

## *Exiguobacterium aurantiacum* virulent pigment producing a novel pathogenic bacteria associated with cases of corneal ulcers

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### Abstract

Blindness is a major public health problem in most developing countries. Corneal ulcer (Keratitis) is a major cause of blindness throughout the world. About 10% cases of blindness are due to corneal ulcer. "Corneal ulcer means loss of corneal substances as a result of infection and formation of raw, excavated area." Bacterial keratitis is an acute or chronic, transient or recurrent infection of the cornea with varying predilection for anatomical and topographical parts of the cornea like marginal or central. This study demonstrates the Novel pathogenic bacteria *Exiguobacterium aurantiacum* with bad pigment production, which are causing corneal ulcer and determine the best possible empirical therapy and specific therapy. Particular attention should be given to this condition as it can progress very rapidly with complete corneal destruction can be occur within 24–48 hours.

**Keywords:** Corneal Ulcer, *Exiguobacterium aurantiacum*, Molecular characterization, Antibiotic treatment, Pigment.

### Introduction

Vision is an important part of our everyday life. Eyes are the organs of vision. The eye which is the window to the soul, it's mainly depends on the clarity of the cornea. Corneal ulceration is major cause of monocular blindness in developing countries. Surveys in Asia and Africa have confirmed these findings.<sup>1,40</sup> Corneal ulcer is a major cause of blindness throughout the world. About 10% cases of blindness are due to Corneal ulcer.<sup>2,40</sup> "Corneal ulcer means loss of corneal substances as a result of infection and formation of raw, excavated area".<sup>3</sup>

Blindness is major public health problem in most developing countries. The World Health Organization (WHO) estimates 45 million blind cases in the world and another 135 million that have severely impaired vision in both eyes. Out of which 5.4 million blind people are from India.<sup>4-6,2,40</sup> In the total disabled population of the Maharashtra state, the proportion of in seeing disabled persons is ranked first.<sup>7,8</sup> It is important to note that corneal diseases including corneal ulcers are among the major cause of vision loss and blindness. It is projected that corneal blindness in India will reach up to 10.6 million by 2020.<sup>9,10,6</sup>

The importance of corneal disease as a major cause of blindness in the world today remains second only to cataract, but its epidemiology is complicated and encompasses a wide variety of infectious and inflammatory eye diseases.<sup>11,4</sup>

Corneal ulcers can be caused by exogenous infections i.e. by bacteria, fungi, viruses or parasites and sometimes it is allergic in nature or it can be due to endogenous infections. Almost any organism can invade the corneal stroma if the normal corneal defence

mechanisms, i.e., lids, tear film and corneal epithelium are compromised.<sup>12,41</sup>

Bacterial keratitis accounts for approximately 65% to 90% of all microbial corneal infections. Tiny tears or scratches on the cornea can make it easier for bacteria to enter the eye and cause infection. A severe ulcer without treatment can lead to blindness in the affected eye. Bacterial keratitis is caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Enterococci*, *Pseudomonas*, *Klebsiella*, *Proteus*, *Bacillus cereus*, *N. gonorrhoeae* and many other bacteria.<sup>12-15,40</sup>

Alteration in the components of tear fluid or the aqueous humour may result in pathological changes in cornea. This study demonstrates The New Study for Keratitis and the isolation of Novel Bacteria which are causing Keratitis. Prompt diagnosis of corneal ulcers and virulence factors and treatment with appropriate antibiotics prevent blindness and devastating visual disability.

### Materials and Methods

The present work was undertaken to characterize the unknown novel pathogenic bacteria from corneal ulcer by performing its isolation, identification, molecular characterization and virulent factors.

