

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF A NEW LOCAL STRAIN OF EIKENELLA CORRODENS ISOLATED FROM CHRONIC PERIODONTITIS

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МОРФОЛОШКА И МОЛЕКУЛАРНА ИДЕНТИФИКАЦИЈА НОВОГ ЛОКАЛНОГ СОЈА EIKENELLA CORRODENS ИЗОЛОВАНОГ ИЗ ХРОНИЧНОГ ПАРОДОНТИТИСА

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ABSTRACT

Objectives. This research focused on the morphological and molecular identification of a new local strain of *Eikenella corrodens*, which was isolated from 25 cases infected with chronic periodontitis.

Methods. A modified Todd Hewitt agar medium was used for the first time locally to isolate the strain. The researchers then examined the isolate to determine its ability for slime layer production and formation of biofilm.

Results. The results of the culture revealed that only one out of the twenty-five cases tested positive for *Eikenella corrodens*, accounting for a 4% occurrence. This positive result was further confirmed by PCR and gel electrophoresis. To identify the strain, the sequence of the 16S rRNA gene of *Eikenella corrodens* was determined and compared with the existing data available at the National Center for Biotechnology Information (NCBI). The sequence was deposited in GenBank under the accession number OQ996282.1. The strain was named MeAm. Additionally, the researchers found that this particular isolate demonstrated a strong ability to form biofilm.

Conclusion. These findings contribute to the understanding of the local strain of *Eikenella corrodens* and its characteristics concerning chronic periodontitis, providing valuable insights for further research and potential treatment strategies.

Key words: *Eikenella corrodens*; biofilms; chronic periodontitis.

INTRODUCTION

The mild early inflammation of the gums occurs as a result of the accumulation of bacteria, and plaque in the mouth and leads to infection, associated with bleeding and swelling of the gums, pain, and may progress to periodontitis when left untreated (1). The accumulation of inflammatory cells across the gingival furrow promotes the growth of Gram-negative bacteria, which are rich in proteolytic enzymes that consume glycoproteins as a main source of energy, virulence factors and metabolic activity (2). In the past, Periodontitis was classified as chronic and aggressive. The chronic form is rapidly evolving and it is

САЖЕТАК

Циљ. Ово истраживање било је фокусирано на морфолошку и молекуларну идентификацију новог локалног соја *Eikenella corrodens*, који је изолован код 25 пацијената који су имали хронични пародонтитис.

Методе. Модификовани Тод Хјуит агар медијум први пут је коришћен локално да би се изоловао сој. Истраживачи су потом испитали изолате да би утврдили њихову способност производње слојева матрикса и формирања биофилма.

Резултати. Резултати микробиолошких култура открили су да је само један од 25 случајева био позитиван на *Eikenella corrodens*, што чини инциденцу од 4%. Овај позитиван резултат додатно су потврдили PCR и електрофореза на гелу. Да би се идентификовао сој, утврђен је низ 16S rRNK гена *Eikenella corrodens* и упоређиван с постојећим подацима, доступним у Националном центру за биотехнолошке информације (NCBI). Секвенца је депонована у GenBank под приступним бројем OQ996282,1. Сој је добио име MeAm. Осим тога, истраживачи су установили да су ови изолати показали снажну способност формирања биофилма.

Закључак. Ови налази доприносе разумевању локалног соја *Eikenella corrodens* и његових карактеристика у вези са хроничним пародонтитисом, пружајући драгоцене увиде за даља истраживања и потенцијалне стратегије лечења.

Кључне речи: *Eikenella corrodens*, биофилмови, хронични пародонтитис

characterized by rapid destruction of the soft and hard tissue attachments of the periodontium, and it is either localized or widespread, depending on the dentist's clinical diagnosis (3). *Eikenella corrodens*, a unique species of the *Eikenella* genus, is a HACEK group of microorganisms (4). which *Eikenella corrodens* is commonly isolated from the human oral cavity and upper respiratory tract, and it belongs to the Neisseriaceae family, genus *Eikenella* (5). This bacterium was first isolated by Eiken in 1958 and originally named "*Bacteroides corrodens*"(6). It is a small fastidious, facultative, anaerobic, gram-negative bacillus (rod), non-motile; slowly growing on chocolate or blood agar at

37°C. The colour of colonies is greyish, and sometimes, on the blood agar, they are surrounded with a greenish discolouration and produce an unpleasant bleach-like odour. Approximately 50% of the strains show pitting or corroding of the agar surface and this feature gave a name to this bacterium (7). *Eikenella corrodens* can cause abscesses in the brain, liver, and thigh. (8). The latest advances in PCR and sequencing technology give a faster identification than that obtained with standard phenotypic procedures that rely on bacterial growth. Sequence analysis of the 16S rRNA gene is considered as the “gold standard” for identification of bacteria (9).

