(RESEARCH ARTICLE)

Phylogenetic tree analysis based on the 16S sequence alignment for *Klebsiella pneumonia* and *Enterobacter aerogenes* Isolated from patients with Ocular eye infection in Wasit province, Iraq

Mukal Assaad Hussein [1], Rana H Raheema [1, *] and Hassan Khalil Melek [2]

[1] Department of Medical Microbiology Faculty of Medicine, University of Wasit, Iraq.
[2] Department of Surgery Faculty of Medicine, University of Wasit, Iraq.

Abstract

In Iraq and other developing countries, ocular infections are a significant public health concern, particularly gram-negative bacterial infections, which are common. The emergence of multidrug resistance is a critical concern because it can lead to treatment failure when bacterial strains become resistant to antimicrobial drugs. This study aimed to investigate, Phylogenetic tree analysis of *Klebsiella pneumonia* and *Enterobacter aerogenes* isolated from patients with Ocular eye infection. The tree provided evidence of a unique species that was represented by a single nucleic acid sequence. This species encompassed both *Klebsiella pneumoniae* and *Enterobacter aerogenes*. Through phylogenetic tree analysis, it was determined that the 16SrRNA gene exhibited effective discriminatory power in identifying these bacterial strains. Based on the sequencing data, it can be inferred that there is a significant sequence similarity between the *Klebsiella pneumonia*e samples and strains from Bangladesh. Similarly, *Enterobacter aerogenes* showed high sequence similarity with strains from India and Iraq, indicating a shared species affiliation.

Keywords: 16S rRNA; *Klebsiella pneumonia*; *Enterobacter aerogenes*; Ocular eye infection

1. Introduction

In the realm of modern healthcare, bacterial infections pose a significant threat to public health, particularly in cases involving the use of interventional materials and post-surgical scenarios. These infections are not only detrimental to patients' well-being but also incur substantial treatment costs [1,2]. In the context of ocular infections, mild cases can manifest as discomforting symptoms such as itching, redness, and sensations of foreign bodies in the eyes, while severe infections can potentially lead to blindness. It is important to note that the appropriate antibiotic treatment varies depending on the specific type of pathogenic microorganism involved. Staphylococcus epidermidis (*S. epidermidis*), Pseudomonas aeruginosa (*P. aeruginosa*), and Staphylococcus aureus (*S. aureus*) are common pathogenic bacteria associated with ocular infections, despite having similar inflammatory responses. Consequently, each of these pathogens requires distinct treatment approaches [3,4].

Eye infections can be attributed to bacteria of both Gram-positive and Gram-negative types. Among the commonly identified bacterial species associated with eye infections are Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus, *Streptococcus pneumoniae, Enterobacteriaceae, Neisseria gonorrhoeae, Haemophilus influenzae, Moraxella spp.,* and *Haemophilus somni.* These bacteria are frequently encountered as causative agents in various eye infections [5]. Include Staphylococci that lack the coagulase [6]. Additionally, S. aureus has been on the rise, posing a serious hazard in the form of eye infections [7].

* Corresponding author: Rana H Raheema

Culture-dependent approaches, used in the earliest studies of ocular surface microbiota (OSM), underestimated 50 percent of the species actually existent [8]. Culture-independent approaches, such as sequencing of bacterial 16S ribosomal ribonucleic acid (rRNA), have recently been developed for the assessment of ocular surface microbiota. This is particularly useful because some microbial species require extremely specific growing conditions [9]. When compared to more conventional approaches to bacterial identification, 16S rRNA gene sequencing has proven to be a speedy and accurate way for diagnosing a wide range of eye illnesses caused by bacteria [10]. The majority of ocular bacterial infections are typically treated empirically using broad-spectrum antibiotics. Unfortunately, this approach has contributed to the development of antibiotic resistance, rendering commonly used antibiotics less effective. Moreover, the misuse of broad-spectrum antibiotics for conditions such as viral infections or as prophylactics has further exacerbated the problem of antibiotic resistance. Antibiotic resistance is a global issue with significant implications [11]. The emergence of bacterial resistance to antimicrobial drugs poses a higher risk of treatment failure. Multidrug resistance has become a particularly concerning problem that is progressively increasing in prevalence [12]. The objective of this study was to investigate the phylogenetic relationship between *Klebsiella pneumoniae* and *Enterobacter aerogenes* isolates obtained from patients with ocular eye infections.

