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In silico Structural Analysis of 16S rDNA Sequences of Bacteria Isolated from Keratitis Patients

[1]Anusha Bhaskar and [2]V. Nithya
[1]PRIST University, Vallam, Thanjavur 613 403, India
[2]Srimad Andavan College, Trichy, India

Corresponding Author: Anusha Bhaskar, PRIST University, Vallam, Thanjavur 613 403, India

ABSTRACT

Keratitis is a condition in which the cornea of the eye becomes inflamed. It is caused by bacteria, viruses, fungi and parasites. The diagnosis of the causative organism of keratitis remains a problem due to lack of advanced techniques. In this study PCR and sequence analysis of 16S rDNA was used to ascertain the bacteria isolated in eye swabs of keratitis patients. The bacterial genes were sequenced and deposited in the GenBank (NCBI) with the accession numbers JN378393, JN378392, HM204502, JQ039348, JN652127, JN652129, JQ039350, JN652128, JQ039349 and HQ404365 for *Streptococcus viridan*, *Staphylococcus aureus*, *Micrococcus* sp., *Moraxella* sp., *Citrobacter koseri*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Propionibacterium* sp. and *Staphylococcus epidermidis,* respectively. Sequences were analyzed and aligned by ClustalX and a phylogenetic relationship was studied. The secondary structure of the 16S DNA was developed using GeneBee which produces secondary structures prediction through the minimization of the potential energy of the system. These results may be useful in characterizing the micro evolutionary mechanisms of the species for researchers.

Key words: 16S rDNA, keratitis, GeneBee, phylogenetic analysis

INTRODUCTION

Microbial keratitis is a leading cause of visual loss in developing countries. It is normally predisposed by ocular trauma and/or contact lens wear. Corneal scarring listed second only to cataracts as an important cause of blindness and visual impairment in many developing countries in Asia, Africa and the Middle East (Whitcher and Srinivasan, 1997). Bacterial keratitis is the most common form of suppurative corneal ulceration. A number of organisms are able to cause infection (Dunlop *et al*., 1994; Upadhyay *et al*., 1991; Wilhelmus, 1996) and analyses of them are essential to understand how they set up the infection.

Approximately 65-90% of all corneal infections is bacterial keratitis (Asbell and Stenson, 1982; McDonnell and Green, 1990; Jones, 1981; Laibson, 1972; Liesegang and Forster, 1980). Although, these bacteria may vary in incidence according to geographical locale (Baum, 1979) the most common organisms include *Staphylococcus aureus*, *Streptococcus epidermis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and other Gram negative bacilli (Morlet and Daniell, 2003; Jones, 1979; Baum, 1978). Of these, *S. aureus* is the predominant pathogen isolated from the majority of cases of keratitis, (Morlet and Daniell, 2003; Baum, 1978) but *P. aeruginosa*, a potentially devastating ocular pathogen, is the most common cause of hypopyon corneal ulcers, (Hyndiuk, 1981) ulcerative keratitis associated with contact lens wear and severe necrotic corneal ulceration (Laibson, 1972).

In this study, 16S rDNA sequences of bacteria isolated from infectious keratitis in eye. Clinic of the General Hospital of Tiruchirappalli Tamil Nadu were sequenced. A comparative phylogenetic analysis of the 16S rDNA sequences were performed by using ClustalX and the phylogenetic tree was constructed using NJ method. The secondary structure of the 16S rDNA was constructed and minimal free energy was calculated by Genebee tool. The ribosomal RNA mainly 16S r DNA has proven to be a stable and specific molecular marker of the identification of bacteria. The 16S rDNA is a common target for the taxonomical purpose, mainly due to the mosaic composition of phylogenetically conserved and variable regions.

MATERIALS AND METHODS

Sample collection: In this study, bacteria were isolated from keratitis patients in Eye Clinic of the General Hospital of Tiruchirappalli Tamil Nadu during 2010-2012 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). Scrapings were performed under magnification of slit-lamp or operating microscope. Leading edge and base of each ulcer were scraped initially and the material obtained were directly inoculated onto the surface of solid media such as sheep blood agar, chocolate agar and Sabouraud Dextrose Agar (SDA) in a row of C-shaped streaks and also deep inoculation in the liquid media such as Brain Heart Infusion (BHI) broth without gentamicin sulphate and thioglycollate medium. Subsequent scrapings were spread onto labelled slides in a thin, even manner for 10% potassium hydroxide (KOH) wet mount and Gram staining.

Culture: All bacterial cultures were incubated aerobically. 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) except SDA which are incubated at 27°C (±1) in BOD incubator. Cultures inoculated in BHI broth were examined for turbidity in similar fashion which was subsequently subcultured and Gram stained for identification. However, liquid media were prone to contamination and were not used for interpretation in isolation. The criteria described by Bharathi *et al.* (2002) were used for determining culture positive samples.

The specific identification of bacterial pathogens was based on microscopic morphology, staining characteristics and biochemical properties using standard laboratory criteria.

PCR and DNA sequencing: Genomic DNA was isolated from sample and loaded on 1% agarose gel. PCR amplified was performed using 16s rDNA primers (PCR amplification gel Photo attached). The PCR product was gel eluted. The purified PCR product was sequenced using 2 primers. The conditions used for PCR amplification of DNA: 1, 16S Forward Primer 400 ng, 16S Reverse Primer 400 ng, dNTPs (2.5 mM each) 4, 10X Taq DNA Polymerase Assay Buffer 10, Taq DNA Polymerase Enzyme (3 U L^{-1}) 1, water X total reaction volume: 100, the two universal primers and 1.5 mM $MgCl_2$. Amplification was done by initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing temperature of primers was 55°C for 30 sec and extension at 72°C for 2 min. Final extension was at 72°C for 15 min All PCR reagents were of Chromous Biotech, Ltd.

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. After purification, the PCR product was submitted for sequencing. Sequences have been deposited in GenBank.

Sequence analysis: Sequences were analyzed and aligned with other sequences deposited in Genbank by ClustalX (Thompson *et al.*, 1997). The secondary structure and minimal free energy were calculated by Genebee tool (Mathews *et al.*, 1999).

RESULTS AND DISCUSSION

Isolation and characterization bacterial of strains: The bacterial colonies differ greatly in their morphologies and these differences can help us in identifying different species of bacteria. The culture containing *Streptococcus viridans*, *Staphylococcus aureus*, *Micrococcus* sp., *Moraxella* sp., *Citrobacter koseri*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Propionibacterium* sp., *Staphylococcus epidermidis* bacteria were and 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 and gas), catalase enzyme activity (degradation of hydrogen peroxide) and non-acidic in nature.