This research aims to highlight one of the most important and virulent new locally-isolated periodontal pathogens using the molecular technique.

MATERIALS AND METHODS

Sample Collection: Twenty-five samples were collected from patients with chronic periodontitis depending on clinical examination by a specialist dentist at Dental Specialized Center / Al-Noor and Mosul General Hospital. Absorbent paper points (size #30) were inserted into the base of pockets with a pocket depth (PD) of ≥ 4 mm for one minute. The absorbent paper points were transferred directly into tubes containing a modified transport medium. The patients (14 male and 11 female with an age of 19-65 years) proved that they had no systemic disease, were not smokers and had not been taking antibiotics for 3 months before sampling (10).

Transport Media: In this study, a modified alternative thioglycollate broth Himedia M010 was used as transport media. 29 g were dissolved in 1000 litres of distilled water, heated if necessary to dissolve the medium completely, and hemin was added in a concentration of 5 µg/ml to modify the medium (11). Then the broth was distributed to the tubes, following by autoclave sterilisation at 121 °C for 15 minutes.

Culture Media: The growth medium used was modified Todd-Hewitt media Himedia M313, which consists of Todd-Hewitt broth medium, 2.0 mg of (KNO₃) potassium nitrate/ml, with 5 µg of hemin/ml. The agar was added to a final concentration of 1.5% (wt/vol) (12). Modified Todd-Hewitt agar supplemented with Twenty-five µg of clindamycin/ml to permit the growth of *Eikenella corrodens*. This culture media was incubated after inoculation at 37°C for 2 to 3 days under microaerophilic conditions, where these conditions had been provided by the use of an anaerobic jar as well as to produce a suitable gaseous atmosphere, by using the CampyGen™ 2.5L Sachet, for growth of Microaerophilic bacteria. It is preferable to prepare the liquid and solid medium one day before taking the sample and place it in the incubator to ensure that there is no contamination.

Culture: After transferring the samples from the specialized centre to the laboratory in the College of Science / Department of Biology, processing of the samples was started by mixing with a vortex, and then 100 µL of each sample was spread on the modified Todd-Hewitt agar. Then the plates with Campy Gen™ 2.5L Sachet were placed in an anaerobic jar at 37°C for 3 to 5 days.

Biochemical Tests

The following Biochemical tests were performed to diagnose the isolated bacteria (10,15):

1. Catalase enzyme production test: Transfer a loop of the bacterial culture broth to a clean, glass slide using a sterile wooden stick, then place a drop of 3% hydrogen peroxide. The result is considered positive with the appearance of gas bubbles.
2. Oxidase enzyme production test: it was performed by oxidase strips.
3. Indole production test: Inoculate the peptone water medium with a bacterial colony and incubate at 37°C for 24-48 hours, then add 5 drops of Kovacs reagent and shake gently, as the appearance of a red ring is evidence of a positive test and the ability of the bacteria to produce indole from the amino acid tryptophan.
4. Urease test: The urea agar medium was inoculated with bacterial isolate, and the tubes were incubated at 37°C for 24-48 hours. The appearance of pink colour indicates the positivity of the test and the ability of the bacterial isolate to produce the enzyme.

Identification of *Eikenella corrodens* by molecular methods: DNA Extraction: The suspected bacteria were cultured on modified Todd-Hewitt agar medium and incubated under microaerophilic conditions for two days, and then extraction of genomic DNA of our bacteria was done using (Presto™ Mini gDNA Bacteria Kit Protocol) provided by Company of Geneaid. The following steps were suggested by the manufacturer. Genomic DNA purity and concentration were measured, and after that, DNA was kept at -20 °C for other uses (14, 15).

