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PHYLOGENETIC ANALYSIS OF 16SrDNA AND 18SrDNA SEQUENCES OF BACTERIA AND FUNGI FROM KERATITIS PATIENTS

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ABSTRACT

Keratitis is a condition in which the eye's cornea, the front part of the eye, becomes inflamed. It is caused by Bacteria, Viruses, Fungi and Parasites. This study investigate that the evaluation the microbial diagnosis and Phylogenetic analysis of 16SrDNA and 18SrDNA sequences of bacteria and fungi from infectious eye sample of keratitis patients. In this study 250 consecutive outpatients 143 males and 107 females were with corneal ulcerations were examined in the Eye Clinic of the General Hospital of Tiruchirappalli. Patients were examined with a slit lamp biomicroscope. The Bacteria and fungus were isolated in eye swabs of keratitis patients. The bacterial and fungal genes were sequenced by using 16S rDNA and 18S rDNA- PCR. These sequences were identified using BLASTn (Basic Local Alignment Search Tool nucleotide) and deposited in the GenBank (NCBI) with the accession numbers *Staphylococcus aureus* (JN378392), *Staphylococcus epidermidis* (HQ404365), *Micrococcus sp.*(HM20450), *Streptococcus viridians*(JN378393), *Moraxella sp.*(JQ039348), *Propionibacterium acnes* (JQ039349), *Acinetobacter baumanii* (JN652129), *Citrobacter koseri* (JN652127), *Pseudomonas aeruginosa*(JQ039350), *Proteus mirabilis* (FJ655896), *Klebsiella sp.*(JN652128) , *Fusarium sp.*(JQ412816), *Aspergillus sp.*(JX204747), *Sebipora aquosa* (submitted) and *Byssochlamys nivea* (Submitted). The Sequences were analysed and aligned by Clustal W. It concluded that the result may be useful in characterizing the micro evolutionary mechanisms of the species for researchers.

KEYWORDS: keratitis disease, CLUSTALW, Blastn, Bacteria.

INTRODUCTION

Microbial keratitis is a major cause of monocular blindness in developing countries. This represents only a small fraction of millions who suffer visual loss as a result of corneal trauma and subsequent microbial keratitis in the world. [1] Keratitis is an inflammation of the cornea, the transparent membrane that covers the colored part of the eye (iris) and pupil of the eye. Keratitis caused by Bacteria, Viruses, Fungi and Parasites. Keratitis is usually diagnosed based on the clinical symptoms of pain and/or redness in the eye along with close examination of the cornea by an eye specialist. Keratitis is a common infectious disease of the cornea[2] that is potentially blinding.[3] Common risk factors for keratitis reported by recent studies in Australia are contact lens wear, ocular surface disease, ocular trauma, and ocular surgery.[4,5] Common causative organisms associated with these case series are Pseudomonas aeruginosa, Staphylococcus aureus, coagulase-negative staphylococci, and Streptococcus pneumoniae.[6,7,8]

Bacterial keratitis accounts for approximately 65% to 90% of all microbial corneal infections. Although these bacteria may vary in incidence according to geographical locale, the most common organisms include *Staphylococcus aureus*, *S. epidermis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and other Gram negative bacilli. Of these, *S. aureus* is the predominant pathogen isolated from the majority of cases of keratitis. [10,11,12,13] but *P. aeruginosa*, a potentially devastating ocular pathogen, is the most common cause of hypopyon corneal ulcers, ulcerative keratitis associated with contact lens wear,[14] and severe necrotic corneal ulceration.[15] The precise role of endogenous bacteria has not been ascertained, although some organisms considered normal flora of the eye can devastate the avascular cornea.

It is important to consider fungus as a possible cause of infectious keratitis because of the devastating ocular damage it can produce if it is not diagnosed and treated promptly and effectively. Unfortunately, delayed diagnosis is common, primarily because of lack of suspicion and even if the diagnosis is made accurately; management remains a challenge because of the poor corneal penetration and limited commercial availability of antifungal drugs. [16]

In this study reports on isolate the common bacterial and fungal causative agents using 16srRNA and 18srRNA type from keratitis patients in eye Clinic of the General Hospital of Tiruchirappalli Tamil Nadu. These sequences were identified by using Blastn and

comparative phylogenetic analysis of the bacterial and fungal sequences were performed by using clustal W.