Molecular characterization of bacteria: Molecular characterization of the bacterial strains was carried out by sequencing their 16S rDNA after its amplification and sequencing. 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 gene was sequenced and it deposited in the GenBank (NCBI) with the accession numbers JN378393, JN378392, HM204502, JQ039348, JN652127, JN652129, JQ039350, JN652128, JQ039349, HQ404365 for *Streptococcus viridan*, *Staphylococcus aureus*, *Micrococcus* sp., *Moraxella* sp., *Citrobacter koseri*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Propionibacterium* sp., *Staphylococcus epidermidis*, respectively are shown in Table 1.

Sequence analyses and phylogenetic tree constructing: The 16S rRNA gene sequences of the bacterial strains studied by us were subject to multiple sequence alignment using ClustalX and the phylogenetic tree was drawn using Neighbour Joining plot. Multiple sequence alignment (Fig. 1) confirmed sequence conservation of the highly conserved regions of 16S rDNA of the causative bacteria of keratitis. The phylogenetic analysis of these genes from the phylogenetic tree (Fig. 2) shows that these genes are closely related.

Table 1: Bacteria isolated from keratitis patients

Microorganism	Accession No.
Gram-negative bacteria	
Pseudomonas aeruginosa	JQ039350
Acinetobacter baumanii	JN652129
Klebsiella sp.	JN652128
Propionibacterium sp.	JQ039349
Citrobacter koseri	JN652127
Gram-positive bacteria	
Staphylococcus aureus	JN378392
Staphylococcus epidermidis	HQ404365
Micrococcus sp.	HM204502
Streptococcus viridians	JN378393
Moraxella sp.	JQ039348

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JN652127    ---------CCTGGCTCAGATTGAGGTGTGGCGGCAGGCCTAACACATGCAAGTCGAACG 51
JN652128    ------------------------------------------------------------
JN378392    ------------------------------------------------------------
HQ404365    ----------------AGGATGAACACTGGCGGCGTGCCTAATACATGCAGGTCGAGCG 43
JQ039348    --------------------------CTGGCGGCAGGCTTAACACATGCAAGTCGAACG 33
JN652129    ------------------------------------------------------------
JQ039350    ------------------------------------------------------------
HM204502    ------------------------------------------------------------A 1
JQ039349    ----------------------------CGGCGTGCTTAACACATGCAAGTCGAACG 29
JN378393    GAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACG 60

JN652127    GTAACAG---GAAGCAGCTTGCTGCTTTGCT---GACGAGTGGCGGACGGGTGAGTAATG 105
JN652128    ------------------------------------------------------------
JN378392    -ACAG----ACGAGAAGCTTGCTTCTCTG----ATGTTANCGGCGGACCGGTGAGTAACA 51
HQ404365    AACAG----ACGAGGAGCTTGCTCCTCTG----ACGTTAGCGGCGGACGGGTGAGTAACA 95
JQ039348    ATGAA------GTCTAGCCTGCT--AGACG----GATTAGTAGCGAACGGGTGAGTAATG 81
JN652129    ----------------------------------------CGAAAGGG--------- 8
JQ039350    ------------------------------------------------------------
HM204502    GAGAA------GCCCAGCTTGCTGCGGGTG----GATTAGTGGCGAACGGGTGAGTAACA 51
JQ039349    GAAAG------GCCCTGCTTTTGTGGGGTG----CTCGAGTGGCGAGCGGGTGAGTGACA 79
JN378393    CACAGTTTATACCGTAGCTTGCTACACCATAGACTGTGAGTTGCGAACGGGTGAGTAACG 120

JN652127    TCTGGG-AAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATA 164
JN652128    ------------------------------------------------------------
JN378392    CGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGTAACCGGAGCTAATACCGGATA 111
HQ404365    CGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATA 155
JQ039348    C-TTAGGAATCTGCCTATTAGTGGGGGATAACGTAGGGAAACTCACGCTAATACCGCATA 140
JN652129    ------------------------------------------ATGCTAATACCGCATA 24
JQ039350    ------------------------------------------------------------
HM204502    CGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATA 111
JQ039349    CGTGAGTAACCTGCCCTTGACTTTGGGATAACTTCAGGAAACTGGCGCTAATACCGGATA 139
JN378393    CGTAGGTAACCTGCCTGGTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATA 180

JN652127    ACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTT--------GCCATCAGATGTGC 216
JN652128    ------------------------------------------------------------
JN378392    ATATTTTGAACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGAT 171
HQ404365    ATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTCACTTATAGATGGAT 215
JQ039348    CGTCTTACGAGAGAAA---GGGGGCCT-TTAGCTCT---------CGCTAATAGATGAGC 187
JN652129    CGTCCTACGGGAGAAAGCAGGGGATCT-CCAAACCTTG-------CGCTAATAGATGAGC 76
JQ039350    ------------------------------------------------------------
HM204502    GGAGCGTCCACCGCATGGTGGGTGTTGGAAAGATTTAT-------CGGTTTTGGATGGAC 164
JQ039349    GGAGCTTCTGCTGCATGGTGGGGGTTGGAAAGCTTCGG-------CGGTTGGGGATGGAC 192
JN378393    ATATTAATTATTGCATGATAATTAATTGAAAGGTGCAATTGCA-CCACTACCAGATGGAC 239
JN652127    CCAGATGGGATTAGCTNGTTGGTGGGGTAACGACTCACCAAGGCGACGATCCCTAGCTGG 276
JN652128    ------------------------------------------------------------
JN378392    CCGCGCTGCATTACCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGA 231
HQ404365    CCGCGCCGCATTAGCTAGTCGGTAGGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGA 275
JQ039348    CTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTAG 247
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Fig. 1: Continue

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JN652129    CTAAGTCGGATTAGTTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACAATCTGTAGCGGA 136
JQ039350    ------------------------------------------------------------
HM204502     TCGCGGCCTATCAGCTTGTCGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGG 224
JQ039349    TCGCAGCTTATCAGCTTGTTGGTGGGGTAGTGGCTTACCGAGGCTTTGACGGGTAGCCGG 252
JN378393    CTGCGTTGTACTAGCTAGTAGGTGAGGTAACGGCTCACCTAGGCGACGATACATAGCCGA 299


JN652127    TCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA 336
JN652128    ------------------------------------------------------------
JN378392    CATGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCT 291
HQ404365     CCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA 335
JQ039348    TCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA 307
JN652129    TCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA 196
JQ039350    ------------------------------------------------------------
HM204502     CCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA 284
JQ039349    CCTGAGAGGGTGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCA 312
JN378393    CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA 359