2. Material and methods

Klebsiella pneumonia and *Enterobacter aerogenes* were collected for a cross-sectional study from patients who were admitted to the Al-Zahra teaching hospital in the Waist province between 1st October 2021 to 1st March 2022. The study included patients of both male and female genders. Aseptic techniques were employed to collect swab samples using sterile cotton-tipped swabs pre-saturated with sterile physiological saline. The swabs were then placed into Amies transport medium and transported to a clinical bacteriology laboratory. The isolated bacteria were identified through a combination of morphological observations, biochemical tests, and the use of Vitek2 system for further characterization and identification.

2.1. Molecular Analysis

DNA extraction from the bacterial samples was performed. The purity and concentration of the extracted DNA were confirmed using a nanodrop spectrophotometer. The measured purity values of the nucleic acid in the samples ranged between 1.8 and 2, indicating good quality DNA. The concentration of the extracted DNA ranged from 50 to 360 mg/μl. Primers were prepared for subsequent PCR experiments. The optimization of the primer annealing temperature was conducted through multiple trials of thermal cycles using a 20x Mastermix. The goal was to identify the optimal conditions for PCR reactions. Further details and results are found in Tables 1, 2, 3 This methodology is supported by the works of Nasser *et al*. [13] and Dahwash *et al.* [14].

Table 1 Primers' sequence of *16SrRNA* genes

NO.	Bacteria	Sequences (5'-3')	Product size (bp)	References
1.	Enterobacter aerogence	F: CTGGAACTGAGACACGGTCC	300	This study
		R: CGGGGATTTCACATCCGACT		
2.	*Klebsiella pneumonia*e	F: TGCCTGATGGAGGGGGATAA	382	
		R: TGCCTGATGGAGGGGGATAA		

2.1.1. Programmable thermal controller

Table 2 Thermal cycling program for 16SrRNA genes of *Klebsiella pneumonia*
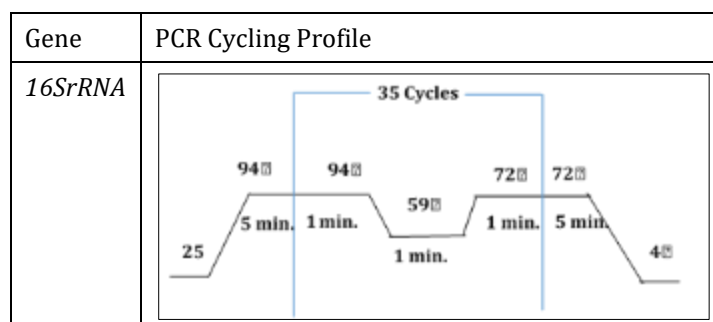
Gene	PCR Cycling Profile
16SrRNA	

Table 3 Thermal cycling program for 16SrRNA genes of *Enterococcus Aerogenes*

Gene	PCR Cycling Profile
16SrRNA	

The PCR product and DNA ladder were loaded into wells of a 1.5-2% agarose gel containing 1 µl of ethidium bromide. This gel electrophoresis technique was employed to separate and visualize the DNA fragments. Subsequently, the gel underwent electrophoresis, applying either 70 or 100 volts, to facilitate the migration of the DNA fragments through the gel. The purpose of this process was to generate results that could be recorded and subsequently analyzed.

2.2. Sequencing of PCR amplicons

The PCR amplicons that were successfully amplified were subjected to sequencing using the Macrogen Corporation - Korea ABI3730XL automated DNA sequencing machine. The same forward and reverse PCR primers were used during the sequencing process. The obtained ABI sequence files were analyzed using BioEdit and MEGA 11 software. Any undesirable nucleotide sequences were removed during the analysis. The NCBI website's BLAST tool was utilized to determine the identity of the nucleotide sequences obtained.

2.3. Interpretation of sequencing data

The sequencing results obtained from the PCR products were compared with their respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The acquired sequences were aligned and compared to identify any variations or similarities. Each identified nucleic acid was assigned a unique number that corresponded to its position within both the PCR amplicons and the reference genome. The NCBI-BLAST program was utilized to retrieve the specific target regions, and sequence matches were identified for each sample. This analysis allowed for the identification and characterization of the sequences obtained from the PCR products.