MATERIALS AND METHODS

Sample collection

In this study 250 consecutive outpatients 143 males and 107 females were with corneal ulcerations were examined in the Eye Clinic of the General Hospital of Tiruchirappalli from January 2009 to February 2012. All patients were examined under a slit-lamp biomicroscope by an ophthalmologist. Corneal scrapings were collected after instillation of 4% lignocaine without preservative under aseptic conditions from each ulcer by an ophthalmologist using a sterile Bard Parker blade (No 15). [17] Subsequent scrapings were spread onto labelled slides in a thin, even manner for 10 % potassium hydroxide (KOH) wet mount and Gram staining.

Isolation of bacteria and fungi from sample

Cultures on blood agar and chocolate agar were evaluated at 24 hours and at 48 hours and then discarded if no growth was seen. All media's were incubated at 35°C (±1) Sabouraud Dextrose Agar (SDA), which are incubated at 27°C (±1) in BOD incubator. Cultures inoculated in Brain heart infusion (BHI) broth were examined for turbidity in similar fashion which was subsequently subcultured, and Gram stained for identification. The criteria described by Bharathi *et al*., [17] were used for determining culture positive samples. [18] The specific identification of bacterial pathogens was based on microscopic morphology, staining characteristics[19,20,21], and biochemical [22] using standard laboratory criteria.

Fungi were identified by their colony characteristics on SDA and by their microscopic appearance in Lactophenol cotton blue. Fungal cultures from cases with smears negative for fungi were discarded as possible contaminants, although Chin *et al.*, (1975) pointed out that negative smears and cultures do not necessarily rule out fungal infection. [23]

PCR and DNA sequencing

To identify the culture based on 16s rDNA and 18s rDNA sequence data. Genomic DNA was isolated from sample and loaded on 1% agarose gel. PCR amplified was performed using 16s rDNA and 18s rDNA primers. The PCR product was gel eluted. The purified PCR product was sequenced using 4 primers. The conditions used for PCR Amplification DNA: 16s Forward Primer 400ng, 5'-AGAGTRTGATCMTYGCTWAC-3', 16s Reverse Primer: 400ng 5'-CGYTAMCTTWTTACGRCT-3', and 18s forward primer 400ng 5'-

TCCGTAGGTGAACCTGCGG-3' 18s reverse primer 400ng 5'-TCCTCCGCTTATTGATATGC-3' dNTPs (2.5mM each) 4 , 10X Taq DNA Polymerase Assay Buffer 10, Taq DNA Polymerase Enzyme (3U/ l) 1, Water X Total reaction volume: 100, the two universal primers and 1.5mM MgCl2. Amplification was done by initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 second, annealing temperature of primers was 55°C for 30 second and extension at 72°C for 2 minute. Final extension was at 72°C for 15 minutes All PCR reagents were of Chromous Make (Chromus Biotech, Chennai).

The PCR product were detected by using 1% agarose gel containing Ethidium bromide and the result recorded by UV transilluminator. Finally, the target PCR product was sliced and recovered by Chromous Make (Chromus Biotech, Chennai).

Sequence analysis

To run, BLAST requires a query sequence to search for, and a sequence to search against (also called the target sequence) or a sequence database containing multiple such sequences. BLAST will find subsequences in the database which are similar to subsequences in the query. In typical usage, the query sequence is much smaller than the database, e.g., the query may be one thousand nucleotides while the database is several billion nucleotides. The sequences were deposited in Genbank with accession numbers.
(http://en.wikipedia.org/wiki/BLAST).

Sequence analysis

CLUSTALW is a widely used multiple sequence alignment computer program. Select Load Sequences from the File menu in the Clustal W window. A new window will appear that displays the user's subdirectories and files. Select a file containing the unaligned sequences. Use the mouse cursor to highlight the filename in the file selection window, and then click the OK button at the bottom of the window. If the selected file contains more than one sequence and these are in one of the seven recognized file formats, then the unaligned sequences will be displayed in the Clustal W window. Sequences were analyzed and aligned with other sequences deposited in Genbank by ClustalW. [24] The parameters in study were used in default.