JN652127    GCAGTGGGGAATATTGCA-CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAA 395
JN652128    ------------------------------------------------------------
JN378392    GCAGTAGGGAATCTTCTTGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAT 351
HQ404365     GCAGTAGGGAATCTTCCG-CAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAT 394
JQ039348    GCAGTGGGGAATATTGGA-CAATGGGCGAAAGCCTGATTCAGCCATGCCGCGTGTGTGAA 366
JN652129    GCAGTGGGGAATATTGGA-CAATGGGGGGAGCCCTGATCCAGCCATGCCGC--------A 247
JQ039350    ------------------------------------------------------------
HM204502     GCAGTGGGGAATATTGCA-CAATGGGCGCAGGCCTGATGCAGCGACGCCGCGTGAGGGAT 343
JQ039349    GCAGTGGGGAATATTGCA-CAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGAT 371
JN378393    GCAGTAGGGAATCTTCGG-CAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAA 418


JN652127    GAAGG-CCTTCGGGTTGTAAA--GTACTTTCAGCGGGGAGGAAG---GTGTTGTGGTTAA 449
JN652128    ------------------------------------------------------------
JN378392    GAAGGTACTTCGGATCGTAAATACTCCTGTTATTAGGGAAGAACATATGTGTAAGT-AAC 410
HQ404365     GAAGG-TCTTCGGATCGTAAA-ACT-CTGTTATTAGGGAAGAACAAATGTGTAAGT-AAC 450
JQ039348    GAAGG-CCTTTTGGTTGTAAA--GCACTTTAAGTAGGGAGGAAA---AGCTTGTGGTTAA 420
JN652129    GCGG------------------------------------------------------- 251
JQ039350    ------------------------------------------------------------
HM204502     GACGG-CCTTCGGGTTGTAAA--CCTCTTTCAGTAGGGAAGAAG---CGAAAGTG----- 392
JQ039349    GACGG-CCTTCGGGTTGTAAA--CCGCTTTCGCCTGTGACGAAG---CGTGAGTG----- 420
JN378393    GAAGG-TTTTCGGATCGTAAA-GCT-CTGTTGTTAGAGAAGAACGGGTGTTAGAGTGGAA 475


JN652127    TAACCACAGCAATTGACGTTACCCGCA-GAAGAAGCACCGGCTAACTCCGTGCCAGCAGC 508
JN652128    ------------------------------------------------------------
JN378392    TGTGCACATCCTTGTACGGTACCTACNGCAGAAAGCCACGGCTAA-------------- 455
HQ404365     TATGCACGTCTTG--ACGGTACCTAAT-CAGAAAGCTACGGCTAACTACGTGCCAGCAGC 507
JQ039348    TACCCACAAGCCCTGACGTTACCCACA-GAATAAGCACCGGCTAGCTCTGTGCCAGCAGC 479
JN652129    ------------------------------------------------------------
JQ039350    ------------------------------------------------------------
HM204502     --------------ACGGTACCTGCA-GAAGAAGCACCGGCTAACTACGTGCCAGCAGC 436
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Fig. 1: Continue

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JQ039349    ---------------ACGATAATGGGT-AAAGAAGCACCGGCTAACTACGTGCCAGCAGC 464
JN378393    AGTTCACACTGTG--ACGGTATCTTAC-CAGAAAGGGACCGCTAACTAAGTGCCAGCAGC 532


JN652127    TGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG 568
JN652128    ------------------------------------------------------------
JN378392    ------------------------------------------------------------
HQ404365    CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGG 567
JQ039348    CGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGTAGG 539
JN652129    ------------------------------------------------------------
JQ039350    ------------------------------------------------------------
HM204502    CGCGGTAATACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGG 496
JQ039349    CGCGGTGATACGTAGGGTGCGAGCGTTGTCCGGATTTATTGGGCGTAAAGGGCTCGTAGG 524
JN378393    CGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAA-CGAGCGCAGC 591


JN652127    CGGTCTGTCAAGTCAGATGTGAAATGCCCGGGCTCAACCTGGGAACTGCATCTGATACTG 628
JN652128    ------------------------------------------------------------
JN378392    ------------------------------------------------------------
HQ404365    CGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAGACTG 627
JQ039348    TGGTCATTTAAGTCAGATGTGAAAGCCCCGGGCTTAACCTGGGAACTGCATCTGATACTG 599
JN652129    ------------------------------------------------------------
JQ039350    ------------------------------------------------------------
HM204502    CGGTTTGTCGCGTCTGTCGTGAAAGTCCGGGGCTTAACCTCGGATCTGCGGTGGGTACGG 556
JQ039349    TGGTTGACCGCGTCGGAAGTGTAATCTTGGGGCTTAACCCTGAGCGTGCTTTCGATACGG 584
JN378393    CGGTTAGATAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAGTACG-CTTTGGAAACTG 650


JN652127    GCAGGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAG 688
JN652128    ------------------------------------------------------------
JN378392    ------------------------------------------------------------
HQ404365    GAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAG 687
JQ039348    GGTGACTAGAGTAGGTGAGAGGGAAGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAG 659
JN652129    ------------------------------------------------------------
JQ039350    ------------------------------------------------------------
HM204502    GCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGAT 616
JQ039349    GTTGACTCGAGGAAGGTAGGGGAGAATGGAATTCCTGGTGGAGCGGTGGAATGCGCAGAT 644
JN378393    TTTAACTTGAGTGCAGAAGGGGAGAGTG-AATTCCATGTGTAGCGGT----AGCGTAGAT 705



JN652127    ATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCG 748
JN652128    ------------------------------------------------------------
JN378392    ------------------------------------------------------------
HQ404365    ATATGGAGGAACACCAGTAGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCG 747
JQ039348    ATCTGGAGGAATACCGATGGCGAAGGCAGCTTCCTGGCATCATACTGACACTGAGGTTCG 719
JN652129    ------------------------------------------------------------
JQ039350    ------------------------------------------------------G 1
HM204502    ATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTACGGAGCG 676
JQ039349    ATCAGGAGGAACGCCAGTGGCGAAGGCGGTTCTCTGGGCCTTTCCTGAC---------- 693
JN378393    ATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCG 765
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Fig. 1: Continue

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JN652127    AAAGCGTGGGGAGCAAACAG---GATTAGATACCCTGATAGTCCACGCCGTAAACGATGT 805
JN652128    ------------------------------------------------------------
JN378392    ------------------------------------------------------------
HQ404365     AAAGCGTGGGGATCAAACAG---GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA 804
JQ039348    AAAGCGTGGGTAGCAAACAG---GATTAGATACCCTAGTAGTCCACGCCGTAAACGATGT 776
JN652129    ------------------------------------------------------------
JQ039350    AAAGCGTGGGGAGCAGACAG---GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT 58
HM204502     AAAGCATGGGGAGTGAACAG---GATTAGATACCCTGGTAGTCCGCGCCGTAAACGTTGG 733
JQ039349    ------------------------------------------------------------
JN378393    AGAGCGTGGGGAGCGAACAGGAAGGTTAGATACCCTGGTAGTCCACGCCGTAAACGATGA 825