**Collection of Samples:** Standard Methods were usually adopted for samples collection.<sup>16,17</sup> Corneal scrapings were taken from patients with infectious keratitis under topical anesthesia using a sterile No. 11 and 15 Bard Parker Surgical blades and by swabbing the lid margins with sterile broth-moistened cotton swabs by ophthalmologist. Scrapings were taken from the edges and base of the ulcer.<sup>12,15,18,40</sup> Samples were collected in sterilized container containing Nutrient broth and

transferred immediately to laboratory for further processing. A total of 300 samples were collected from clinically diagnosed patients of corneal ulcers, admitted in Government Medical College and Hospital, Ophthalmic Hospitals and Clinical Laboratories of various districts and towns of Maharashtra state like Akola, Jalna, Buldhana, Chikhli etc. The hospitals were selected on the basis of geographical proximity, feasibility of conducting the study and availability of the samples.<sup>40,41</sup>

**Enrichment of Samples:** Samples were collected in sterile container containing 0.5ml of Brain Heart Infusion Broth (BHI) as enrichment culture medium which supports the growth of bacteria and then transferred immediately to laboratory for further processing. Samples were then incubated at 37 °C for 24 hours for propagation.<sup>15,40,41</sup>

**Isolation and Identification of Pathogenic Bacteria by Phenotypic Methods:** After 24 hours of incubation, a loopful of each enriched culture was taken and streaked on CLED agar and Nutrient agar plates in aseptic conditions and were incubated at 37 °C for 24 hours. Colonies with different morphological characters (Size, Shape, Margin, Texture, Opacity, Elevation, Colour, Motility and Gram's (Positive) characters were selected and inoculated on respective selective media viz. Milk agar, Mannitol salt agar. All the plates were incubated at 37 °C for 24 hours. All the suspicious screened colonies of the isolates were then analyzed for

**Table 1: Primer details**

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	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 2 min and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, 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. Include a positive control (*E.coli* genomic DNA) and a negative control in the PCR.<sup>40</sup>

**Purification of PCR Products:** Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit

**Table 2: Sequencing primer details**

Primer Name	Sequence Details	Number of Base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTCAATTCMTTTRAGTTT	20

Sequence data was aligned and analyzed for Identifying the Sample.<sup>40</sup>

**16S rRNA Sequence Analysis:** Database homology searching was performed with BLAST on the NCBI (National Center for Biotechnology Information).

their conventional biochemical tests such as Carbohydrate fermentation, IMViC, Enzyme tests etc.<sup>19,40,41</sup>

**Identification of Pathogenic Bacteria by Genotypic Methods:** Molecular studies were carried out by using 16S rRNA analysis at Yaazh Xenomics (Crack the life code), DNA sequencing service, Madurai (Chennai Branch), Tamil Nadu, (India).<sup>40</sup> The molecular characterization of different pathogenic bacteria was done in following steps:<sup>40</sup>

**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 micro centrifuge tube. Centrifuge it for 1 minute at 10,000–12,000 rpm to remove the supernatant. Add 200 µl of Insta Gene matrix to the pellet and incubate at 56°C for 15 minutes. 5. 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. 6. Finally, vortex the content at high speed for 10 seconds and Spin at 10,000–12,000 rpm for 2 minutes. 7. In result, 20µl of the supernatant was used per 50 µl PCR reaction.<sup>40</sup>

**PCR Protocol:** Using below 16S rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler.

(Millipore). 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).<sup>40</sup>

**Sequencing Protocol:** Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labelled 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.<sup>40</sup>

**Antimicrobial Susceptibility Testing:** After molecular characterization, the pathogenic bacteria were subjected for antibiogram. The antimicrobial susceptibility testing was done by the agar Disk Diffusion Method.<sup>20,21,40</sup> Antibiotic disc were placed on a lawn culture of the

isolate under test on Mueller Hinton Agar (MHA).<sup>40</sup> The Antibiotics used were:

**β-Lactam Antibiotics:** β-Lactamase production in all the isolates was detected by Disc Diffusion Method and Well Diffusion Method by using three groups of antibiotics.<sup>40</sup> 1) **Penicillins:** Amoxicillin (10 mcg), Amoxyclav (20μg Amoxicillin and 10μg Clavulanic acid) (30 mcg), Ampicillin (10 mcg), Methicillin (10 mcg). Penicillin G (10 mcg). Cephalosporins: Cefalexin (30 mcg), Cefixime (30 mcg), Cefpodoxime (30 mcg), Ceftazidime (30 mcg), Cefotaxime (30 mcg), Cephazolin (5%), Cefadroxil (30 mcg), Ceftriaxone (30 mcg) 3) **Carbapenems:** Imipenem (10mcg), Meropenem (10mcg).