RESULTS AND DISCUSSION

Isolation and characterization bacterial of strains: The results observed previously, bacterial colonies can differ greatly in their morphologies. These differences can help us in identifying different species of bacteria. The bacterial culture containing *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus sp.*(HM20450), *Streptococcus viridians*, *Moraxella sp.*, *Propionibacterium acnes*, *Acinetobacter baumanii*, *Citrobacter koseri*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Klebsiella sp* were isolated from patients and identified by using biochemical tests. It shows that it was cocci shaped and Gram positive and Gram negative bacteria and it has glucose, lactose, sucrose fermentations (acid & gas), catalase enzyme activity (degradation of hydrogen peroxide), non-acidic in nature.

Corneal scraping with Lactophenol cotton blue staining showed fungal elements in keratitis patients. *Fusarium sp. Aspergillus sp. Sebipora aquosa, and Byssochlamys nivea*, were isolated. The results for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of KOH preparation, Gram stain, and culture for identification of fungi.

DNA sequencing

16S rDNA and 18S rDNA gene of the bacterial and fungal strains were conducted BLAST searches against GenBank. Most strains showed high sequence identities with the same species on GenBank, and some strains showed high identities with other species. This analysis made the identification of reference strains more reliable. The Bacterial and fungal genes were sequenced and it deposited in the GenBank (NCBI) with the accession numbers *Staphylococcus aureus* (JN378392), *Staphylococcus epidermidis (*HQ404365), *Micrococcus sp.*(HM20450), *Streptococcus viridians*(JN378393), *Moraxella sp.(*JQ039348), *Propionibacterium acnes* (JQ039349), *Acinetobacter baumanii (*JN652129), *Citrobacter koseri* (JN652127), *Pseudomonas aeruginosa*(JQ039350), *Proteus mirabilis* (FJ655896), *Klebsiella sp.*(JN652128) , *Fusarium sp.*(JQ412816), *Aspergillus sp.*(JX204747), *Sebipora aquosa* (submitted) and *Byssochlamys nivea* (Submitted) are shown in table- 1.

Sequence analyses and phylogenetic tree constructing

The 16S rDNA and 18s rDNA gene sequences were obtained from BLAST hits, using multiple sequence alignment programme ClustalW. A phylogenetic tree was drawn, using neighbor-joining method for this alignment by using ClustalW (http://www.ebi.ac.uk/Tools/clustalW). Because the similarity for most of the sequences with

those of known rumen bacteria and fungus were identify the sequence as representing a particular taxon, a phylogenetic tree was constructed to investigate the taxonomic placement.

Sequence alignment was achieved using multiple sequence alignment software CLUSTAL W Version 1.81. [25] Fig. 1 shows that CLUSTALW sequence alignment of the bacterial and fungal genes of keratitis patients. The phylogenetic tree was constructed by the neighbour-joining method [26] and the tree was evaluated using the bootstrap test [27] are shown in fig.3. It represents that Neighbour-joining method of phylogenetic tree for progressive alignment with distances scores using CLUSTALW. Here, the fifteen sequences and the order in which they will be aligned according to a neighbor-joining tree.

Table 1. Bacteria and fungus were isolated from keratitis patients and deposited in Genbank with accession number

Microorganisms	Amplicon	Accession number
Bacteria		
Staphylococcus aureus	1355	JN378392
Staphylococcus epidermidis	1472	HQ404365
Micrococcus sp	1396	HM20450
Streptococcus viridians	1184	JN378393
Moraxella sp.	1445	JQ039348
Propionibacterium acnes	699	JQ039349
Acinetobacter baumanii	251	JN652129
Citrobacter koseri	1497	JN652127
Pseudomonas aeruginosa	702	JQ039350
Proteus mirabilis	1268	FJ655896
Klebsiella sp.	649	JN652128
Fungus		
Fusarium sp.	436	JQ412816
Aspergillus sp.	918	JX204747
Sebipora aquosa	459	Submitted
Byssochlamys nivea	332	Submitted

Although, all bacteria have shared the more-or-less uniform phenotypic being acidophilic, obligately chemolithoautotrophic, Gram-negative rods for energy generation, they do exhibit considerable genetic variation. In the past two decades, some work was done to investigate these genotypic variations. [28,29,30] Recently, using RAPD techniques to assess intrespecific variability, similarity coefficients between various isolates which were obtained from different mine ores in China ranged from 44% to 83%. [31] Our results showed that the some similar sequences in bacteria when compared with fungal sequences in phylogentic groups.