JN652127    CGACTTGGAGGTTG-TGCCCTTGAGGCG-TGGCTTCCGGAGCTAACGCGTTAAG-TCGAC 862
JN652128    -GATTCGGAGGTTG-TGCCCTTGAGGCAGTGGCTTCCGGAGCTAACGCGTTAAAATCGAC 58
JN378392    ------------------------------------------------------------
HQ404365     GTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAG-TGCTGCAGCTAACGCATTAAG-CACTC 862
JQ039348    CTACCAGTCGTTGGGTCTCCTGAAG--ACTTAGTGACGCAGTTAACGCAATAAG-TAGAC 833
JN652129    ------------------------------------------------------------
JQ039350    CGACTAGCCGTTGG-GATCCTTGAGATCTTAGTG-GCGCAGCTAACGCGATAAG-TCGAC 115
HM204502     GCACTAGGTGTGGGGACCATTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAG-TGCCC 792
JQ039349    ---CTAGGC------------------------------------------- 699
JN378393    GTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAG-TGCCGCAGCTAACGCATTAAG-CACTC 883


JN652127    CGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAGTTGACGGGGGCCCGCACAAG 922
JN652128    CGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG 118
JN378392    ------------------------------------------------------------
HQ404365     CGCCTGGGGAGTACGACCGCAAGGTTGAAGCTCAAAGGAATTGACGGGGACCCGCACAAG 922
JQ039348    CGCCTGGGGAGTACGGCCGCAAAGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG 893
JN652129    ------------------------------------------------------------
JQ039350    TGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG 175
HM204502     CGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG 852
JQ039349    ------------------------------------------------------------
JN378393    CGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG 943


JN652127    CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAGGAATCTTACCTGGTCTTGACATCC 982
JN652128    CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCC 178
JN378392    ------------------------------------------------------------
HQ404365     CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCC 982
JQ039348    CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATAG 953
JN652129    ------------------------------------------------------------
JQ039350    CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGC 235
HM204502     CGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGT 912
JQ039349    ------------------------------------------------------------
JN378393    CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC 1003
JN652127    ACAG-AAGTTKTCAGAGATGAGMATGTGCCTTCGGGA--ACCGTGAGACAGGTGCTGCAT 1039
JN652128    ACAG-AACTTAGCAGAGATGGTTTGGTGCCTTCGGGA--ACTGTGAGACAGGTGCTGCAT 235
JN378392    ------------------------------------------------------------
HQ404365     T-CTGACCCCTCTAGAGATAGAGTTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCAT 1041
JQ039348    TGAGGATC-CTGCAGAGATGCGGGAGTGCCTTCGGGA--ATCCACATACAGGTGCTGCGT 1010
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Fig. 1: Continue

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JQ039350    TGAG-AACTTTCCAGAGATGGATTGGTGCCTTCGGGA--ACTCAGACGCAGGTGCTGCAT 292
HM204502     TCTCGATCGCCGTAGAGATACGG-TTTCCCCTTTGGG--GCGGGTTCACAGGTGGTGCAT 969
JQ039349    ------------------------------------------------------------
JN378393    CGATGCCCGCTCTAGAGATAGAGTTTT--ACTTCGGTACATCG-GTGACAGGTGGTGCAT 1060


JN652127    GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT 1099
JN652128    GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT 295
JN378392    ------------------------------------------------------------
HQ404365     GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTCAAGTCCCGCAACGAGCGCAACCCTT 1101
JQ039348    GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT 1070
JN652129    ------------------------------------------------------------
JQ039350    GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTTCCGTAACGAGCGCAACCCTT 352
HM204502     GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTC 1029
JQ039349    ------------------------------------------------------------
JN378393    GGTTGTCGTCAGCTCGTGTCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT 1120


JN652127    ATCCTTTGTTGCCAGCGGTCCG-GCCGGGAACTCAAAGGAGGCTGCCAGTGATAAACTGG 1158
JN652128    ATCCTTTGTTGCCAGCGGTTCG-GCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGG 354
JN378392    ------------------------------------------------------------
HQ404365     AAGCTTAGTTGCCA--TCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGG 1159
JQ039348    TTCCTTAGTTAACAGCACTTCG-GGTGGGAACTCTAAGGATACTGCCAGTGACAAACTGG 1129
JN652129    ------------------------------------------------------------
JQ039350    GTCCTTAGTTACCAGCACCTCG-GGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGG 411
HM204502     GTTCCATGTTGCCAGCACGTCGTGGTGGGGACTCATGGGAGACTGCCGAAGTCAACTCGG 1089
JQ039349    ------------------------------------------------------------
JN378393    ATTGTTAGTTGCCA--TCATTCAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGG 1178


JN652127    AGGAAGGTGGGGATGACGTCAAGTCGTCATGGCCCTTACGACCAGGGCTACACACGTGCT 1218
JN652128    AGGAAGGTGGAAATGACGTCAAGTCATCATGGCCCTTATGACCAGGGCTACACACGTGCT 414
JN378392    ------------------------------------------------------------
HQ404365     AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCT 1219
JQ039348    AGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGAGCTACACACGTGCT 1189
JN652129    ------------------------------------------------------------
JQ039350    AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCT 471
HM204502     AGGAAGGTGAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCT 1149
JQ039349    ------------------------------------------------------------
JN378393    AGGAAG------------------------------------------------- 1184