2.4. Comprehensive phylogenetic tree construction

In this study, the cladogram building method introduced by Hashim et al. [15] was employed to construct a specific and comprehensive tree. The discovered variations in the sequences were compared to their neighboring homologous reference sequences using the NCBI-BLASTn service. This comparison allowed for a comprehensive analysis of the relationships and similarities between the identified sequences and their corresponding reference sequences. [16]. The Clustal Omega tool was used to make multiple sequence alignments on the retrieved nucleic acid sequences [17]. The neighbor-joining strategy was then used to build an inclusive tree, which was then visualized in BioEdit and MEGA 11. Both the detected variations and their associated reference sequences were incorporated into the full cladogram.

2.5. Statistical Analysis

In order to conduct the statistical analyses, version 9.1 of the Statistical Analysis System (SAS)[18] was utilized. Also, to analyze the differences in percentages, a Chi-square test was carried out. P 0.05 is considered to be acceptable significance threshold in statistics.

3. Results and discussion

The results of the identification of *Klebsiella pneumoniae* indicated the presence of pink, lactose fermenter, and mucoid colonies on MacConkey agar. Positive results were obtained for Voges-Proskauer and Simmons citrate tests, while

negative results were observed for oxidase, methyl red, motility, indole, and TSI (acid/acid with gas) tests(Fig 1, 2, 3, 4) Similar findings have been previously reported by Raheema and Mahood [19] as well as Jassim *et al*. [20].

Enterobacter aerogenes, on the other hand, appeared as pink to red colonies on MacConkey agar, indicating lactose fermentation. This isolate exhibited motility and gave negative results for indole, methyl red, oxidase, and H2S production tests. Positive results were obtained for Simmons citrate, Voges-Proskauer, and motility tests. The TSI result was acid/acid with gas. Similar results have been documented by Martins *et al*. [21]and Iqbal *et al.* [22].

One of the samples included in the study had a ribosomal fragment of approximately the expected length. The sequence of this fragment was confirmed by using NCBI BLAST to identify the amplified product. The NCBI BLASTN engine revealed a significant sequence similarity between the sequenced samples of *Klebsiella pneumoniae* and *Enterobacter aerogenes* with their respective target sequences. The analysis showed a high homology of approximately 99% between the obtained sequences and the expected targets, specifically within the designated regions of the rRNA gene sequences.
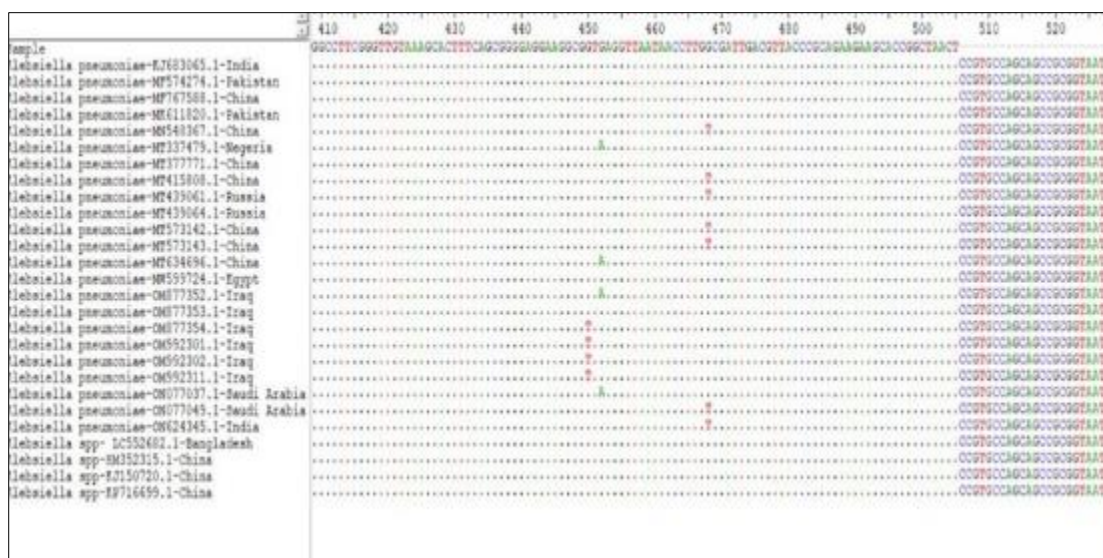

