

Characterization of *Staphylococcus* Spp. From Patients' Sputum and Determination of Its Antibiotic Resistance/ Susceptibility

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ABSTRACT

Background: The occurrence of multi drug resistance is quite prevailing in the species of *Staphylococcus aureus* since a very long time. Due to this, it poses a threat as most of the antibiotics used against it are not effective due to multiple antibiotic-resistant mechanisms. Although there has been the development of new treatments and advancements in control of infections, but the persistence of resistance is still at a rise. **Objective:** In this paper, an effort is done to isolate a strain of *Staphylococcus* species from sputum to further analyse the organism's capacity for antimicrobial resistance/sensitivity. **Methodology:** The isolation was done using sputum sample collected randomly from ten patients suffering from nosocomial infections (both male and females) of Kamti Healthcare and Diagnostics, Siliguri. Microbiological and microscopic techniques were used. The biochemical tests were performed, and Polymerase Chain Reaction (PCR) was done to characterize the species of the organism. To confirm the species, 16s ribosomal sequencing and phylogenetic analysis was performed. Antibiotic resistance test was performed using disk diffusion method to test the antibiotic resistance/susceptibility of the organism. Statistical analysis of the zone of inhibition was done using ANOVA. **Results:** 10 isolates were obtained that were characterized as Gram positive and Catalase positive. The isolates 1, 2, 4, 6, 9 and 10 were positive to carbohydrate fermentation test and negative for citrate utilization and oxidase test. These isolates were confirmed as *Staphylococcus aureus* species as analysed from PCR test and previous studies. These isolates were found susceptible to some antibiotics while resistant to gentamycin, amoxycylav/augmentin, erythromycin, rifampicin, vancomycin, meropenem, levofloxacin and amoxycillin with p-value ≤ 0.05 . **Conclusion:** The study confirms the isolated strain as *Staph aureus*, as no study has indicated the antibiotic susceptibility/ resistance to these antibiotics till present. The need of the hour is to identify other such strains so that certain preventive measures could be attained to treat the infections caused by such strains using alternative methods.

Keywords: Multi drug resistance, antibiotics, *Staphylococcus* spp., antibiotic susceptibility

INTRODUCTION

Staphylococcus aureus is the most significant pathogen worldwide and can spread through direct and indirect contacts. It is commonly found in skin, mucous membranes, gastrointestinal tract. The major illnesses caused by the bacterium from folliculitis and food poisoning to endocarditis and pneumonia (Askari et al, 2012). Once infected, it colonises within the bloodstream and becomes contagious. The problem of multi drug resistance is quite prevailing in this species since a very long time. The presence of several genetic factors like methicillin resistance and PVL toxin increases the risk factor associated with the species. The strains of MRSA are usually non-susceptible to β -lactam antibiotics but not all categories of penicillins, cephalosporins, carbapenems and β -lactamase inhibitors (Cretenet et al, 2011; Magiorakos et al, 2018). Due to this, it poses a threat as most of the antibiotics used against it are not effective due to multiple antibiotic-resistant mechanisms (Thapar et al, 2025).

Numerous studies have been conducted to search for alternative solutions towards managing the antibiotic resistance. Although there has been the development of new treatments and advancements in control of infections, but the persistence of resistance is still at a rise. WHO stated the increase of MRSA incidence in hospitals by 20% in all nations and 80% in few countries including Nepal and India (Locke et al, 2025). In India, the prevalence of Methicillin resistant (MRSA) in humans was found to be highest in 8 different regions. At present, there has been a consistent rise in new strains of *Staphylococcus* species that possess antibiotic resistance to other antibiotics than the strains of MRSA (Bhola and Bhadekar, 2019; Thapar et al, 2026). It is estimated that by 2050, there will be rise in fatalities by 10 million per year due to antimicrobial resistance, which shall result in loss of around 100 trillion million dollars (Ghareeb et al, 2024; Xu et al, 2024).

In this paper, an effort is done to isolate a strain of *Staphylococcus* species from sputum to further analyse the organism's capacity for antimicrobial resistance/sensitivity.

MATERIALS AND METHODS

Sample collection:

A sputum sample at morning (around 10:00 am) was collected randomly from ten patients (both male and females) suffering from nosocomial infections of Kamti Healthcare and Diagnostics, Siliguri into a sterile specimen container.

Isolation of *Staphylococcus* spp. from Sputum samples:

The serial dilutions till 10^{-7} from sputum samples were spread plated on nutrient agar media (Hi Media, Mumbai), incubated for 24 h at 40°C. To obtain the purified culture, it was streaked on the nutrient agar slants. The isolates were designated as numbers.

