

Original Research Article

Molecular characterization of corneal ulcers causing *Staphylococcus aureus*

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ABSTRACT

Background: The human eye is one of the most remarkable sensory systems. Human beings gather most of the information about the external environment through their eyes and thus rely on sight more than on any other sense, with the eye being the most sensitive organ we have. Molecular characterization of *Staphylococcus aureus* from the cases of corneal ulcers.

Methods: A total of 300 samples of corneal ulcers collected from various ophthalmology hospitals, government hospital and clinical laboratories of different places of Maharashtra, India. The Isolates were identified based on the colony morphology and biochemical reaction. The isolates were subjected for antibiotic sensitivity test and perform its molecular characterization.

Results: In present study, 39 coagulase positive *Staphylococcus aureus*, pathogenic bacteria isolated from corneal ulcers.

Conclusions: *Staphylococcus aureus* is one of the most significant pathogens in bacterial keratitis. Early diagnosis and prompt treatment are needed to minimize the possibility of permanent vision loss and reduce structural damage to the cornea.

Keywords: Antibiotic sensitivity test, Corneal ulcer, Molecular characterization, *Staphylococcus aureus*

INTRODUCTION

The human eye is one of the most remarkable sensory systems. Human beings gather most of the information about the external environment through their eyes and thus rely on sight more than on any other sense, with the eye being the most sensitive organ we have.

Cornea is a clear transparent front part of the eye with a smooth shining surface. That covers Iris, Pupil and anterior chamber. The cornea with the anterior chamber and lens reflects light with the cornea accounting for approximately two-third of the eye's total optical power. "Corneal Ulcer means loss of corneal substances as a

result of infection and formation of raw, excavated area".¹ Blindness is major public health problem in developing countries. WHO estimates 45 million blind cases in the world. Out of which 5.4 million blind people are in from India. It is important to note that corneal diseases including corneal ulcers are among the major cause of vision loss and blindness. It is projected corneal blindness in India will reach up to 10.6 million by 2020.² Corneal ulcer is a major cause of blindness throughout the world. About 10% cases of blindness are due to corneal ulcer.³

The eye is a unique organ that is virtually impermeable to most environmental agents. During normal ocular

development in mammals, the eyelids grow across the eye, fuse, and subsequently reopen.⁴ An epithelium layer of cornea provides an effective barrier against most microorganisms, and therefore, bacterial keratitis occurs rarely in the normal eye.⁵ However, predisposing factors such as corneal injury, corneal abnormalities, chemical burns, trauma and overuse of contact lenses may alter the local defence mechanisms of the ocular surface and allow bacteria to invade the cornea through epithelial defects that occur.⁶

Bacterial keratitis, because of its high incidence and potential complications, is one of the most visually threatening ocular infectious pathologies. Eighty percent of bacterial corneal ulcers are caused by *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Pseudomonas* species. Certain bacterial toxins and enzymes help in the digestion and degradation of the corneal matrix. Since keratitis is not included in the five target diseases of WHO for blindness prevention, most of the data regarding keratitis is from individual publications. Bacterial keratitis is one of the most important causes of corneal opacifications, which is the second common cause of legal blindness world-wide after cataracts. The pattern of microbial keratitis varies with geographic region and according to the local climate.⁷

Staphylococcus aureus is a gram-positive bacterium, characterised by individual cocci, which divide in more than one plane to form grape-like clusters and discovered in 1880's has been shown to be a potential pathogen causing infections such as minor skin infections and post-operative wound infection.⁸ *Staphylococci* have a special relationship with the eye. They cause severe eye infections which may result in irreversible blindness.⁹

S. aureus is the most common cause of bacterial keratitis and an important cause of other ocular infections.^{10,11} *S. aureus* is well known for its ability to evolve mechanisms of antibiotic resistance, making these infections among the most difficult to treat, and antibiotic resistance has increased since 2000.¹⁰ Bacterial corneal ulceration is serious ocular infectious disease that can lead to significant vision loss. The goal of this study was to isolate the pathogenic *Staphylococcus aureus*, determine antibiotic resistance pattern and perform its molecular characterization.

METHODS

In assessment to isolate and identify the pathogenic bacteria *Staphylococcus aureus* responsible for corneal ulcers in clinical settings and perform its molecular characterization, present work was under taken.

Collection of samples

A total of 300 samples were collected from various ophthalmology hospitals, government hospital and clinical laboratories of different places.