Figure 1 The process of aligning the nucleic acid sequence of a *Klebsiella pneumonia* sample with the most appropriate genomic sequence deposited in a database was performed
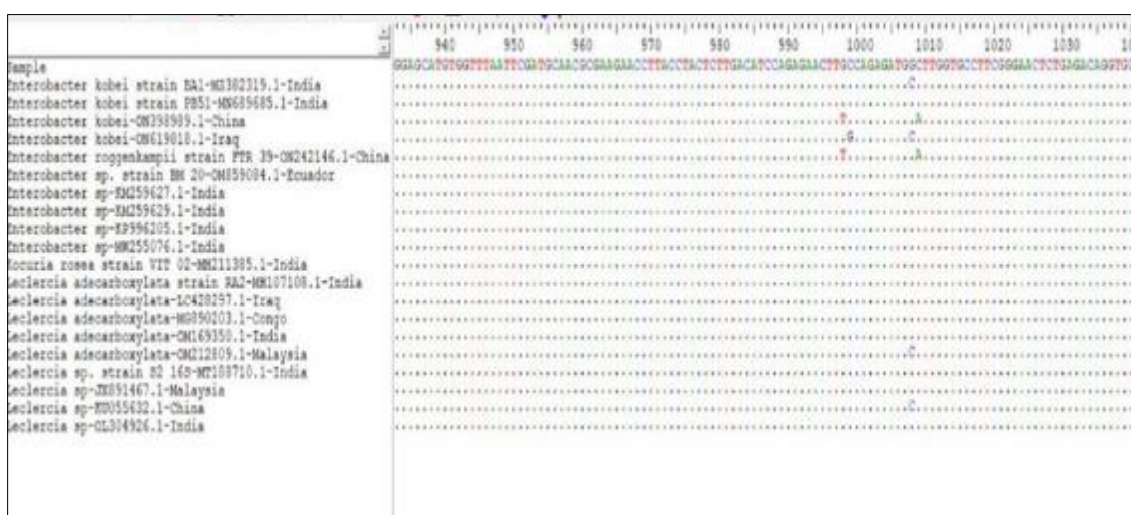

Figure 2 The process of aligning the nucleic acid sequence of the *Enterobacter aerogenes* sample with the most suitable deposited genomic sequence was carried out

Using the *16S ribosomal nucleic* acid sequences of the bacterial samples, a phylogenetic tree was constructed by linking our screened samples with other deposited DNA sequences in a neighbor-joining fashion. The generated phylogenetic tree demonstrated the existence of a unique and distinct species, which was represented by the sole included nucleic acid sequence. The analysis revealed that the sequence belonged to a distinct lineage separate from other known species, indicating its distinct taxonomic identity within the evolutionary tree. Both *Klebsiella pneumoniae* and *Enterobacter aerogenes* belonged to this species. The BLAST program showed that these samples were closely related to Gene Bank accession numbers from various international sources, including two accession numbers of Lc552682.1 and MK611820.1, which were from strains of *Klebsiella pneumoniae* in Bangladesh and Pakistan, respectively, and had a high degree of similarity with the sample.