Polymerase Chain Reaction (PCR) Protocol (14, 15)

A 25 µL volume reaction of PCR was fulfilled by using GoTaq G2 Green Master Mix provided by an American Company called Promega. The following sequence of 27F AGAGTTTGATCMTGGCTCAG and 1522R AAGGAGGTGATCCARCCGCA, which are the universal primers, were taken for amplifying the 16S rRNA gene (14). The two primer concentrations, which were (1 µM for each); also, and the template total amount of DNA which was (100 ng), were used as advised by the company. PCR cycling conditions for the gene of 16S

rRNA are illustrated in Table (1) below. On 2% agarose gel, the products of PCR were taken separately, adding the stain of Midori Green Advance DNA. Finally, a DNA marker of 100 base pairs was used in this process.

Sequencing of DNA and Homology Search: Products of PCR for the gene of 16S rRNA were delivered for processing at the Company of Psomagene sequencing (United States of America). Returning sequences were examined for homology against published genes that are present in GenBank via using the tool BLAST at NCBI.

Investigation of Slime Layer Production: The ability of bacteria to produce a slime layer was studied by modifying Congo red agar medium by adding the Congo Red (CR) dye (0.8 g/l of distilled water and sucrose 36 g to the modified Todd-Hewitt agar medium). This modification was performed for the first time. Modified Todd-Hewitt agar medium and Congo Red dye were sterilized separately by autoclave, while sucrose was sterilized by filtration as a concentrated aqueous solution. After modified Todd-Hewitt agar medium cooled to 45-50 °C, sugar and CR dye were added. The samples were inoculated on CR- CR-modified Todd-Hewitt agar medium and incubated at 37 C for 48 hours, then left for 48 hours at room temperature.

Estimation Biofilms Formation by *Eikenella corrodens* (13): In this experiment, a bacterial culture of three-day-old *Eikenella corrodens* was utilized to obtain a suspension of modified Todd-Hewitt broth medium, which was then prepared to match McFarland 0.5 in the absence of antibiotics. The next step involved pouring 200 µl of bacterial broth into each well of a 96-flat bottom well microtiter plate, creating a "mono-microbial population." The plates were then closed and incubated under anaerobic conditions for four days. After the incubation period, the contents of the wells were discarded, and the wells were rinsed multiple times with distilled water to remove any non-adherent cells. To stain the adherent biofilm, 0.1% crystal violet (100 µl) was added to each well and left for 15 minutes, followed by several washes with distilled water. To stabilize the attached bacteria, 200 microliters of 95% ethanol were added to each well and left for 15 minutes before being poured out and allowed to dry. The optical density of the wells was then measured using an ELISA reader at a wavelength of 630 nm. This measurement was used to assess the efficiency of the isolates in producing biofilm, with different ranges of optical density (OD) values indicating varying degrees of biofilm formation. According to the provided equation, an OD value less than or equal to ODC indicated that the isolate was non-adherent or non-biofilm forming. An OD value greater than ODC but less than or equal to 2 times ODC indicated weak biofilm formation, while an OD value greater than 2 times ODC but less than or equal to 4 times

ODC indicated moderate biofilm formation. Finally, an OD value greater than 4 times ODC indicated strong biofilm formation.

RESULTS

The results of primary isolation revealed that 22 out of 25 specimens (88%) of periodontal cases showed positive bacterial growth on modified Todd-Hewitt agar medium with diverse morphologies (Figure 1).

The suspected colonies of *Eikenella corrodens* on modified Todd-Hewitt agar and clindamycin were small, grey (older colonies may become light yellow), translucent, and slightly speckled (Figure 1A), to show the different levels of corrosion, or show the pitting of the agar surface, and distinguishable from other bacteria (Figure 1B).

The results of microscopic examination revealed that our isolate was a small straight gram-negative rod, that can be appeared in a pleomorphic or coccobacilli shape. It is non-motile, non-spore-forming, and also, does not contain a capsule (Figure 2A). After the purification process, the result of bacterial growth of *Eikenella corrodens* on the modified Todd-Hewitt agar medium plus clindamycin as pure culture (Figure 2B).

Biochemical Tests: This bacterium shows a small number of positive results of biochemical methods, which may face difficulty in determining this kind of bacterium from other morphologically similar bacteria (Table 2).