Microscopic and Biochemical identification of the Isolates:

The microscopic identification of the isolates was performed using Gram staining. Some characteristics according to biochemical performances were noted as follows:

Catalase test: The catalase test was performed by adding 2-3 drops of 30% hydrogen peroxide (H_2O_2) over the colonies to observe for the formation or absence of bubbles.

Fermentation of glucose, dextrose and mannitol: The fermentation of carbohydrates- glucose, fructose and mannitol was determined by acid formation. 10 ml of sterile test tube containing glucose broth along with Durham's tube was inverted and dipped with strains, then incubated at 40°C for 24 h.

Citrate utilization test: The isolates were inoculated in the Citrate agar medium (Hi Media, Mumbai) and incubated at 40°C for 24 h to observe was the presence or absence of change in the colour of the media.

Oxidase test: The isolates were inoculated into 1% tetramethyl p- phenylenediamine dihydrochloride (Kovac's reagent) and incubated for 24h at 37°C to observe was the presence or absence of change in the colour of the reagent.

Molecular Identification of Isolates Using Polymerase Chain Reaction:

The bacterial DNA was isolated. The cells were grown in Nutrient agar media (Hi Media, Mumbai) and incubated at 37°C for 24 h. The cells were then lysed using EDTA and lysozyme; centrifugation was done at 12,000 rpm for 15 min. Removed the supernatant and pellets were denatured using protease enzyme. Precipitation was done using organic solvents like phenol. Centrifuged at 14000 rpm for 10 min and removed the pellets. The supernatant was precipitated using ethanol. Measured the bands with electrophoresis on 1.5% agarose gel and stained with ethidium bromide. Further, polymerase chain reaction was performed to confirm the species as *Staphylococcus aureus*. The procedure was followed as per the method described by the Franco

et al, 2007. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

16s Ribosomal Sequencing for species Confirmation

The consensus sequence of the rDNA gene was generated from forward and reverse sequence data using aligner software. The rDNA gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA XI. The phylogeny was inferred using the Maximum Likelihood method and Tamura-Nei (1993) model [II of nucleotide substitutions. The initial tree for the heuristic search was selected by choosing the tree with the superior log-likelihood between a (NJ) tree and a Maximum Parsimony (MP) tree. The NJ tree was generated using a matrix of pairwise distances computed using the p-distance. The analytical procedure encompassed 5 coding nucleotide sequences using 1st, 2nd, 3rd, and non-coding positions with 1,495 positions in the final dataset. Evolutionary analyses were conducted in MEGA12 utilizing up to 4 parallel computing threads.

Antibiotic susceptibility/resistance test to confirm *Staphylococcus* species:

Antibiotic susceptibility/ resistance test was performed using disk diffusion method. 50 µg disks of 12 antibiotics- moxifloxacin, amoxycillin, erythromycin, levofloxacin, imipenem, amikacin, meropenem, amoxyclav/ augmentin, vancomycin, nitrofurantoin, gentamicin, rifampicin (Hi-Media, Mumbai) were used to detect susceptibility and resistance of the isolated strain of *Staphylococcus* spp. The plates were then incubated for 24 h at 40°C to observe for the presence or absence of zone of inhibition. Statistical analysis of the zone of inhibition was done using ANOVA- single factor.

RESULTS AND DISCUSSION

Colony characteristics and microscopic identification of the isolates:

10 isolates were obtained. These were represented in numbers from 1-10. The colony characteristics of all the isolates- yellowish coloured, opaque, convex, slightly circular with smooth margin but irregular edged colonies. The isolates were observed as violet colour, cocci shaped and in chains. This confirmed the strain as Gram positive (Figure1).

Similar results were obtained in a study by Kulkarni et al, 2016. The isolates of *S. aureus* from urine sample showed opaque, circular and smooth margin colonies with microscopic characteristics as Gram positive.

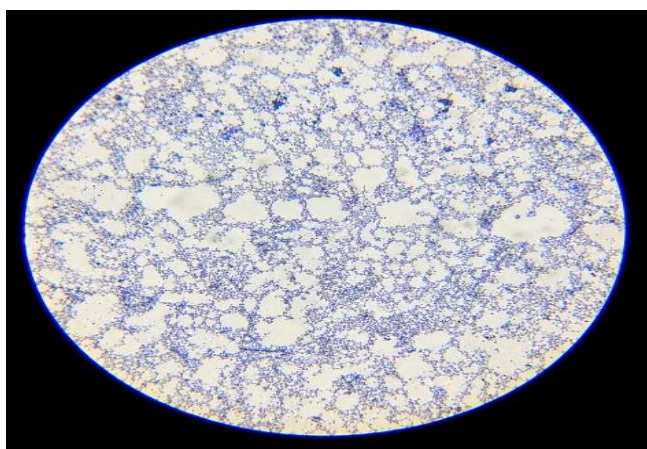


Figure 1: Gram positive cocci cells

Biochemical characteristics of the isolates:

Catalase test: After adding 30% hydrogen peroxide (H₂O₂) over the colonies in 10 isolates, no bubbles were observed, the strain was characterized as catalase positive.