The *Enterobacter aerogenes* sample obtained in this study was found to have matches with two Gene Bank accession numbers, MH107108.1 and Lc428297.1. These accession numbers corresponded to strains originating from India and Iraq, respectively. The comparison analysis indicated a high level of similarity, approximately 94%, between our isolate and the bacterial isolates present in the NCBI database. The dendrogram analysis further confirmed that our isolate formed a distinct cluster separate from all other similar sequences, indicating its unique genetic profile within the studied population.
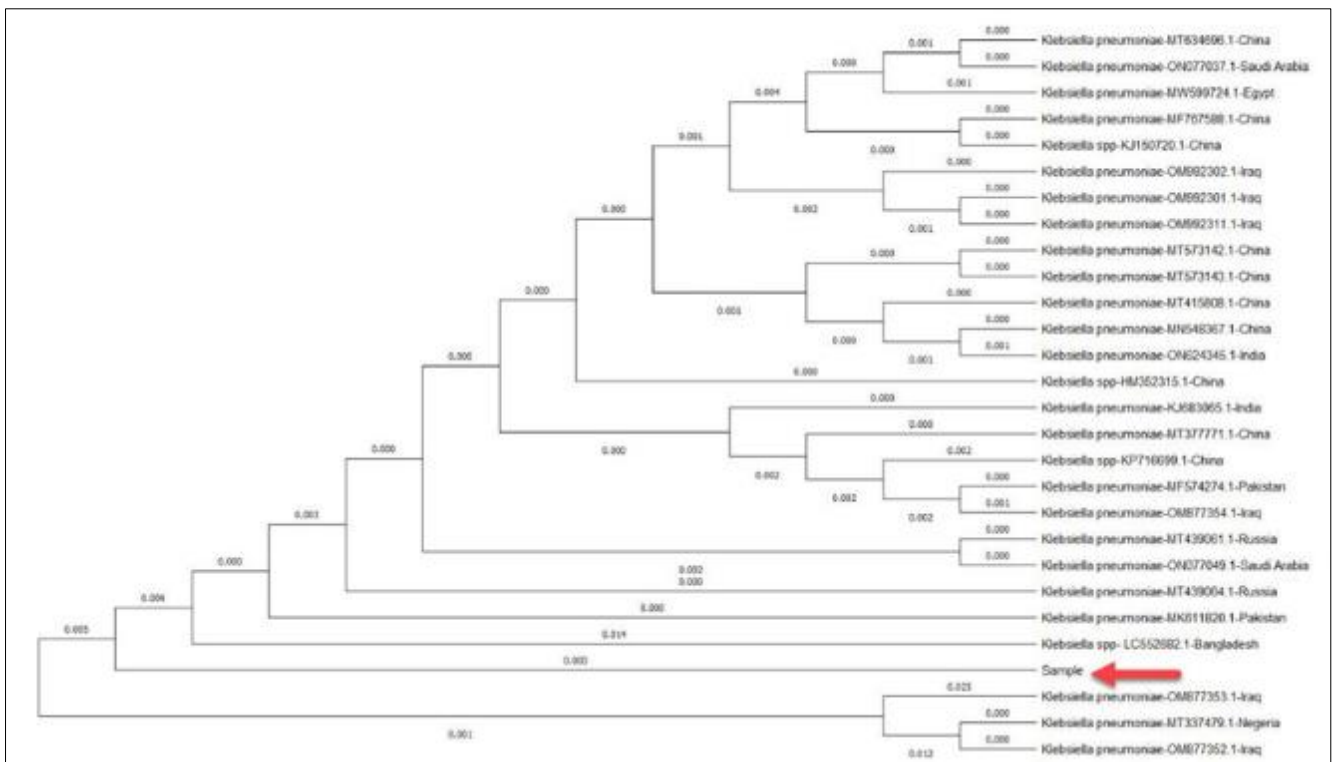