After DNA had been extracted by genomic DNA extraction kit, the concentration was 90.14 ng/µl and purity 1.763. The 16S rRNA gene amplification of the bacterial genomic DNA was done. PCR result was confirmed by gel electrophoresis, it was positive through the presence of the band belonging to the genomic DNA of *Eikenella corrodens* (Figure 3). The 16S rRNA gene sequence was determined for of *Eikenella corrodens*, and compared with that of (NCBI) National Center Biotechnology Information. Deposition the sequence of 16S rRNA of *Eikenella corrodens* at GenBank, under the accession number OQ996282.1, then this strain has given MeAm name. Thus, this is the first locally isolated strain.

Slime Layer Production: After inoculation of CR-modified Todd-Hewitt agar medium with *Eikenella corrodens* isolates (Figure 4), there was a change in colour to black, which is an indication that this bacterium can create a slime layer.

Formation of Biofilm: The result of the detection of biofilm formation indicated its ability to form biofilm. This ability was estimated by the Micro-Titer-Plate method (MTP). The results showed that this bacterium has strong adherence after applying the previously mentioned equations.

Table 1. PCR cycling conditions.

| Steps | Temp. | Time | Cycles |
|----------------------|-------|--------|--------|
| Initial denaturation | 95 °C | 5 min | 1 |
| Denaturation | 94 °C | 30 sec | 30 |
| Annealing | 57 °C | 30 sec | |
| Extension | 72 °C | 1 min | |
| Final extension | 72 °C | 3 min | 3 |

Table 2. The results of biochemical tests.

| Biochemical Tests | Result |
|-------------------|----------|
| Oxidase | Positive |
| Catalase | Negative |
| Urease | Negative |
| Indole | Negative |
| Nitrate Reduction | Positive |