**Non β-Lactam antibiotics:** 1) **Tetracycline:** Tetracycline (30 mcg), Chloramphenicol (30 mcg). 2) **Aminoglycosides:** Bacitracin (30 mcg), Kanamycin (20 mcg), Gentamicin (10 mcg), Erythromycin (15 mcg), Tobramycin (1.33%), Vancomycin (30mcg). 3) **Fluoroquinolones:** Ofloxacin (5 mcg), Moxifloxacin (0.5%). 4) **Quinolones:** Ciprofloxacin (10 mcg), Norfloxacin (10 mcg).

#### Combination Therapy or Dual Therapy

1. Ceftazidime + Ciprofloxacin
2. Cephazolin + Vancomycin
3. Cephazolin+ Tobramycin
4. Gentamicin + Cefuroxime

#### Pigment Extraction and its Assessment as Virulent

**Factor:** Seed cultures were prepared by inoculating 100 ml Erlenmeyer flask containing 30 ml nutrient broth by a loopful from a single pigmented colony and shaken at 150 rpm at 30 °C till bacterial growth reached OD 550 nm = 1. The seed culture was introduced into 1000 ml Erlenmeyer flasks containing 500 ml of sterile nutrient broth, a growth medium with pH adjusted to 7. Inoculated flask was incubated in a rotary shaker at 180rpm, 37°C for three days. After three days of incubation, cells were harvested by centrifugation at 8,000 rpm for 15minutes. Then the pellet was washed with sterile distilled water and again centrifuged for 15 minutes at 4,000rpm. Then 5 ml of methanol was added to the pellet and suspended it. Then it was incubated in a water bath at 60°C for 15 minutes until all visible pigments were extracted and centrifuged at 4,000 rpm for 15 minutes. The process was repeated till the cell pellets became colourless. The coloured supernatant was separated and then it was filtered through Whatman filter paper no. 1.<sup>21,23</sup>

**Pigment Identification by FTIR:** The qualitative identification of pigments was done by Infrared spectroscopy.

**Assay of Nitric Oxide Scavenging Activity:** According to the method of<sup>24,25,42</sup> the assay of scavenging activity was done by taking reaction mixture (5.0 ml) containing sodium nitroprusside (10mM) in phosphate buffered saline (pH 7.3), was mixed with different concentrations (25, 50, 100μg/ml) of methanol extract of each pigment were dissolved in

methanol and incubated at 30°C for 3 hours. The same reaction mixture without the extract but the equivalent amount of methanol served as the control. The NO radical thus generated interacted with oxygen to produce the nitrite ion (NO) which was assayed at 30 min intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). The absorbance was measured at 546 nm. The nitrite generated in the presence of the pigment extract was estimated using a standard curve based on sodium nitrite solutions of known concentrations. Each experiment was carried out at least three times and the data presented as an average of three independent determinations.

**Antioxidant Activity Assay:** The percentage inhibition of the extract was calculated and recorded.<sup>24,25,42</sup> The percentage nitrite radical scavenging activity of the methanol extracts was calculated using the following formula:

Percentage nitrite oxide radical scavenging activity:

$$\% \text{ inhibition} = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100$$

Where,  $A_{\text{control}}$  = absorbance of control sample and  $A_{\text{test}}$  = absorbance in the presence of the samples of extracts.

#### Statistical Analysis

Data analysis was performed using SPSS software (Statistical Package for the Social Sciences). By using ANOVA (Analysis of variance) test (using the number of independent variables in one way and two way) levels of significance were determined.  $P \leq 0.05$  values were considered to be statistically significant difference.

#### Results

In present research study 300 samples were collected during period of January 2014 to January 2017 from clinically diagnosed patients of corneal ulcers, admitted in government medical college, ophthalmic hospitals and clinical laboratories of various cities and towns.<sup>40</sup> Out of 300 samples, bacteria were isolated from 212 samples. The patients selected were belonging to both genders and age groups varying from 11 to 80 years from rural and urban areas. Highest prevalence of bacterial corneal ulcers was found to be in the patients belonging to the age group of 31-40 years. Male patients were noted to be at an increased risk of corneal ulcers as compared to female patients. The distribution of patients as per the place of resident reveals that 61% of cases were found from urban areas and 39% cases were obtained from rural area. As per the etiology of corneal ulcer cases, noted in these investigations, the significant number (32%) of isolates were obtained from the patients with contact lens users, followed by 21% isolates from traumatic patients. The severity distribution of bacterial corneal ulcers reveals