JN652127    ACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGT 1278
JN652128    ACAGTGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGT 474
JN378392    ------------------------------------------------------------
HQ404365     ACAATGGACAATACAAAGGGCAGCGAAACCGCGAGGTCAAGCAAATCCCATAAAGTTGTT 1279
JQ039348    ACAATGGTTGGTACAAAGGGTTGCTACACAGCGATGTGATGCTAATCTCAAAAAGCCAAT 1249
JN652129    ------------------------------------------------------------
JQ039350    ACAATAGTCAATACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGAT 531
HM204502     ACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGT 1209
JQ039349    ------------------------------------------------------------
JN378393    ------------------------------------------------------------
```

Fig. 1: Continue

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JN652127    CGTAGTCCGGATTGGAGTCTGCAGCTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTG 1338
JN652128    CGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTA 534
JN378392    ------------------------------------------------------------
HQ404365     CTCAGTTCGGATTGTAGTCTGCAACTCGACTATATGAAGCTGGAATCGCTAGTAATCGTA 1339
JQ039348    CGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCACTAGTAATCGCA 1309
JN652129    ------------------------------------------------------------
JQ039350    CGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTG 591
HM204502     CTCAGTCCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA 1269
JQ039349    ------------------------------------------------------------
JN378393    ------------------------------------------------------------


JN652127    GATCAG-AATGCCACGGTGAATACGTCCCCGGGCCTTGTACAACCG-CCCGTCACACCAT 1396
JN652128    GATCAG-AATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACGGCCCGTCACACCAT 593
JN378392    ------------------------------------------------------------
HQ404365     GATTAGCATG-CTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCAC 1398
JQ039348    GATCAG-AATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT 1368
JN652129    ------------------------------------------------------------
JQ039350    AATCAG-AATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT 650
HM204502     GATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCGGTCAAGTCAC 1329
JQ039349    ------------------------------------------------------------
JN378393    ------------------------------------------------------------


JN652127    GGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGT 1456
JN652128    GGGAGTGGGTTGCAAAAGAGGTAGGTAGCT-AACCTTCGGGAGGGCGCTTACCAAGG--- 649
JN378392    ------------------------------------------------------------
HQ404365     GAGAGTTTGTAACACCCGAGGCCGGTGGAGTAACCATTCGGAGCTAGCTGTCGAAGGTGG 1458
JQ039348    GGGAGTTGATCTCACCAGAAGTGGTTAGCCTAACGCAAGAGGGCGATCACCACGGTGGGG 1428
JN652129    ------------------------------------------------------------
JQ039350    GGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCG-CAAGGGAACGG-TACCA------ 702
HM204502     GAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCAAAGTCGGTAACACCCAAGCCGGTG 1389
JQ039349    ------------------------------------------------------------
JN378393    ------------------------------------------------------------


JN652127    GATTCATGACTGAGGTGAAGTCGTATCAAGGTAACCGTAGG 1497
JN652128    ----------------------------------------
JN378392    ----------------------------------------
HQ404365     AACAAATGATTGGG-------------------------- 1472
JQ039348    TCGATGACTGGAGTGGA----------------------- 1445
JN652129    ----------------------------------------
JQ039350    ----------------------------------------
HM204502     GCCTAAC-------------------------------- 1396
JQ039349    ----------------------------------------
JN378393    ----------------------------------------
```

Fig. 1: Multiple sequence alignment of 16S rDNA sequences of bacteria causing kertatitis

GeneBee-secondary structure prediction: In the multiple alignment the algorithm used by GeneBee produces structures through the minimization of the potential energy of the system. The algorithm divides a sequence S, into subsequences (Sij) and calculates the minimal energy for two cases. W(i, j) defines the minimal free energy of all allowed structures formed from Sij and V(i, j)

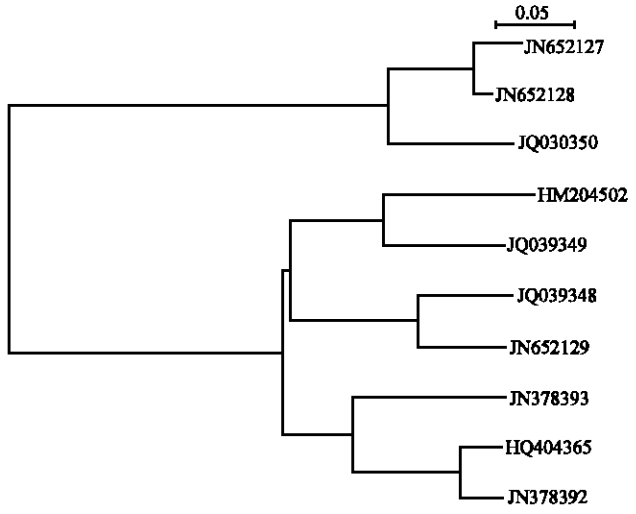

Fig. 2: Phylogenetic analysis of 16S rDNA sequences of bacteria causing keratitis

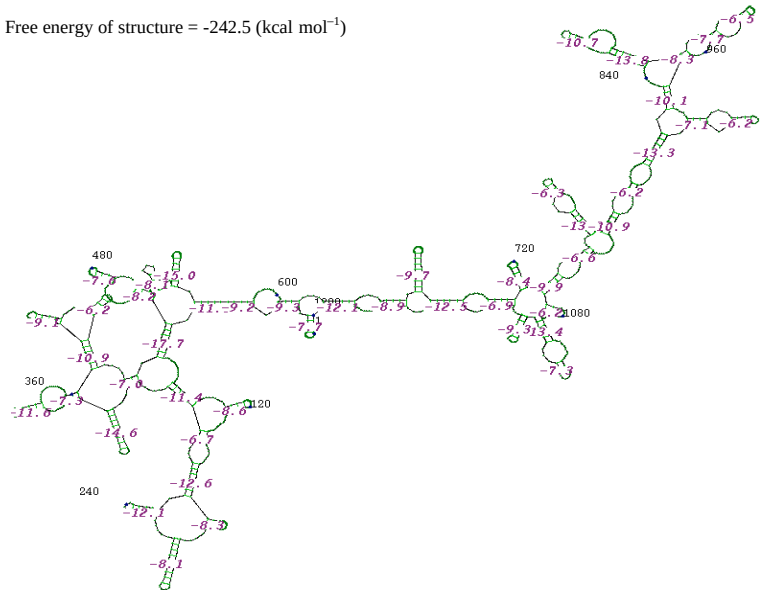

Fig. 3: Structure of *Streptococcus viridan*

the minimum energy if Si and Sj pair with each other. The second term is set to infinity if Si and Sj cannot base pair. Starting with sequences of five nucleotides, W(i, j) and V(i, j) are calculated recursively for longer and longer sequences, selecting the optimal structure at each step. The final computation considering the entire sequence is the result. W(i, j) and V(i, j) are calculated based upon the stability of base pairs formed and the resulting structures: hairpin loops, stacking regions, bulge loops, interior loops and bifurcation loops as defined by Zuker and Stiegler (1981).