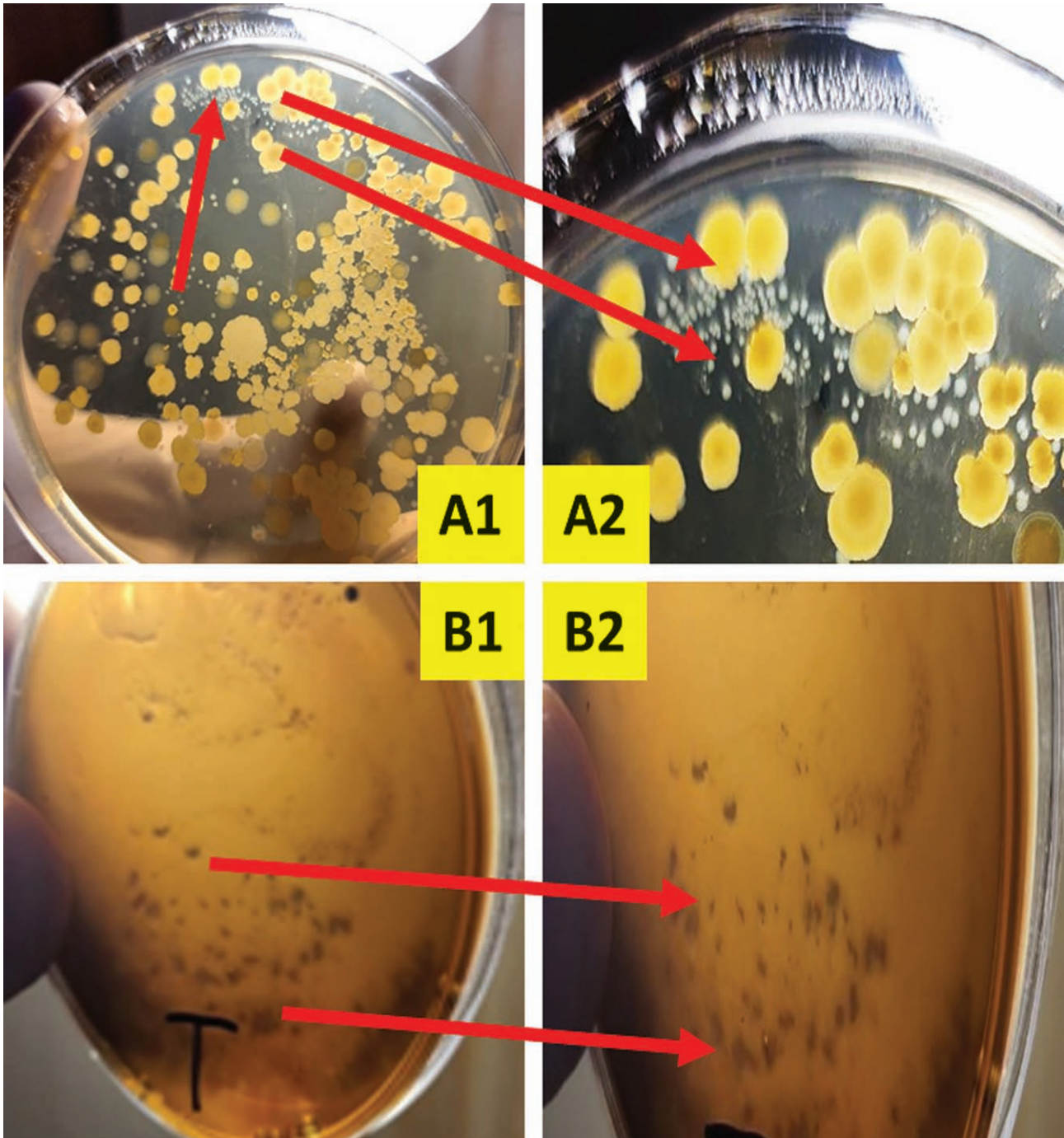


Figure 1. Representative images of culture petri dishes (A) Mixed growth on modified Todd-Hewitt agar medium (B) Corrosion or pitting of the agar surface.