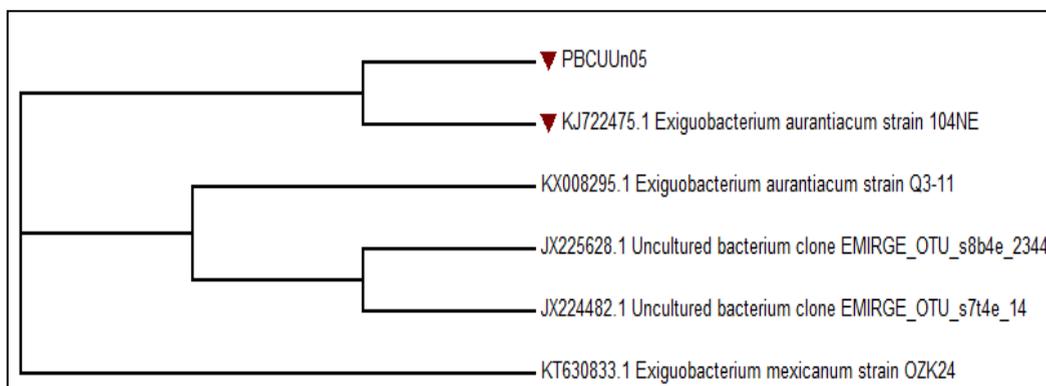
34.57% cases of mild, 29.25% cases of moderate and 36.17% cases of severe.

From 212 total positive clinical samples 278 different kinds of bacteria were isolated. Out of 278 isolates, 07 isolates were identified as *Exiguobacterium aurantiacum* (coded as PBCUUn05). PBCUUn05 isolates were Gram positive, motile and bacilli in shape. They showed yellow to orange coloured colonies on nutrient agar and mannitol fermenting yellow coloured colonies on mannitol salt agar.<sup>41</sup> PBCUUn05 fermented Mannitol, maltose and sucrose sugars with acid and gas production. These isolates were indole positive, amylase, Catalase, gelatinase and nitrate reduction positive. These isolates have ability to hydrolyze DNA and utilize it as a source of carbon and energy for growth by showing DNase enzyme test positive.

We have confirmed our findings again and again. To acquire the accuracy in the assignment of genus and species, these 07 Isolates of *Exiguobacterium aurantiacum* were further confirmed by 16SrRNA molecular analysis.<sup>40</sup> 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. By using the program MUSCLE 3.7, the sequence was compared with sequences of closely related species in GenBank by multiple sequence alignment.<sup>26,40</sup> Phylogenetic relationships were determined by using the neighbour joining method.

#### Sequences producing significant alignments:

Name of isolate	Maximum score	Total score	Query cover	E value	Total Identity	Accession No.
<i>Exiguobacterium aurantiacum</i>	2760	2760	87%	0.0	99%	KJ722475.1



**Fig. 1: Phylogenetic tree of *Exiguobacterium aurantiacum* isolate (PBCUUn05) and related species**

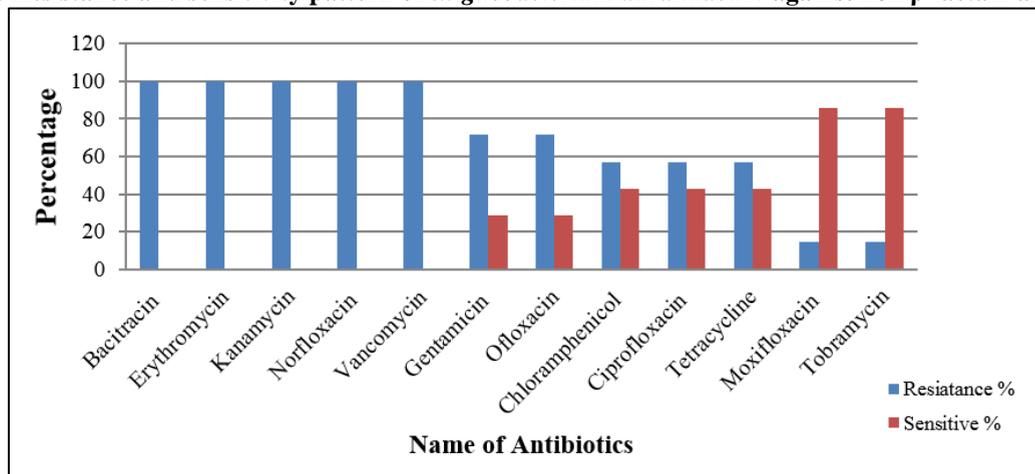
Sequencing of the 16S rRNA of the isolates showed that there was 99% similarity between the 16S rRNA sequences of PBCUUn05 isolates of present study and *Exiguobacterium aurantiacum* strain 104NE (GenBank accession no. KJ722475.1). This provided us taxonomy report with 99% identity in BLAST analysis. The 16S rRNA was made up of 1706 base pairs.