Enrichment of samples

Samples were collected in sterile container containing 0.5ml of brain heart infusion broth (BHI) as enrichment culture medium that supports the growth of bacteria and then transferred immediately to laboratory for further processing. Then samples were incubated at 37°C for 24 hours for propagation.^{12,13}

Isolation and identification of pathogenic bacteria

After incubation, a loopful of each enriched culture was streaked on CLED agar and Nutrient agar plates were incubated at 37°C for 24 hours. Colonies with different morphological characters and Gram's characters were selected and inoculated on respective selective media viz. Mannitol salt agar, Milk agar, Baird parker agar. All the plates were incubated at 37°C for 24 hours. All the suspicious screened colonies of *Staphylococcus* isolates were then analyzed for their biochemical character viz. Carbohydrate fermentation, IMViC, coagulase enzyme test etc. by inoculating into respective media. Further their identification was confirmed by morphological, biochemical and cultural characteristics.

Antimicrobial susceptibility testing

After identification, the coagulase positive isolates of *Staphylococcus* isolates were subjected to antibiotic sensitivity testing. It was done by the agar disk diffusion method as described by NCCLS 2002 and Kirby Bauer disk diffusion method, now known as the Clinical and Laboratory Standards Institute (CLSI).^{14,15}

The Antibiotics used were bacitracin (30mcg), ceftazidime (30mcg), cephazolin (5%), chloramphenicol (30mcg), ciprofloxacin (10mcg), gentamicin (10mcg), imipenem (10mcg), kanamycin (20 mcg), moxifloxacin (0.5%), norfloxacin (10mcg), ofloxacin (0.3%), vancomycin (30mcg), tobramycin (1.33%), tetracycline (30mcg). Antibiotic discs were placed on a lawn culture of the isolate under test on Mueller Hinton Agar (MHA).

Molecular characterization

After observing the antibiotic sensitivity and resistance pattern of *Staphylococcus* isolates, were subjected for molecular characterization. The procedure is as follows:

DNA extraction

- Bacterial Genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit- as per the kit instruction below procedure followed.
- An isolated bacterial colony was picked and suspend in 1ml of sterile water in a microfuge tube.
- Centrifuge it for 1 minute at 10,000-12,000 rpm to remove the supernatant.
- Add 200µl of Insta Gene matrix to the pellet and incubate at 56°C for 15 minutes.

- Vortex at high speed for 10 seconds and place the tube in a 100°C in heat block or boiling water bath for 8 minutes.
- Finally, vortex the content at high speed for 10 seconds and Spin at 10,000-12,000 rpm for 2 minutes.
- In result, 20µl of the supernatant was used per 50µl PCR reaction.

PCR protocol

Using below 16S rRNA universal primers gene fragment was amplified using MJ research Peltier thermal cycler.

Table 1: Primer details.

Primer name	Sequence details	Number of base
27F	AGAGTTTGATC MTGGCTCAG	20
1492R	TACGGYTACCTT GTTACGACTT	22

Add 1µL of template DNA in 20µL of PCR reaction solution. Use 27F/1492R primers used for bacteria, and then PCR reaction performed with below conditions: Initial Denaturation 94°C for 2minutes and then 35 amplification cycles at 94°C for 45 seconds, 55°C for 60 seconds, and 72°C for 60 sec. Final extension at 72°C for 10 min. DNA fragments are amplified about 1,400bp in the case of bacteria. Purification of PCR products removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR clean up kit (Millipore).⁴ The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ terminator cycle sequencing kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Sequencing protocol Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Table 2: Sequencing primer details.

Primer name	Sequence details	Number of base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTC AATTCMTTTRAGTTT	20

Sequence data was aligned and analyzed for Identifying the sample.

Bioinformatics protocol

The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our

sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

RESULTS

In this research work a total of 300 samples were collected from various ophthalmology hospitals, government hospital and clinical laboratories of different places. The patients were of both sex and age groups varying from 20 to 70 years. Out of 300 samples, 92 *Staphylococcus* isolates isolated. They were identified based on the colony morphology and biochemical reaction. *Staphylococcus* isolates were confirmed based on yellowish colony coloration and pigmentation on Mannitol salt agar, golden yellow colonies on Milk agar and black coloured colonies on Baird parker agar. From these isolates 39 were showing coagulase enzyme test positive and 53 were showing coagulase enzyme test negative. Coagulase is a protein enzyme produced by several microorganisms that enables the conversion of fibrinogen to fibrin. In the laboratory, it is used to distinguish between different types of *Staphylococcus* isolates (Figure 1).