Figure 3 A detailed dendrogram was created by analyzing the 16S rRNA sequences of *Klebsiella pneumonia* in conjunction with those present in the NCBI database
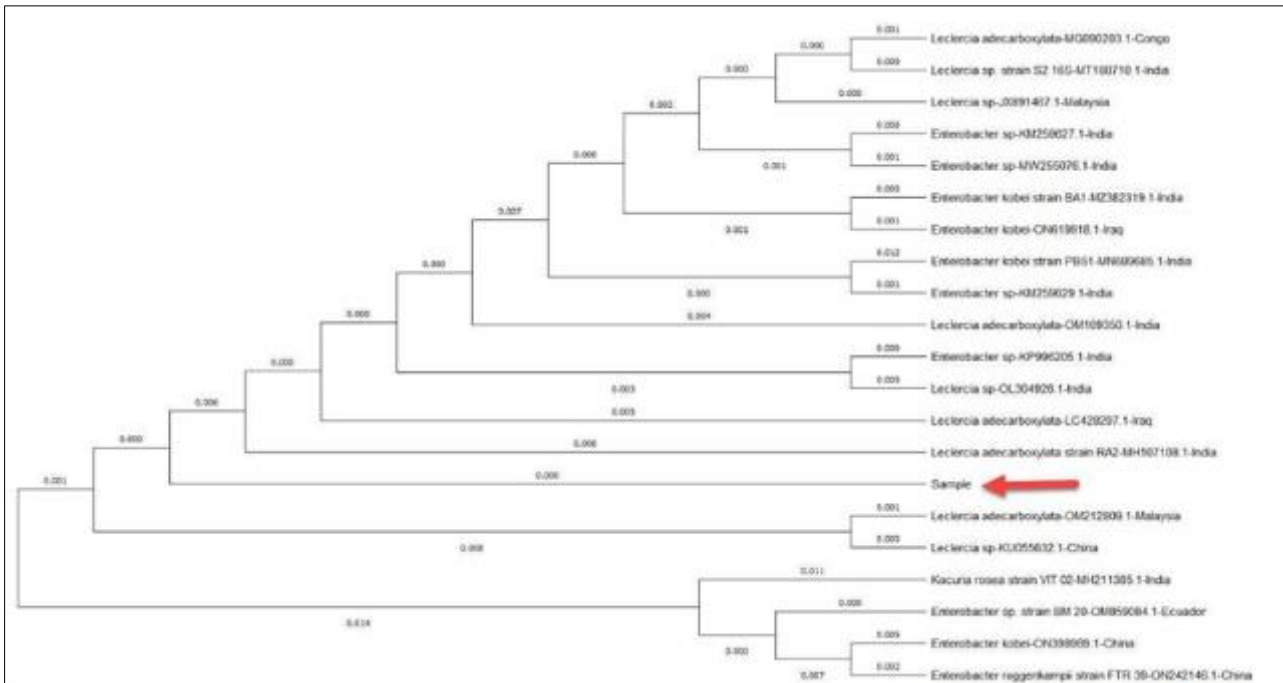
Figure 4 A detailed dendrogram was constructed by analyzing the *16S rRNA* sequences of *Enterobacter aerogenes* with those of other similar sequences in the NCBI database, highlighting their evolutionary relationships

4. Conclusion

Klebsiella pneumoniae and *Enterobacter aerogenes* were identified as significant pathogens associated with ocular eye infections. The phylogenetic tree analysis demonstrated that the 16SrRNA gene was effective in accurately identifying these bacteria. Based on the sequence analysis, it was observed that the sequenced samples of *Klebsiella pneumoniae* showed a high degree of sequence similarity with strains originating from Bangladesh and Pakistan. Similarly, the sequenced samples of *Enterobacter aerogenes* exhibited a significant sequence similarity with strains from India and Iraq, indicating their relatedness within the same species.

Compliance with ethical standards

Acknowledgments

The authors like to thank to the Al-Zahra teaching hospital in the Waist province for their cooperation and help.

Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

Ethical approval for this study was done.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

References

[1] Topçu, Sibel, Mustafa Kemal Sezgintürk, and Erhan Dinçkaya. Evaluation of a new biosensor-based mushroom (Agaricus bisporus) tissue homogenate: investigation of certain phenolic compounds and some inhibitor effects. Biosensors and Bioelectronics 20.3 (2004): 592-597.

[2] Han, Haijie, et al. Biofilm microenvironment activated supramolecular nanoparticles for enhanced photodynamic therapy of bacterial keratitis. Journal of Controlled Release 327 (2020): 676-687.

[3] Bose, Sunil Kumar, et al. Therapeutic potential of nanolipoidal α-terpineol in combating keratitis induced by Pseudomonas aeruginosa in the murine model. International Journal of Pharmaceutics 594 (2021): 120175.

[4] Chen, Hao, et al. Synergistic Chemotherapy and Photodynamic Therapy of Endophthalmitis Mediated by Zeolitic Imidazolate Framework-Based Drug Delivery Systems. Small 15.47 (2019): 1903880.

[5] Miller, E.J. and Brines, C.M. (2018) 'Canine diabetes mellitus associated ocular disease', Topics in companion animal medicine, 33(1), pp. 29–34.