In present study, antibiotic susceptibility studied for *Exiguobacterium aurantiacum* isolated from corneal ulcers and examined towards a panel of 27 antibiotics of several group (*viz.*, 12 non  $\beta$ -lactam, 15  $\beta$ -lactam), 04 combination of antibiotics using Kirby Bauer's disc diffusion method on Mueller Hinton Agar. The results of antibiotic susceptibility testing were categorised as sensitive, intermediately sensitive and resistant on the basis of zone of inhibition (Scale: <10: Resistant, 10-12: Intermediate, >12: Sensitive).

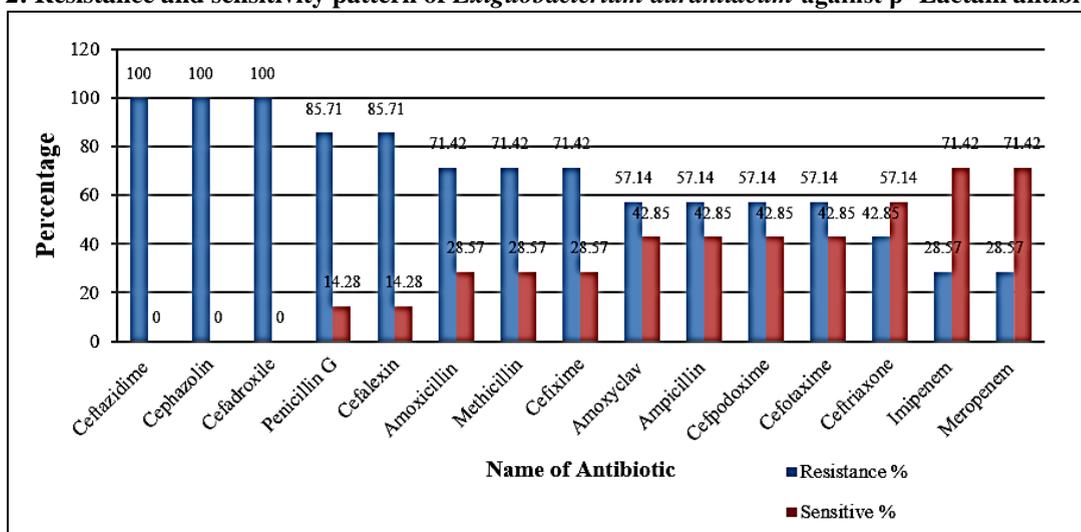
*Exiguobacterium aurantiacum* showed 100% resistance to Bacitracin, Erythromycin, Kanamycin, Norfloxacin and Vancomycin antibiotics. They had

shown sensitivity towards Moxifloxacin, Tobramycin (Graph 1). Ceftazidime, Cephezolin, Cefadroxile showed 0% effectiveness on *E. aurantiacum* isolates while Ceftriaxone, Imipenem, Meropenem showed 57 to 72% sensitivity (Graph 2). These isolates have shown 57.14% resistance towards Ceftazidime + Ciprofloxacin, intermediate resistances towards Cephazolin + Vancomycin and Gentamicin + Cefuroxime combinations but 85% isolates were sensitive to Cephazolin+ Tobramycin combinations (Graph 3).