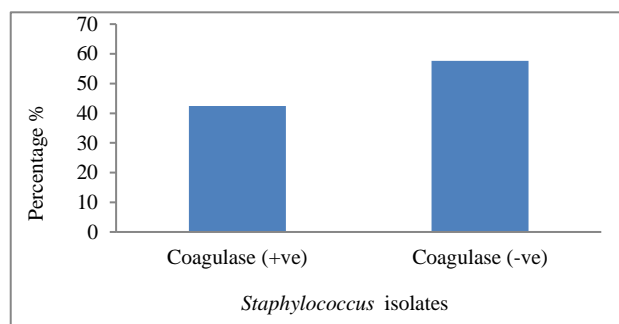


Figure 1: Frequency of coagulase enzyme test.

Table 3: Resistance and sensitivity pattern of coagulase (+ve) *Staphylococcus* isolates against several antibiotics.

Name of antibiotics	No. of Resistance	%	No. of Sensitive	%
Vancomycin	38	97.43	01	02.56
Tetracycline	36	92.30	03	07.69
Bacitracin	36	92.30	03	07.69
Kanamycin	35	89.74	04	10.25
Gentamicin	34	87.17	05	12.82
Cephazolin	34	87.17	05	12.82
Norfloxacin	31	79.41	08	20.51
Ceftazidime	26	66.66	13	33.33
Ciprofloxacin	23	58.97	16	41.02
Ofloxacin	15	38.46	24	61.53
Moxifloxacin	14	35.89	25	64.10
Tobramycin	11	28.20	28	71.79
Chloramphenicol	09	23.07	30	76.92
Imipenem	05	12.82	34	87.17

The sensitivity and resistance pattern of *Staphylococcus* isolates against several antibiotics were observed by disc diffusion method on Mueller Hinton agar (MHA- Hi-media) such as vancomycin, tetracycline, bacitracin, kanamycin, gentamicin, cephazolin, norfloxacin, ceftazidime, ciprofloxacin. The isolates showed 59% to 98% resistance to these antibiotics. The ofloxacin was 62%, moxifloxacin was 65%, tobramycin was 72%, chloramphenicol was 77%, and imipenem was 88% sensitive to coagulase (+ve) *Staphylococcus* isolates (Table 3).

After observing the antibiotic resistance pattern of *Staphylococcus* isolates, the selected isolates were subjected for molecular studies. The primary identification of obtained *Staphylococcus* isolates was carried out based on cultural and biochemical characteristics but to acquire accuracy in the assignment of genus and species, Molecular characterization was carried out by using 16S rRNA at Yaazh Xenomics (Crack the life code), DNA sequencing service, Madurai (Chennai Branch), Tamil Nadu, (India).

Present study was carried out to do molecular characterization of *Staphylococcus aureus*. Isolates were sequenced by using universal primers by giving name such as PBCUS64. The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. NCBI database provide us more information regarding nucleotide sequences.

The sequences of *Staphylococcus aureus* were compared with sequences of closely related species in GenBank by multiple sequence alignment, using the program MUSCLE 3.7.¹⁶ Phylogenetic relationships were determined by using the neighbor-joining method. Sequencing of the 16S rRNA of the isolates showed that there was 94% similarity between the 16S rRNA sequences of *Staphylococcus aureus* isolates of present study and methicillin resistant *Staphylococcus aureus* BMB9393 (GenBank accession no. CP005288). This accession number in NCBI, provide us taxonomy report with 94% identity in BLAST analysis (Figure 2).

Table 4: Sequences producing significant alignments.

Description	Max score	Total score	Query cover	E-value	Identity	Accession
<i>Staphylococcus aureus</i> , complete genome	1657	8250	87%	0.0	94%	CP005288

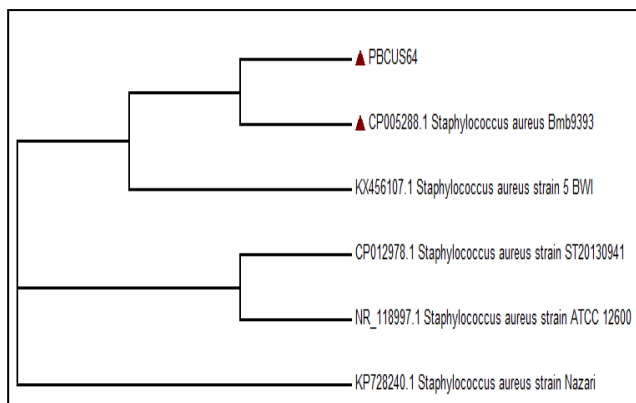


Figure 2: Phylogenetic tree showing the relationships of the corneal-scraping *Staphylococcus aureus* isolates from our patients to related species.