The thermostabilities of the secondary structure were calculated with online software (http://www.genebee.msu.su/services/rna2_reduced.html) (Fig. 3-12). The thermostability energy was calculated as -242.5, -74.4, -307.3, -275.2, -326.3, 46.7, -161.2, -234.8, -131.7 and -293.4 kcal mol^{-1}, for *Streptococcus viridians*, *Staphylococcus aureus*, *Micrococcus* sp.,

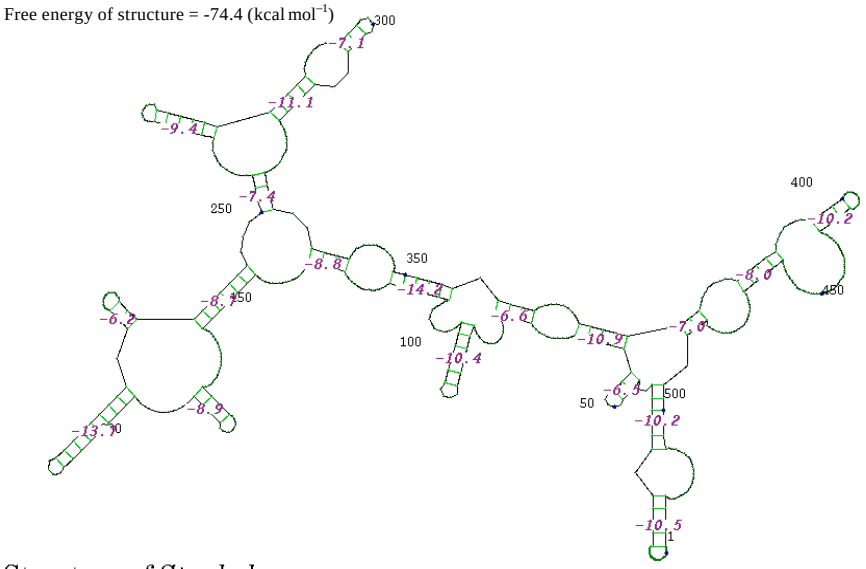

Fig. 4: Structure of *Staphylococcus aureus*

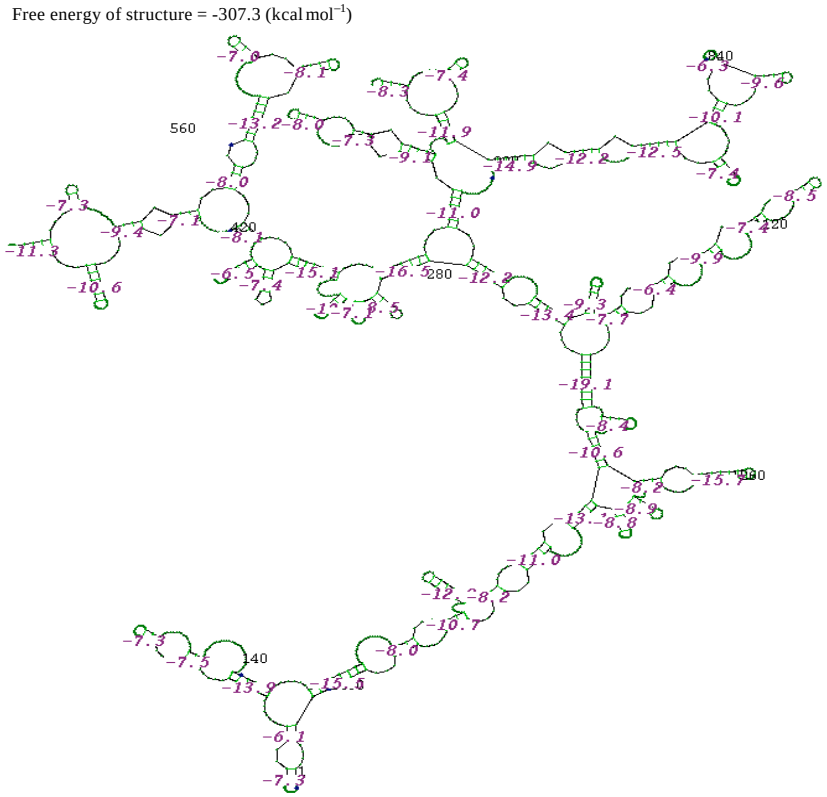

Fig. 5: Structure of *Micrococcus* sp.

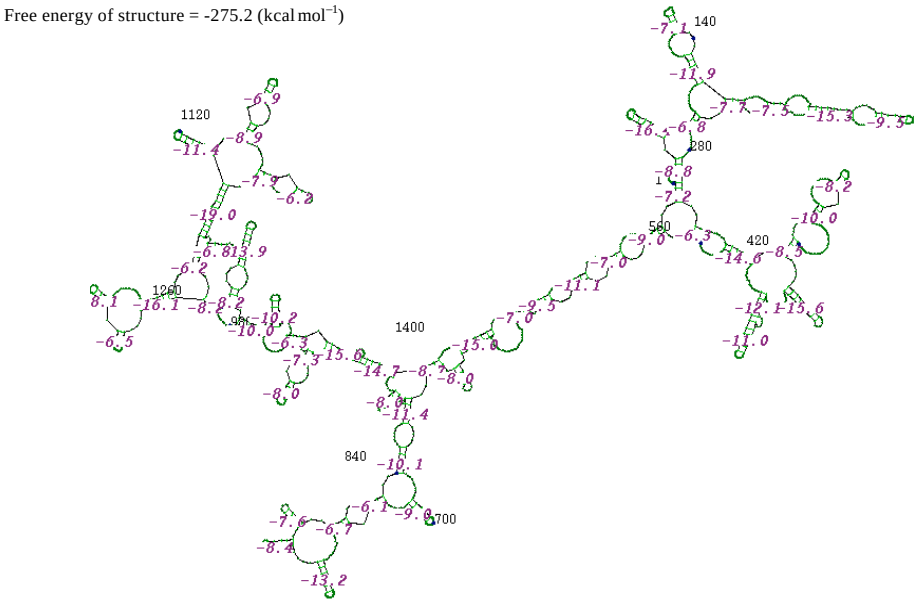

Fig. 6: Structure of *Moraxella* sp.