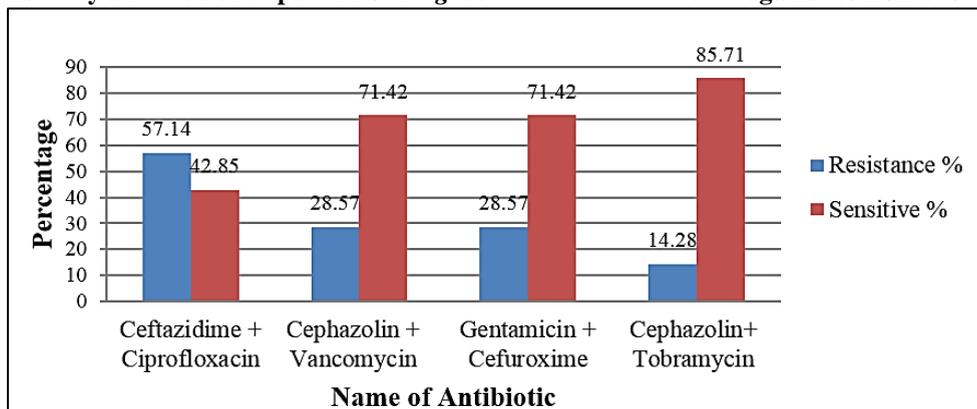
**Graph 1: Resistance and sensitivity pattern of *exiguobacterium aurantiacum* against non  $\beta$ -lactam antibiotics**



**Graph 2: Resistance and sensitivity pattern of *Exiguobacterium aurantiacum* against  $\beta$ -Lactam antibiotics**



**Graph 3: Sensitivity and resistance pattern of *Exiguobacterium aurantiacum* against combination therapy**



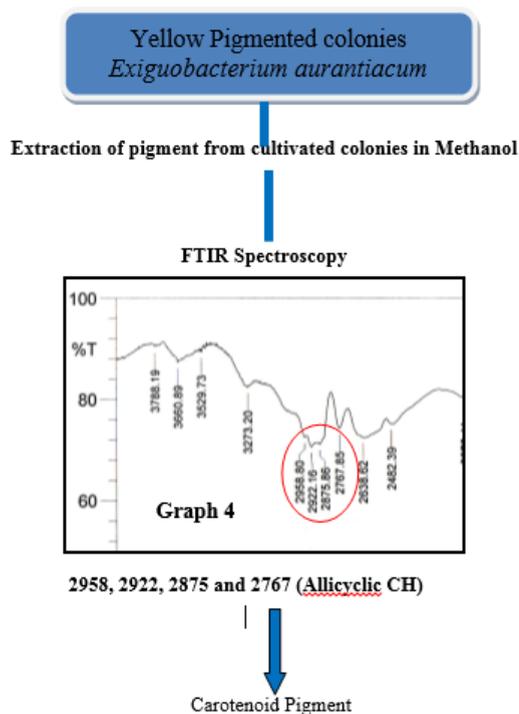
In present study, *Exiguobacterium aurantiacum* was found to be the novel bacterium and not reported earlier from the cases of corneal ulcers. As a matter of inquisitiveness, these novel isolates were

further subjected to virulence studies, which constitute one of the mechanisms for survival.

The pigment production is also one of the mechanisms among bacterial pathogens to increase their virulence. In the present studies, Pigment

identification was done by studying characteristic peaks in FTIR spectrum, which confirms the presence of structural functional groups (Graph 4).

FTIR spectrum of dried powder of isolated pigments confirmed that *Exiguobacterium aurantiacum* produces Carotenoid pigment.



#### Scavenging effect of Carotenoid on NO production:

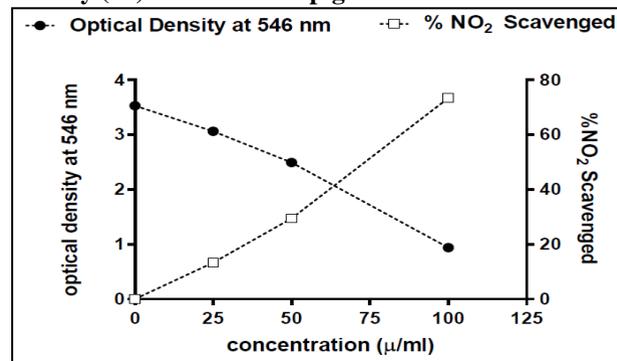
Nitric oxide released from sodium nitroprusside has a strong NO<sup>+</sup> character which can alter the structure and function of many cellular components.<sup>42</sup> The methanol extract of Carotenoid pigments isolated from *Exiguobacterium aurantiacum* exhibited well NO scavenging activity leading to the reduction of the nitrite concentration in the assay medium. The NO scavenging capacity was concentration dependent.<sup>42</sup> In this study, with 100 μg/ml scavenging was found most efficient. The present study showed a potent nitric oxide scavenging activity of Carotenoid pigments. The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthyl ethylenediamine dihydrochloride was measured at 546 nm (Table 1).<sup>42</sup>