DISCUSSION

Bacterial keratitis is a serious ocular infectious disease that can lead to severe visual disability.¹⁷ The severity of the corneal infection usually depends on the underlying condition of the cornea and the pathogenicity of the infecting bacteria. Many patients have a poor clinical outcome if aggressive and appropriate therapy is not promptly initiated.¹⁸

In recent years, the literature extensively addressed this issue but only a few publications reviewed large series of patients with bacterial keratitis.¹⁹ On the frequency of factors predisposing to bacterial keratitis a very little information is available. In present study *Staphylococcus* isolates were associated with different aetiological risk factors; but these isolates were more often associated with contact lens wear and trauma. Trauma was most common predisposing factor and contact lens (CL) wear was the second most common risk factor. Those were encountered in 32 % and 21% (Figure 3).

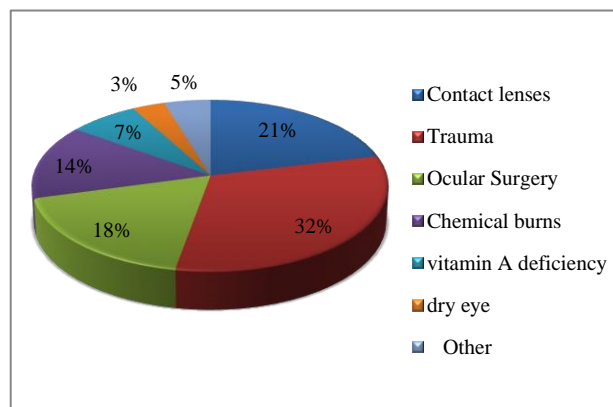


Figure 3: Aetiological risk factors.

The elderly group (age was 65 years or older) affected more with bacterial keratitis by trauma risk factor, had severe, central ulcers with a poor visual outcome.²⁰

Multiple organisms have been reported from bacterial keratitis in human eyes all over the world.^{6,21} The mechanisms involved in the initiation of keratitis are not yet understood. *S. aureus* has been shown to bind to human corneal cells by the fibronectin-binding protein which is present on the bacterial surface.¹¹ Binding to the cornea can also be mediated by a collagen binding adhesion on the *S. aureus* surface.²² Keratitis infrequently develops due to this binding ability and the availability of organisms from the flora surrounding the eye.

Present results have shown that *Staphylococcus aureus* as the major cause of corneal infection, and the need to increase public education about corneal ulcer causing risk factors. *Staphylococcus aureus* are Gram-positive cocci typically cause round or oval ulceration with greyish white stromal infiltrates having distinct borders.²³ Bourcier et al, in 2003 believe that the emerging resistance of Gram positive organisms to second generation fluoroquinolones indicates the necessity to perform pre-treatment cultures of corneal ulcers in all patients or to use third or fourth generation quinolones characterized by a superior Gram positive profile.⁶ In present study Moxifloxacin, a fourth generation quinolone exhibited higher sensitivity. Also, tobramycin, aminoglycoside group showed 72% sensitivity on *Staphylococcus aureus*.

Ofloxacin (0.3%) can be substituted for ciprofloxacin as a monotherapy in situations of unknown organisms or a new case or where there is no growth on first culture. Increasing resistance to the fourth-generation fluoroquinolones has been reported amongst *Staphylococcus* species in the USA.²⁴

Bouhenni et al, in 2015 have highlighted some of the proteomic technologies that have been used to identify virulence factors and the host response to infections of bacterial keratitis in order to understand the disease process and develop improved methods of diagnosis and treatment. They have shown that *S. aureus* is one of the most significant pathogens in bacterial keratitis. Virulence factors produced by *S. aureus* in keratitis include α -toxin as the major factor, with β and γ -toxins to a lesser extent.²⁵

The ability of an organism to adhere the edge or base of an epithelial, defect and signature its pathogenicity. Membrane appendages such as fibrillae in Gram-positive organism help these organisms adhere to damaged epithelial cells and stroma.

Nucleotide sequence accession number

The 16S rRNA complete genome sequence of the isolate of *Staphylococcus aureus*, gram-positive cocci recovered

from the patients has been deposited at the NCBI GenBank sequence database under accession numbers LC216327 (PBCUS64).

CONCLUSION

In conclusion, present study results showed the presence of *Staphylococcus aureus*, the pathogenic bacteria associated with the cases of corneal ulcer (bacterial keratitis). It evolves mechanisms of antibiotic resistance, making these infections among the most difficult to treat, and antibiotic resistance has increased day by day. Particular attention should be given to pathogenic bacterial keratitis condition as it can progress very rapidly, leading to complete corneal destruction. Early diagnosis and prompt treatment are needed to minimize the possibility of permanent vision loss and reduce structural damage to the cornea.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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