Similar results were obtained in a study by Kulkarni et al, 2016. The isolates of *S. aureus* from urine were identified as Catalase positive.

Carbohydrate fermentation test: In the fermentation of carbohydrates- glucose, fructose and mannitol, gas production was noted in isolates no. 1, 2, 4, 6, 9 and 10 within the hollow space of the Durham's tube resulting as positive (Figure 2a), whereas negative result was obtained for the isolates 3, 5, 7 and 8.

In a study by Kulkarni et al, 2016, the species of *Staphylococcus aureus* isolated from urine produced gas during fermentation by glucose and dextrose.

Citrate utilization test: After incubation for citrate utilization, there was change in the colour of the media for all the isolates. This resulted in positive test for citrate utilization (Figure 2b).

In a study by Hallabjaiy et al, 2014, the isolates of *Staphylococcus aureus* from urine and stool showed negative citrate test.

Oxidase test: When the isolates were inoculated into 1% tetramethyl p- phenylenediamine dihydrochloride, no colour change was observed in the isolates no. 1, 2, 4, 6, 9 and 10 whereas for isolates 3, 5, 7 and 8, a change in colour was observed.

In a study by Hallabjaiy et al, 2014, the isolates of *Staphylococcus aureus* from urine and stool showed negative oxidase test.

The observations of the biochemical tests are summarized in Table 1.

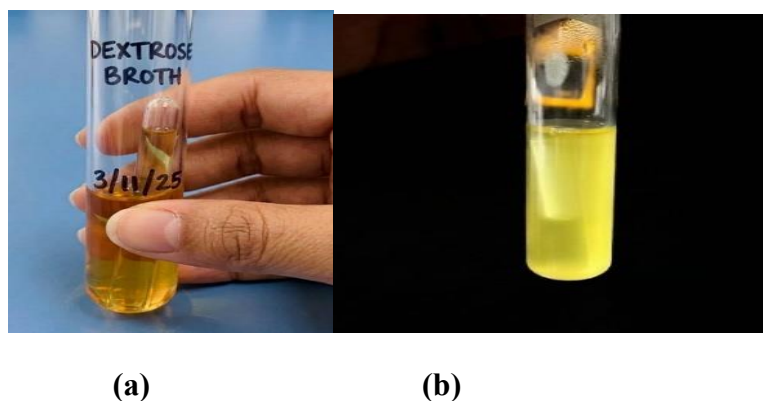


Figure 2: Biochemical examination of the isolates a. Carbohydrate fermentation test b. Citrate utilization test

Table 1- Characterization of the Isolates

Isolates No.	Microscopic identification	Gram positive/ Negative	Catalase test	Carbohydrate fermentation test	Citrate utilization test	Oxidase test
1	Violet colour, cocci shaped and in chains	Gram positive	-	+	+	-
2	Violet colour, cocci shaped and in chains	Gram positive	-	+	+	-
3	Violet colour, cocci shaped and in chains	Gram positive	-	-	-	+
4	Violet colour, cocci shaped and in chains	Gram positive	-	+	+	-
5	Violet colour, cocci shaped and in chains	Gram positive	-	-	-	+
6	Violet colour, cocci shaped and in chains	Gram positive	-	+	+	-

7	Violet colour, cocci shaped and in chains	Gram positive	-	-	-	+
8	Violet colour, cocci shaped and in chains	Gram positive	-	-	-	+
9	Violet colour, cocci shaped and in chains	Gram positive	-	+	+	-
10	Violet colour, cocci shaped and in chains	Gram positive	-	+	+	-

Polymerase Chain Reaction and 16S ribosomal sequencing:

Based on nucleotide homology and phylogenetic analysis, the isolates 1, 2, 4, 6, 9,10 showed high similarity with *Staphylococcus aureus* (Figure 3-4). The sequence identified as:

TTTATGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGA
GCGAACGGACGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGATAA
CCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGAACCGC
ATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTAGT
TGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCG
AAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAG
GGAAGAATTAGCATGTAAGTAACTGTGCACATCTTGAC