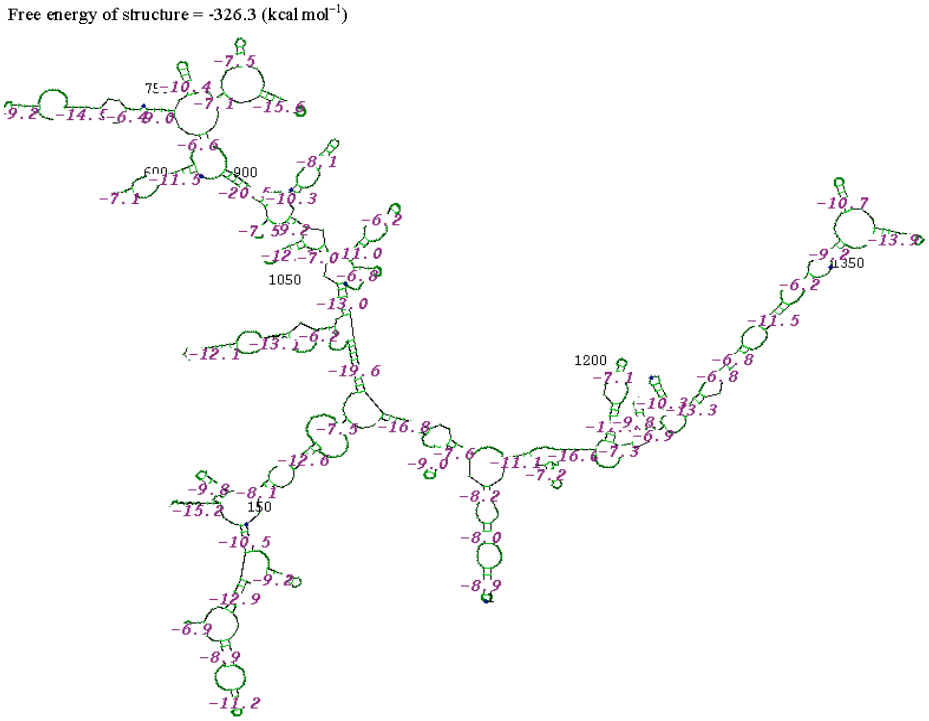

Fig. 7: Structure of *Citrobacter koseri*

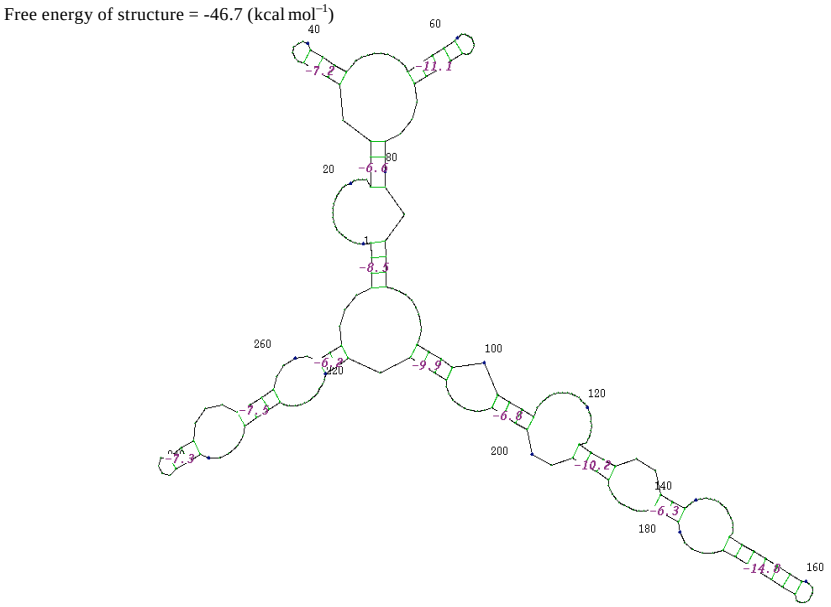

Fig. 8: Structure of *Acinetobacter baumannii*

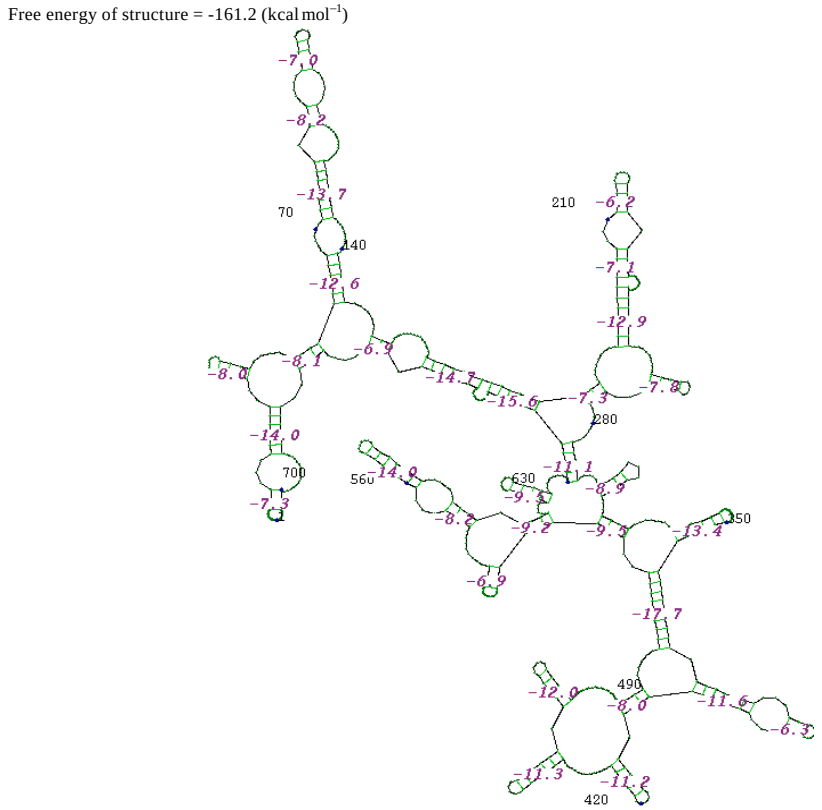

Fig. 9: Structure of *Pseudomonas aeruginosa*

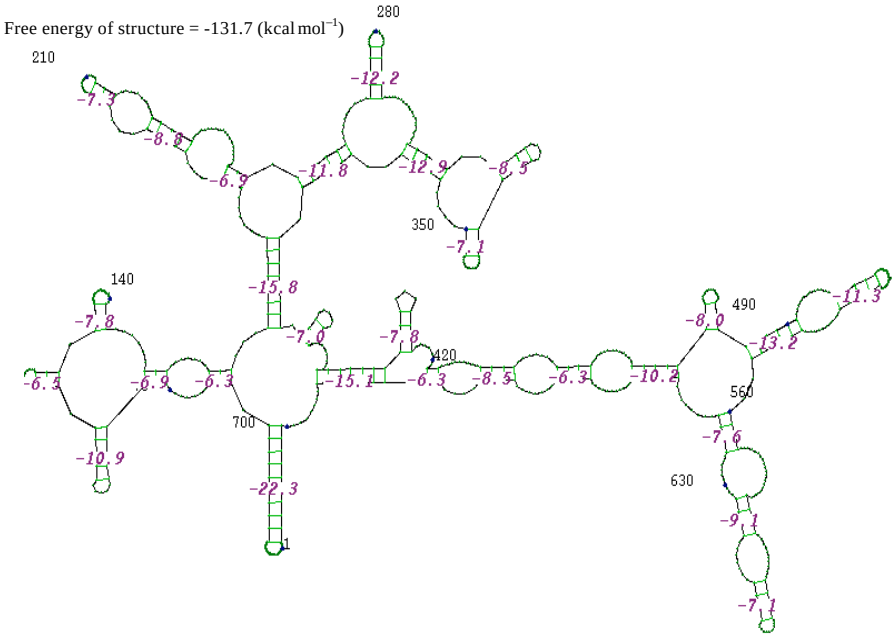

Fig. 10: Structure of *Propionibacterium* sp.