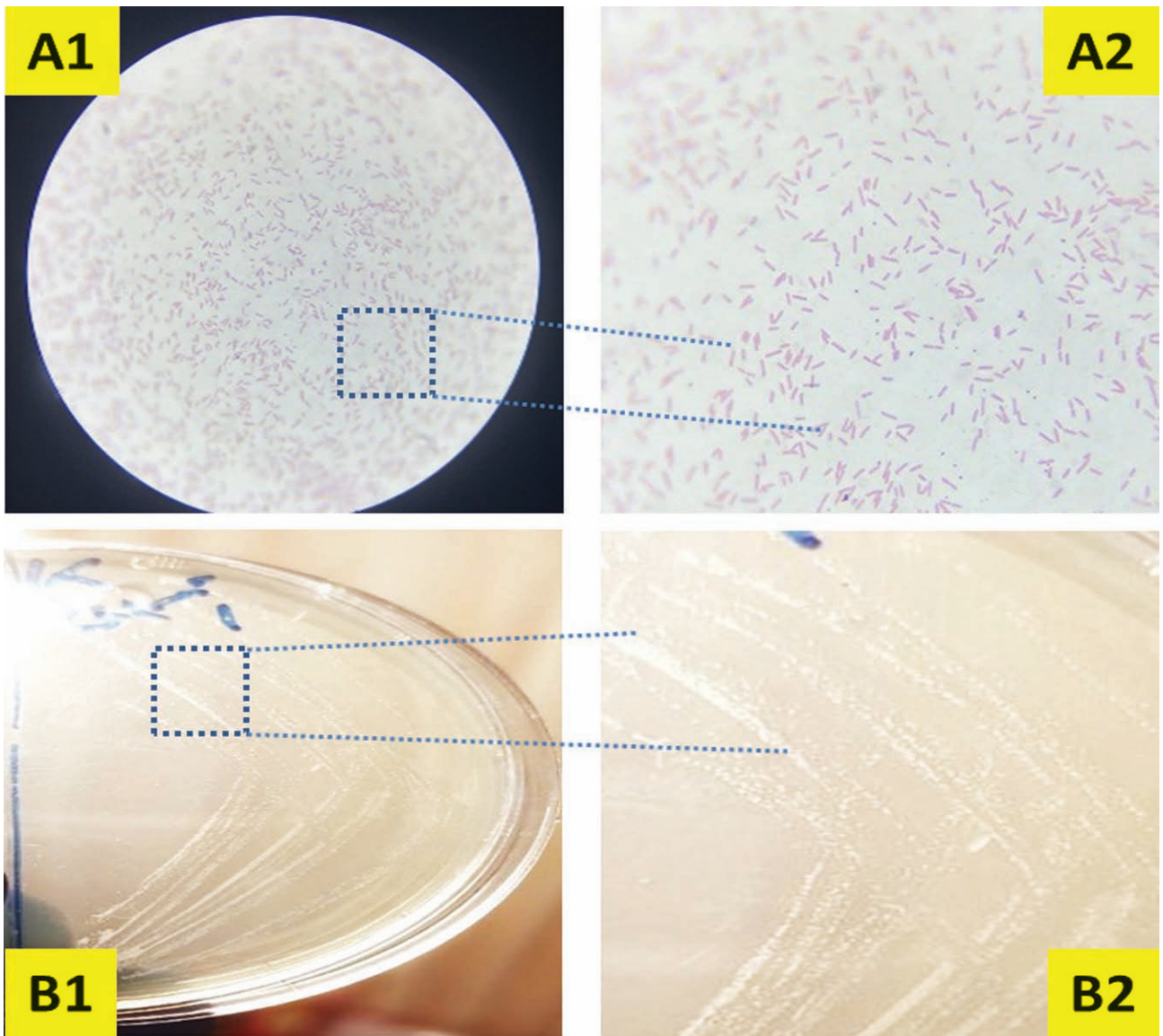


Figure 2. Representative images of (A) Gram negative cells (100x), (B) Growth of *Eikenella corrodens* on modified Todd-Hewitt agar as pure culture.

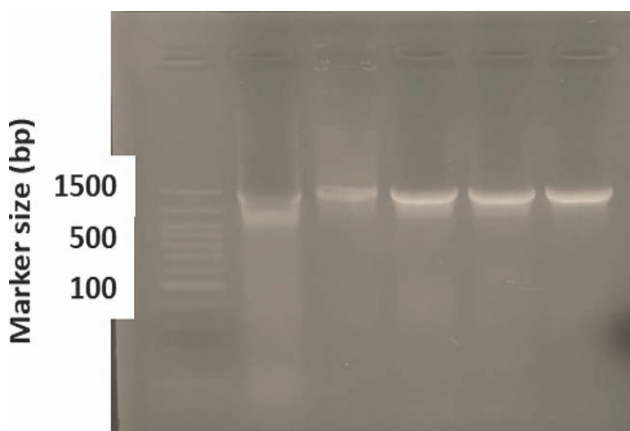


Figure 3. Amplification of the 16S rRNA gene from *Eikenella corrodens* genomic DNA which isolated from chronic periodontitis.

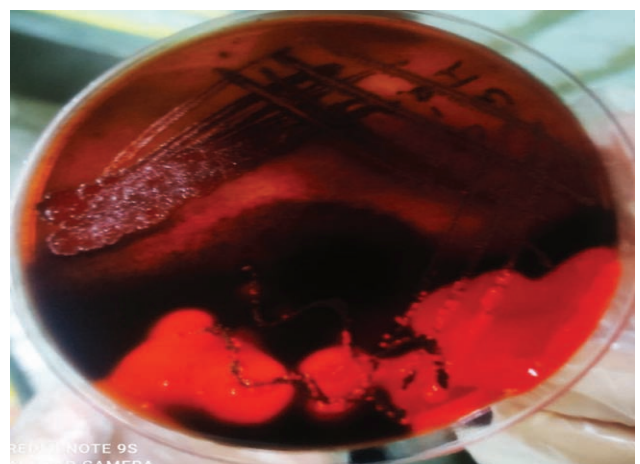


Figure 4. Slime layer production on CR- modified Todd-Hewitt agar medium by *Eikenella corrodens*.

DISCUSSION

The results of the culture revealed that only one out of the twenty-five cases tested positive for *Eikenella corrodens*, accounting for a 4% occurrence. The proportion of its isolation was low due to its slow-growing features of primary isolation and the fastidious characteristics of *Eikenella corrodens*. *Eikenella corrodens* grows slowly and requires an atmosphere containing 5%-10% CO₂, which may result in it being missed on routine culture (16). Additionally, it may be overgrown by other organisms, further hindering its isolation (17). These factors contribute to the low recovery rate of *Eikenella corrodens*.

To achieve pure isolates, each phenotypic form is subcultured on a modified Todd-Hewitt agar medium for routine purification and subjected to molecular identification to assign the pathogens. More than 5 µg/ml of clindamycin Concentrations clearly partially show the inhibition of the *Eikenella corrodens* growth. Adding clindamycin 25 µg of /ml to the modified Todd-Hewitt agar medium allowed the *Eikenella corrodens*. Despite this, it is expected that some isolates of *Eikenella corrodens* are lost in this concentration (12). On the blood agar, a slight greenish discolouration can be produced by colonies, producing an bleach-like odour, and this is confirmed by a study conducted by Rodríguez-Rojas and associates (7).