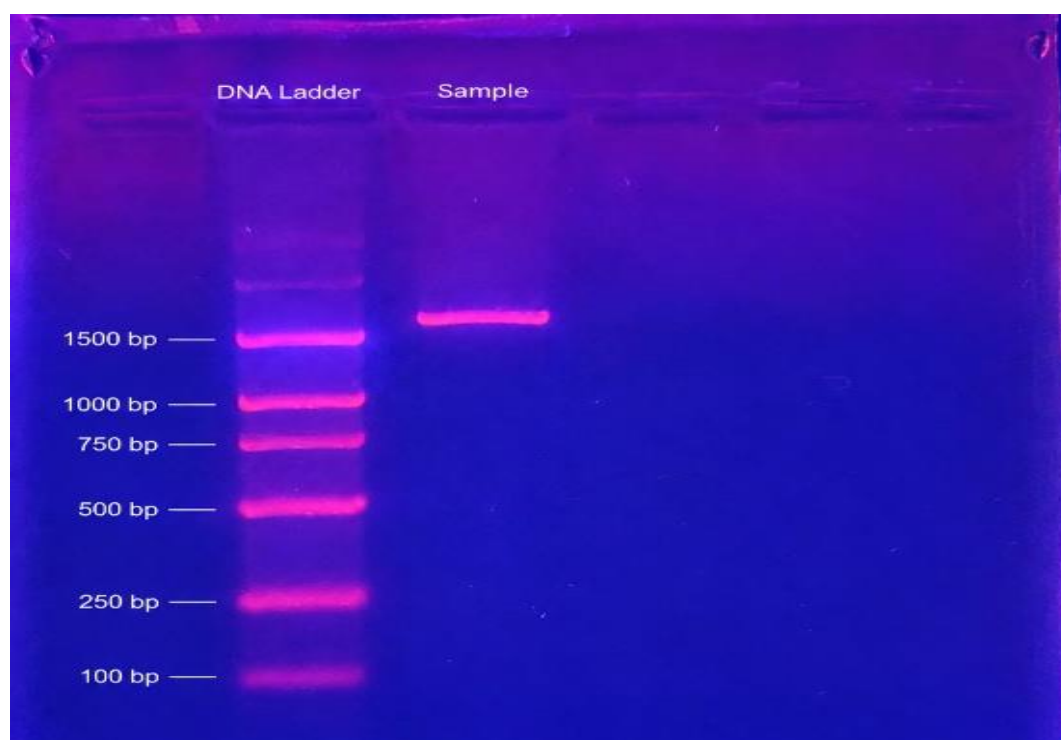
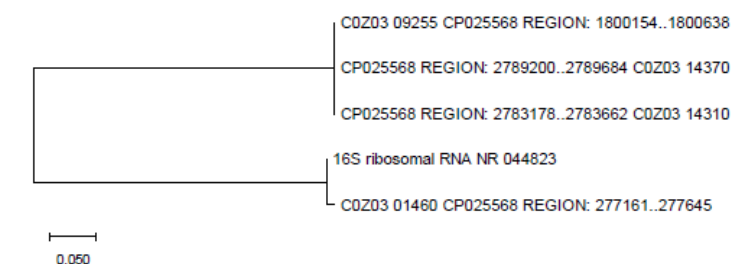


Figure 3: Amplified DNA bands of *S. aureus*

The phylogentic tree with the highest log likelihood (-2,598.37) is shown in Figure 4a. The MP tree had the shortest length among 10 MP tree searches; each performed with a randomly generated starting tree- C0Z03 09255 CP025568 REGION: 1800154..1800638; CP025568 REGION: 2789200..2789684 C0Z03 14370; CP025568 REGION: 2783178..2783662 C0Z03 14310; 16S ribosomal RNA NR 044823C0Z03 01460 CP025568 REGION: 277161..277645.

The numbers of base substitutions per site from between sequences are shown in Figure 4b. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2- parameter model. The

analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA11.