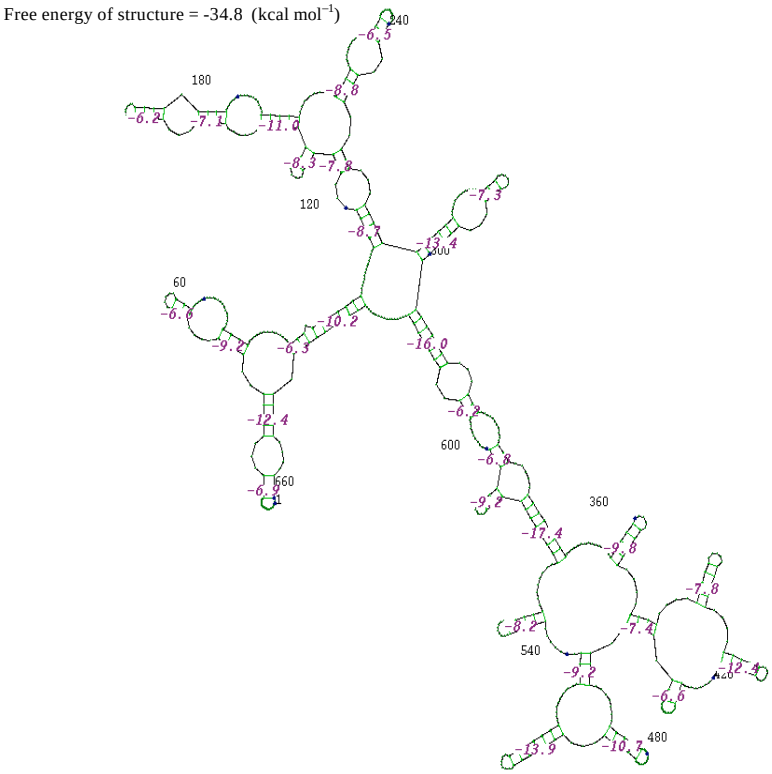

Fig. 11: Structure of *Klebsiella* sp.

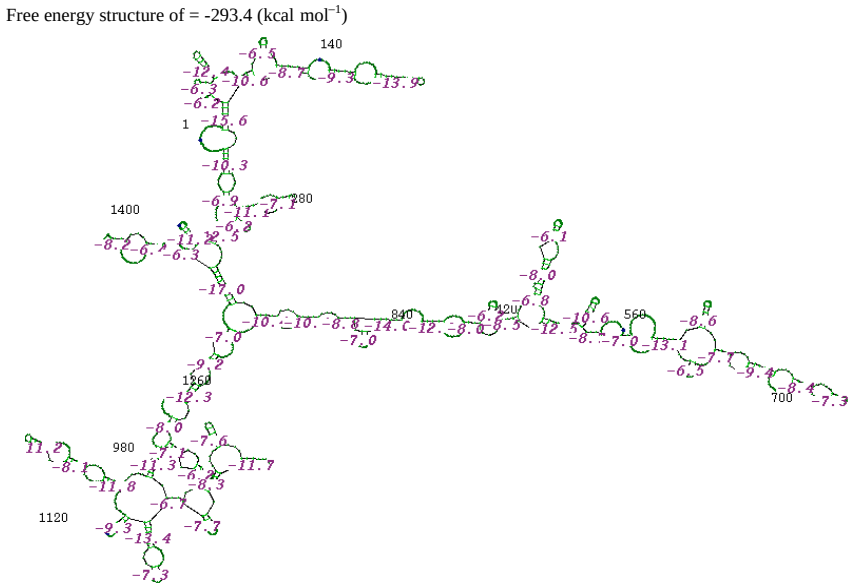

Fig. 12: Structure of *Staphylococcus epidermidis*

Table 2: Secondary structure prediction of rDNA for bacterial sequences by using GeneBee tool

Microorganism	Free energy of structure (kkal mol^{-1})
Streptococcus viridians	-242.5
Staphylococcus aureus	-74.4
Micrococcus sp.	-307.3
Moraxella sp.	-275.2
Citrobacter koseri	-326.3
Acinetobacter baumannii	-46.7
Pseudomonas aeruginosa	-161.2
Klebsiella sp.	-234.8
Propionibacterium sp.	-131.7
Staphylococcus epidermidis	-293.4

Moraxella sp., *Citrobacter koseri*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Propionibacterium* sp., *Staphylococcus epidermidis*, respectively (Table 2). The determination of the structure of the RNA molecule is very important in the determination of its activity.

In the secondary structure prediction of RNA the fold with the more negative free energy, is more stable. From the result, the *Citrobacter koseri,* (-326.3 kkal mol^{-1}) has lower minimal free energy. When compared with others species whereas the *Acinetobacter baumannii*, (-46.7 kkal mol^{-1}) has high minimal free energy when compared with other species. RNA molecule must be energetically stable to be able to maintain their structure and perform their functions.

rDNA-based analysis is a central method in microbiology, used as a method for bacterial strain identification. Molecular methods, including PCR, genotyping and automated DNA sequencing, are becoming more available and hence their adoption into routine medical microbiology is increasing. Therefore, 16S rDNA is known to be highly conserved in species and it could also be used for the

verification of the thermodynamic stability on the basis of conserved secondary structure of RNA. The structure of RNA plays an important role in the life cycle of bacteria and provides the ability to understand evolution and stability (Zuker and Stiegler, 1981). Earlier studies reported that Molecular Characterization and Sequencing of a Gene Encoding Mannose Binding Protein in an Iranian Isolate of *Acanthamoeba castellanii* as a major agent of *Acanthamoeba* keratitis by PCR techniques (Niyyati *et al.*, 2008).

In this study, we have characterized the bacteria causing keratitis and analyzed the phylogenetic relationship between them. The Multiple sequence analysis reveals that this sequence is highly conserved as all the organism share a region which is highly similar. The rDNA has also been modeled and their free energies have been predicted.

CONCLUSION

All bacterial species possess at least one copy of the 16S rDNA gene which contains highly conserved as well as hyper variable nucleic acid sequences. Therefore, the PCR targeting of the conserved nucleic acid sequence of 16S rDNA gene of bacterial isolate is used as a molecular tool to identify bacteria causing keratitis.

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. The sequence analysis of the gene fragment characterized in this study is still under investigation. This technique provides the opportunity to identify non-cultivable, uncommon, or even unknown causative bacteria.

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