

Staphylococcus aureus drug resistance was evaluated using 20 antibiotic discs. All four *Staphylococcus aureus* strains were resistant to ampicillin, cefixime, cefepime, ceftazidime,

methicillin, and penicillin. Cefuroxime is least resistant. Cefpodoxime and cefotaxime are resistant to all four clinical isolate strains. 35% of *Staphylococcus aureus* urinary tract infections are resistant to methicillin, penicillin, and other cephalosporin medicines (Lubelchek and Weinstein, 2008). Similarly, antibiotics such as azithromycin, linezolid, ofloxacin, teicoplanin and vancomycin were 100% sensitive (Table 3). Gentamycin and tetracycline were least sensitive. MRSA strains are multidrug resistant (Esimone *et al.* 2010). Methicillin-resistant *S. aureus* was responsive to linezolid, teicoplanin, and vancomycin (Mohanasundaram *et al.*, 2011; Sharma *et al.*, 2013). All isolates, including biofilm-producing ones, were susceptible to vancomycin. This supports earlier reports of Percival *et al.* (2015). Highly resistant *Staphylococcus aureus* was 0.45 for many drugs. Multiple drugs resistance index measures spread of *Staphylococcus aureus* resistant in a particular community (David and Daum, 2010). These environmental pathogens behave as opportunistic

Table 3: Disc diffusion pattern of *Staphylococcus aureus*

Antimicrobial agent	No. of Resistant samples	Resistant percentage	No. of Intermediate samples	Intermediate percentage	No. of Sensitive samples	Sensitive percentage
Ampicillin(10 mg)	4	100%	-	-	-	-
Azithromycin(15 mg)	-	-	-	-	4	100%
Cefepime(30)	4	100%	-	-	-	-
Cefixime (5 mg)	4	100%	-	-	-	-
Cefpirome (30 mg)	-	-	1	25%	3	75%
Cefpodoxime (10 mg)	-	-	4	100%	-	-
Ceftazidime(30 mg)	4	100%	-	-	-	-
Ceftizoxime(30 mg)	2	50%	2	50%	-	-
Cefuroxime(30 mg)	1	25%	3	75%	-	-
Cephalthin(30 mg)	2	50%	2	50%	-	-
Cefotaxime(10 mg)	-	-	4	100%	-	-
Doxycycline (30 mg)	-	-	1	25%	3	75%
Gentamycin (10 mg)	-	-	3	75%	1	25%
Linezolid (30 mg)	-	-	-	-	4	100%
Methicillin(5 mg)	4	100%	-	-	-	-
Ofloxacin (5 mg)	4	-	-	-	4	100%
Penicillin(10 mg)	4	100%	-	-	-	-
Teicoplanin (30 mg)	-	-	-	-	4	100%
Tetracycline (30 mg)	-	-	3	75%	1	25%
Vancomycin (30 mg)	-	-	-	-	4	100%

Table 4: Minimum Inhibitory Concentration activity of Nanocomposites against *Staphylococcus aureus*

S. No.	Nanocomposites	1000 µg	500 µg	250 µg	12.5 µg	62.5 µg	31.2 µg	15.6 µg
1.	MoSe ₂	R	R	R	R	R	R	R
2.	Mn ₃ O ₄	R	R	R	R	R	R	R
3.	Nb ₂ O ₅	S	S	S	I	I	I	I
4.	ZnO-SnO ₂	R	R	R	R	R	R	R

R – Resistant; S – Sensitive; I – Intermediate

bacteria to induce clinical infections resistant to penicillin and cephalosporins (Hughes *et al.*, 2005; Mohanasundaram *et al.*, 2011).

Alkaline lysis and electrophoresis were used to examine multidrug-resistant plasmid profiles. Only lane 1 had plasmid band. Three lanes lost plasmid. *Staphylococcus aureus* was tested for plasmids because it was resistant to ampicillin, cefepime, ceftazidime, methicillin, and penicillin (Dias Neto *et al.*, 2003; Tenney *et al.*, 2018). Nosocomial infections are a global concern. Multidrug-resistant strains are plasmid-encoded

and resistant to all antibiotics, allowing facile transmission of plasmids encoding multidrug-resistant genes (Mah and O'Toole, 2001; Kollef, 2009).