[6] Mohammed, A.A., Ali, M.M. and Zenebe, M.H. (2020) 'Bacterial etiology of ocular and periocular infections, antimicrobial susceptibility profile and associated factors among patients attending eye unit of Shashemene comprehensive specialized hospital, Shashemene, Ethiopia', BMC ophthalmology, 20, pp. 1–8.

[7] Vola, Maria Eugenia, et al. Prevalence and antibiotic susceptibility of methicillin-resistant Staphylococcus aureus in ocular infections. Arquivos brasileiros de oftalmologia 76 (2013): 350-353.

[8] Matysiak, A., Kabza, M., Karolak, J.A., Jaworska, M.M., et al. (2021) 'Characterization of ocular surface microbial profiles revealed discrepancies between conjunctival and corneal microbiota', Pathogens, 10(4), p. 405.

[9] Avetisov, S.E., Abramova, N.D., Gogoleva, N.E., Gusev, O.A., et al. (2020) 'Rational strategy for studying microbiome of the ocular surface of people using hard contact lenses by method of 16S rRNA gene metabarcoding', Vestn. Oftalmol, 136(3), pp. 3–9.

[10] Omi, M., Matsuo, Y., Araki-Sasaki, K., Oba, S., et al. (2022) '16S rRNA nanopore sequencing for the diagnosis of ocular infection: a feasibility study', BMJ Open Ophthalmology, 7(1), p. e000910.

[11] Montagnani, F., Malandrini, A., Stolzuoli, L., Lomurno, L., et al. (2008) 'Microbiological features of acute bacterial conjunctivitis in a central Italian area.', The new microbiologica, 31(2), p. 291.

[12] Amsalu, A., Abebe, T., Mihret, A., Delelegne, D., et al. (2015) 'Potential bacterial pathogens of external ocular infections and their antibiotic susceptibility pattern at Hawassa University Teaching and Referral Hospital, Southern Ethiopia', Afr J Microbiol Res, 9(14), pp. 1012–1019.

[13] Nasser, Dhilal Dhiaa, Rana H. Raheema, and Ahmed Ibrahim Ansaf. Phenotypic and molecular characterization of β-lactamases among uropathogenic Escherichia coli from pediatric patients in wasit province. Biochem. Cell. Arch.2022; Vol. 22, No. 1, pp. 332-341.

[14] Dahwash, Sarah Mohsin, et al. Distribution of phylogenetics and virulence genes of uropathogenic Escherichia coli among urinary tract infection in pregnant women. Biochem. Cell. Arch 21.1 (2021): 449-456.

[15] Hashim, Hayder O., et al. Infection with different strains of SARS-CoV-2 in patients with COVID-19. Archives of Biological Sciences 72.4 (2020): 575-585.

[16] Zhang, Zheng, et al. A greedy algorithm for aligning DNA sequences. Journal of Computational biology 7.1-2 (2000): 203-214.

[17] Sievers, Fabian, and Desmond G. Higgins. Clustal Omega, accurate alignment of very large numbers of sequences. Multiple sequence alignment methods (2014): 105-116.

[18] SAS Institute. SAS/ETS 9.1 User's Guide. SAS Institute, 2004.

[19] Raheema, R.H. and Mahood, S.A. karium. (2016) The Effect of Partial Purified Plantaricin against Urinary Tract Infection (UTI) Induced by Escherichia Coli and Proteus Mirabilis in Experimental Rat Model. European Journal of Pharmaceutical and Medical Research, 3(8), pp., 623–630. https://www.researchgate.net/publication/324416223

[20] Jassim, A.T., Raheema, R.H. and Melek, H.K. (2022) Characterization of Multidrug Resistant Bacteria Isolated from Patients with Diabetic Foot Ulcers in Wasit Province. Biochem. Cell. Arch, 22(1), pp., 000–000. https://connectjournals.com/03896.2022.22.000

[21] Martins, Nathana Beatriz, et al. Dermatopathy Caused by *Enterobacter aerogenes* and Pseudomonas aeruginosa in Boa constrictor amarali. Acta Scientiae Veterinariae 45 (2017): 4-4.

[22] Iqbal, Hamid, et al. Pathogenic bacteria and heavy metals toxicity assessments in evaluating unpasteurized raw milk quality through biochemical tests collected from dairy cows. Asian Pacific Journal of Tropical Disease 6.11 (2016): 868-872.