**Antioxidant Activity:** The percentage inhibition of the Carotenoid pigment extract was calculated and recorded. The Carotenoid pigments showed a very good concentration-dependent inhibition of nitric oxide. Graph 5 is showing that, as concentration of pigments is increasing, % if inhibition is increasing and absorbance decreasing. These pigments are showing virulent factor with an antioxidant action because these pigments neutralize the oxidants.

**Table 1: Absorbance and Nitric oxide scavenging activity (%) of Carotenoid pigment**

Concentration (μg/ml)	Optical density at 546nm	% NO <sub>2</sub> Scavenged
0	3.53	0
25	3.06	13.31
50	2.49	29.46
100	0.94	73.37

**Graph 5: Absorbance and nitric oxide scavenging activity (%) of carotenoid pigment**



#### Discussion

Corneal ulceration has only recently been recognized as a 'silent epidemic' in developing countries. Corneal scarring and vision loss are possible consequences. Bacterial corneal ulcer is a therapeutic challenge and vision threatening serious ocular condition.

Bacterial Corneal infection can affect people of all ages. The age groups with a higher prevalence of disease are likely tied to risk factors, in the first group (<40 years) who are more likely to be contact lenses wearers and/or sustain ocular trauma, and those in the second group (>50 years) who are more likely to undergo eye surgery. In this study, majority of the patients (49%) were in 3rd and 4th decade which is the crucial stage of the life. In a study by Panda et al.,<sup>27</sup> performed on thousand eyes of thousand patients, 50% of the patients with corneal ulcer were aged between 36 and 65 years. The present study showed similar age distribution.

In present study, the incidence of bacterial keratitis was higher in males (54%) than in females (46%) with Male to Female Ratio 1.18:1. This ratio was very close to the ratio 1.6:1 of previous study reported by Srinivasan et al., in 1997,<sup>28</sup> Maske et al., 1986<sup>29</sup> and 1.9:1 by Narsani et al., 2009.<sup>30</sup> The outcome of the study according to male female distribution was found in male due to predisposing factors like traumatic condition and use of contact lens.

Earlier several bacteria viz. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* were isolated and identified in the cases of corneal ulcers.<sup>41</sup> *Exiguobacterium aurantiacum* was not reported earlier from the cases of corneal ulcers as per

the literature. In present studies, same organism was isolated from the patients associated with the cases of corneal ulcers with the isolation rate of 02.51%.

The genus *Exiguobacterium* was first described in 1983 by Collins *et al.*, who characterized the species *E. aurantiacum*. It is facultative anaerobic and varied in shape from rods to almost coccoid forms. In some respects it resembled certain coryneform bacteria but its true taxonomic position was not determined. *Exiguobacterium aurantiacum* founds from potato-processing effluent.<sup>31</sup>

*Exiguobacterium* spp. are alkaliphilic, halotolerant, non-spore-forming Gram-positive bacilli, hitherto uncharacterised from human infections. Six isolates of *Exiguobacterium aurantiacum* were obtained from patients with bacteraemia.<sup>32</sup>

In present study, the 16S rRNA sequencing of the isolates (PBCUUn05) confirmed the presence of *Exiguobacterium aurantiacum*, which is a new bacterium for corneal ulcers.

Shatila *et al.*,<sup>33</sup> have characterized *Exiguobacterium aurantiacum* but its 16S rRNA sequence was made up of 1500 base pairs. These estimates were slightly different from present study.

In present study comparison of different classes of antibiotics resistance was done by calculating % antibiotics resistance pattern. *E. aurantiacum* showed higher resistance to Aminoglycoside followed by Quinolones and Cephalosporin and higher sensitive to Cephazolin+ Tobramycin combinations.