By dilution, Mueller-Hinton broth's minimum inhibitory concentration was determined. Four nanocomposites, MoSe₂, Mn₃O₄, Nb₂O₅, and ZnO-SnO₂, were tested for MIC using Mueller-Hinton broth. Nb₂O₅ inhibited *Staphylococcus aureus* the least at 15.6 µg (Table 4). Mueller-Hinton agar was used for agar well diffusion tests on test organisms. Wells were constructed in agar and

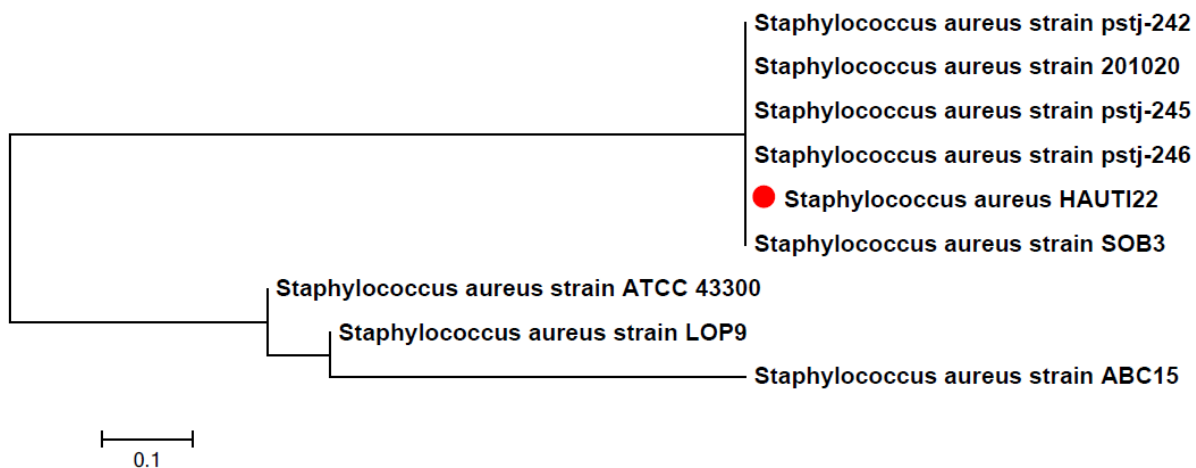


Fig.1: Phylogenetic relationship of *Staphylococcus aureus*.

different amounts of Nb₂O₅ nanocomposite were added. 24 h incubation at 37°C was performed. After incubation, 1000 µg and 500 µg ZI were measured. Nb₂O₅ nanocomposite compounds at 10 mg/ml were used to suppress *Staphylococcus aureus* biofilm. 100 g nanocomposite demonstrated full suppression. The Nb₂O₅ nanocomposite was more effective than cefotaxime and other cephalosporins. Nanocomposites penetrate multidrug-resistant bacteria and disrupt cell membranes, causing antibacterial activity (Panacek *et al.*, 2006). *Staphylococcus aureus* biofilm production varies on environment, nutritional availability, geographical origin, specimen type, surface adherence, and genetic composition (Cha *et al.*, 2013; Poudel *et al.*, 2015). Biofilms are therapeutically relevant because biofilm bacteria are antibiotic-resistant (Hassan *et al.*, 2011; Wu *et al.*, 2013). Early identification and screening of *Staphylococcus aureus* biofilm producers is critical for selecting a chemotherapeutic drug.

Staphylococcus aureus HAUT122 genomic DNA was amplified for 16S rRNA gene. *Staphylococcus aureus* HAUT122 16S rRNA gene product was 1500 bp. GenBank accession number of *Staphylococcus aureus* HAUT122 is MG263510. Phylogenetic study comprised *Staphylococcus aureus* 16S rRNA and NCBI 16S rRNA sequences. MEGA 5.05 aligned 16S rRNA gene sequences. Neighbor joining created individual dendrograms.

Gene tree length and branching order determine phylogenetic groupings and subgroups. The phylogenetic study of *Staphylococcus aureus* 16S rRNA gene data resulted in a tree congruent with contemporary knowledge of the relatedness among *Staphylococcus* species, based on DNA sequence homology (Fig. 1). Neighbor-Joining inferred evolutionary history (Saitou and Nei, 1987). The ideal tree has 1.43806643 total branch length. The tree's branch lengths match the evolutionary distances utilised to estimate the phylogenetic tree. 9 nucleotide sequences were analysed. 1st+2nd+3rd+Noncoding codons added. We deleted any gaps and missing data. Final dataset has 35 locations. MEGA5 evolution analyses (Tamura *et al.*, 2011). Alignment of nucleotide sequences is important in phylogenetic research, especially for diverse taxa. Phylogenetic analysis of sequences starts with proper alignment of the data, yet alignment is one of the most challenging and poorly understood aspects of molecular data processing. Phylogenetic tree analysis requires genomic sequence alignments. Phylogenetic analysis looks for directional selection in molecular evolution (Lamers *et al.*, 2012).

Conclusion

The current investigation indicated that multidrug-resistant *Staphylococcus aureus* caused urinary tract infections. Multidrug-resistant *Staphylococcus aureus* is a serious worldwide issue

and a chronic problem in human societies. Multidrug-resistant microorganisms are spreading without proper controls. Nanoparticles and nanomaterials may destroy multidrug-resistant bacteria. Nanocomposites are multi-elements that may kill pathogenic bacteria owing to their high reactivity, reducing biofilm-related infections and antibiotic-resistant bacteria. Using bioinformatics methods, 16sRNA sequences of *Staphylococcus aureus* were linked with *Staphylococcus* species to investigate molecular evolution and population structure.

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