(a)

Distance Matrix:

	1	2	3	4	5
1. 16S ribosomal RNA NR 044823					
2. C0Z03 01460 CP025568 REGION: 277161..277645	0.0083544107				
3. C0Z03 09255 CP025568 REGION: 1800154..1800638	154.5377554685	165.2552374231			
4. CP025568 REGION: 2783178..2783662 C0Z03 14310	154.5377554685	165.2552374231	0.0000000000		
5. CP025568 REGION: 2789200..2789684 C0Z03 14370	154.5377554685	165.2552374231	0.0000000000	0.0000000000	

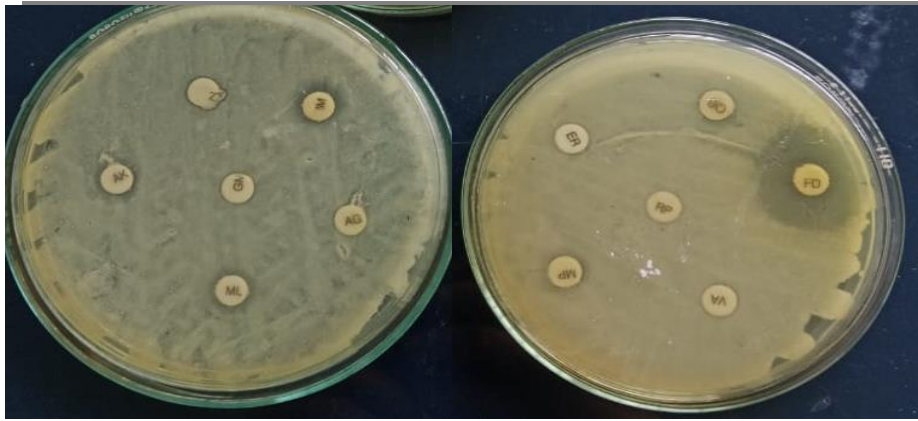
b)

Figure 4: Phylogenetic Analysis

Antibiotic susceptibility/resistance test for *Staphylococcus* species

The antibiotic susceptibility test showed that the isolates no. 1, 2, 4, 6, 9 and 10 are susceptible to the antibiotics nitrofurantoin, imipenem, moxifloxacin and amikacin while resistant to the antibiotics gentamycin, amoxycylav/augmentin, erythromycin, rifampicin, vancomycin, meropenem, levofloxacin and amoxycillin (Figure 5 a, b). The isolates no. 3,5,7 and 8 were found to be sensitive to all experimental antibiotics. The diameter of zone of inhibition for the antibiotic sensitive isolates is mentioned in Table 2. The p- value obtained for zone of inhibition is 0.05 which indicates no significant difference at $p \leq 0.05$.

According to Kulkarni et al, 2016 the strain of *Staphylococcus aureus* isolated from a patient sputum was found to be sensitive to antibiotics linezolid and teicoplanin while resistant to erythromycin, clindamycin, tetracyclin, penicillin and trimethoprim/ sulfamethoxazole. In a study by Dhanalakshmi et al, 2012, the strains of *S. aureus* were found to be resistant to penicillin, amoxicillin/clavulanic acid, gentamycin, netilmicin. Studies by Farhadian et al, 2014 showed resistance of *S. aureus* strains against erythromycin, clindamycin and low resistance to ciprofloxacin.



(a) (b)

Figure 5 a,b: Sensitive/ Resistance to antibiotics by isolated strain of Staphylococcus

Table 2- Zone of Inhibition (cm)

Isolate No.	Zone of inhibition (cm)									p-value
	gentamycin	amoxycillin	augmentin	erythromycin	rifampicin	vancomycin	meropenem	levofloxacin	amoxycillin	
1	0.2	0.2	1	0.1	0.1	0.1	0.1	0.1	0.1	0.05
2	0.19	0.19	0.99	0.11	0.11	0.11	0.11	0.11	0.11	
4	0.22	0.22	1	0.1	0.1	0.1	0.1	0.1	0.1	
6	0.2	0.2	0.98	0.12	0.12	0.12	0.12	0.12	0.12	
9	0.21	0.21	1	0.11	0.11	0.11	0.11	0.11	0.11	
10	0.21	0.21	1	0.1	0.1	0.1	0.1	0.1	0.1	

CONCLUSION

The infections caused by *Staphylococcus aureus* have become difficult to treat in health care centres and hospitals, especially the strains isolated from urine and blood since the prevalence of MRSA. But the challenge is to prevent the outbreak of infections by other strains that are also becoming antibiotic resistant (Chandran et al, 2022). In the present study, a strain of *Staphylococcus aureus* is identified that has shown resistance to antibiotics like penicillin, amoxicillin/clavulanic acid, gentamycin, netilmicin while susceptibility to nitrofurantoin, imipenem, moxifloxacin and amikacin no other strain of *Staph aureus* has indicated the antibiotic susceptibility/ resistance to these antibiotics till present. The need of the hour is to identify other such strains so that certain preventive measures could be attained to treat the infections caused by such strains using alternative methods.

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Ethical approval:

Not applicable

Conflict of Interest:

The authors declare that there is no conflict of interest.

Data availability:

All data is mentioned in the manuscript.