Microbial pigments have numerous beneficial properties. On the other hand, Pigment production may increase virulence in bacterial pathogens. These pigments have been shown to increase resistance to oxidative stress, killing by immune cells, and mutagenesis. Pigments seem to increase virulence of pathogens by increasing survival in immune cells, invasiveness and size of local abscesses.<sup>34,35</sup>

In present study, *Exiguobacterium aurantiacum* was found to be Novel Bacteria and not reported earlier for the corneal ulcers were subjected to virulence study, which was done by testing pigment extraction. As per the literature, no researcher has reported about this bacterium from the cases of corneal ulcers.

Production of pigments appeared to be more significantly associated with multi-drug resistance, presence of virulence-associated genes, and expression of certain virulence factors, most notably elastase, protease, siderophore and DNase activity. Since pigment production is easy to determine, this might to be a good starting point to identify the virulence status of an isolate.<sup>36</sup>

The FTIR analysis of methanolic extracts of *Exiguobacterium aurantiacum* confirmed that *Exiguobacterium aurantiacum* produced Carotenoid. Carotenoid may have evolved to reinforce bacterial membranes and when it inserted into a membrane, conjugated double bonds provide rigidity, serve to

stabilize it. Carotenoids are serving mechanical functions into pathogenic bacteria.

Several researchers have reported that some pigmented bacteria, which are rich in carotenoids, have been resistant to radiation when subjected to sub-lethal and lethal doses of ionizing radiations, due to the accumulation of the radio-protective pigments in the outer membrane.<sup>37,38</sup>

Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to bacteria against various innate and adaptive immune defence mechanisms as well as antibiotics.

The present study showed that Carotenoid pigments have a potent nitric oxide scavenging activity. Carotenoid pigments showed a very good concentration-dependent inhibition of nitric oxide. These pigments showed virulent factor with an antioxidant action because these pigments neutralize the oxidants. These carotenoid pigments may be responsible for virulence of *Exiguobacterium aurantiacum* associated with the cases of corneal ulcers. Bacteria pigments protect themselves from oxidative immune attack.

In present study, with respect to Carotenoid pigment, the higher radical scavenging activity was found with 100 µg/ml concentrations with 73.37 values. Result of current study showed that, as the concentration of pigments increases, % inhibition increases with fall in absorbance. According to Balraj *et al.*, [39], radical scavenging activity increases with concentrations of carotenoid pigment.

In present study, Data analysis was performed by using SPSS software (Statistical Package for the Social Sciences). By using ANOVA (Analysis of variance) test, the levels of significance were determined using the number of independent variables in one way and two way.

To show there is significance difference among antibiotic resistance of different organism we have used two way anova, the p value was 0.005 which was less than 0.01 which shows that different antibiotics having different resistance on different organisms. Comparing the resistance of the drug except the Meropenem & Imipenem having the lesser resistance than the other drugs

In present study we checked the association between the Severity of the Bacterial Ulcers and number of organisms. The p value was found 0.115 which was greater than 0.05 which shows that there is no association between severity and number of organism. So it revealed that the severity is due to type of bacteria.

## Conclusion

The corneal ulcer is an ocular emergency that raises high-stakes questions about diagnosis and management. Rapid bacterial isolation and treatment with intensive ocular antibiotics represent decisive steps

in the management of such pathologies. Fluoroquinolones appeared to be the therapy of choice for bacterial keratitis but based upon these *in vitro* studies, some strains were resistant. Proper diagnosis and antibiotic selection requires for successful treatment of a corneal ulcer. For certain cases of bacterial keratitis fluoroquinolone antibiotics may be appropriate as empirical monotherapy treatment. As per the present studies observations, these days antibiotic resistance has been increased therefore combination therapies are suggested for the initial treatment of corneal ulcers. In present study, as per the literature referred, the bacteria *Exiguobacterium aurantiacum* was found to be Novel Bacteria which was not reported earlier for the cases of corneal ulcers and produced BAD colour.

**Accession Number of Nucleotide Sequence:** The complete genome sequence of *Exiguobacterium aurantiacum* deposited at the NCBI GenBank sequence database and got accessed as LC217383.

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