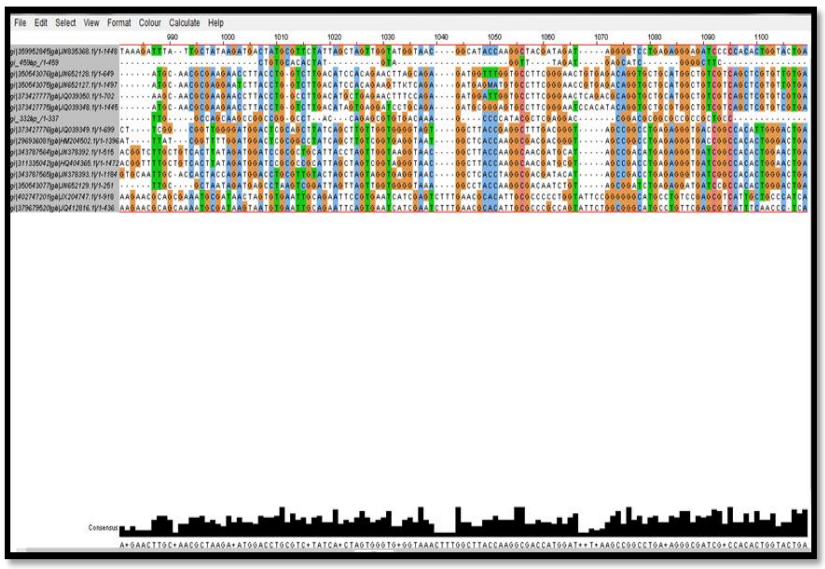

Fig. 1: CLUSTALW sequence alignment of the bacterial and fungal genes of keratitis patients

In general, the 16S rDNA and 18S rDNA sequences can be used as a molecular marker to distinguish the subspecies. In this study, we identified 11 common bacteria of 16S rDNA and 4 common fungus of the 18S rDNA sequences from keratitis patients.

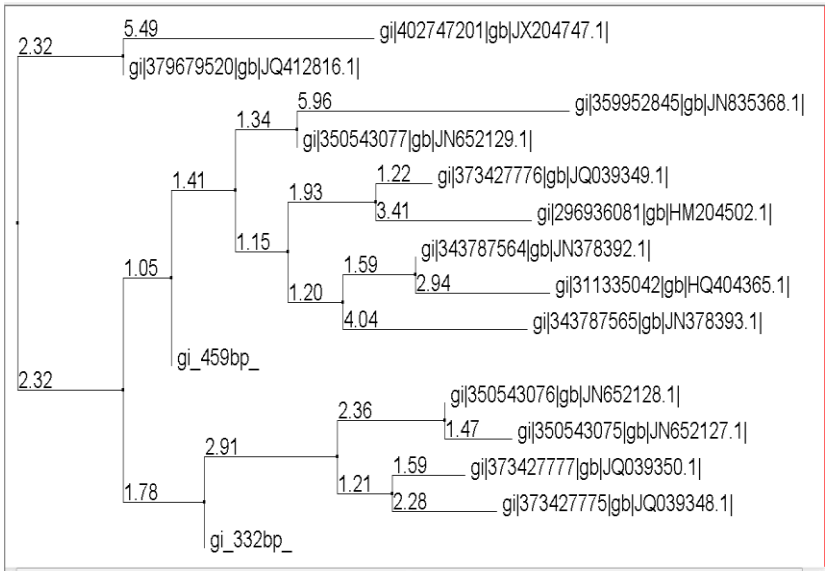

Fig. 3: Neighbour-joining method of phylogenetic tree for progressive alignment with distances scores using CLUSTALW. Here we show the fifteen sequences and the order in which they will be aligned according to a neighbor-joining tree.

CONCLUSION

In conclusion, identification and sequencing of this important gene is the first step to pursue future research such as developing better therapeutic agents, immunization of population at

risk or even developing a rapid diagnostic tool by PCR techniques. This technique provides the opportunity to identify common, or even unknown causative bacteria. phylogenetic analysis between 11 bacteria of 16S rDNA and 4 fungus of the 18S rDNA sequences in keratitis patients by using software CLUSTALW align all nucleotide sequences in a line and show maximum parsimony to minimum parsimony species to species. Phyllogram show evolutionary relationship between all species by comparing them and concluded that less uniform phenotypic variations.

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