The results of the biochemical tests have confirmed *Eikenella corrodens* is biochemically inactive. It does not ferment glucose and other carbohydrates or produce acid. It is negative for catalase, urease, arginine dehydrogenase, and indole, but is positive for nitrate reduction, as well as oxidase and lysine decarboxylase (18). A study by Chen and Wilson mentioned that *Eikenella corrodens* has a small number of positive results of biochemical methods, which may present difficulty in differentiating this bacterium from other morphologically similar bacteria (19). It is an extremely heterogeneous species. First, there is considerable inter-strain variation concerning biochemical activity. Secondly, *Eikenella corrodens* exhibits marked variation in sensitivity to serum bactericidal activity. Recently *Eikenella corrodens* was identified and diagnosed according to the MALDI-TOF MS system (20).

The methods used to detect the production of mucous substance and the ability to form a biofilm are different, mainly including Congo red agar medium CRA and MTP method (21). The study of Mathur et al.(2006)(22) showed a lack of a relationship between the two methods and that the MTP method is the most sensitive and realistic, as shown by Samie and Nkgau (2012) study that the MTP method is the most sensitive and easy to detect biofilm formation among strains (23). The CRA method relies on

showing the difference in the morphological pattern of isolates which produce slim layers with high virulence, and that detection of this pattern may help in distinguishing between strong and weak biofilm-producing strains that reflect the degree of infecting and helping in the determination the primary treatment, and the different production of multiple polysaccharide adhesion (PIA) causes this different degree in the production of biofilm, which indicates changes in the genetic regulation (22). Bacteria growing in the biofilm show differences morphologically from the original one growing on the culture, these differences involve movement change, high production of extracellular polysaccharides and high antibiotic resistance (24). The method of MTP is very important in the study of the early stage of biofilm formation and classification of the degree of infection into low, medium and high, as it is the production of the biofilm is an important virulent factor, as it supports bacteria with protection for from phagocytosis, lead to the continuation of the infection (25).

In conclusion, the findings of the present study significantly enhance our comprehension of the specific strain of *Eikenella corrodens* prevalent in the local context and shed light on its distinctive characteristics in the context of chronic periodontitis. By delving into the intricacies of this pathogen, we have gained invaluable insights that not only expand our knowledge, but also paved the way for future research endeavours. Moreover, these findings hold the potential to revolutionize treatment strategies for chronic periodontitis, as they provide a solid foundation upon which novel therapeutic interventions can be developed. Consequently, this research serves as a vital stepping stone in the ongoing quest to combat the detrimental effects of *Eikenella corrodens* and its impact on periodontal health. Moving forward, further investigations must be conducted to build upon these findings, with the ultimate goal of improving patient outcomes and ensuring enhanced oral health for individuals affected by chronic periodontitis.

REFERENCES

1. Belay AS, Achimano AA. Prevalence and risk factors for periodontal disease among women attending antenatal care in public hospitals, Southwest Ethiopia, 2022: a multicenter cross-sectional study. Clin Cosmet Investig Dent 2022; 14: 153-70.
2. Abusleme L, Hoare A, Hong BY, Diaz PI. Microbial signatures of health, gingivitis, and periodontitis. Periodontology 2000 2021; 86: 57-78.
3. Albuquerque-Souza E, Schulte F, Chen T, et al. Maresin-1 and resolvin E1 promote regenerative properties of periodontal ligament stem cells under inflammatory conditions. Front Immunol 2020; 11: 585530.

4. Matheus MP, Vecilla DF, Gutiérrez MJ, Muro FE, Hidalgo GI. Bacteremia caused by *Eikenella corrodens* in a patient with pelvic inflammatory disease. *Rev Esp Quimioter* 2023; 36: 329.
5. Jaramillo-Lanchero RD, Suarez-Alvarez P, Teheran-Sierra L. Effect of respiratory inhibitors and quinone analogues on the aerobic electron transport system of *Eikenella corrodens*. *Sci Rep* 2021; 11: 8987.
6. Stormo KA, Nygaard RM, Bruvold TS, et al. *Eikenella exigua* sp. nov., isolated from brain abscess and blood. *Int J Syst Evol Microbiol* 2020; 70: 1478-88.
7. Rodríguez-Rojas L, Suarez-López A, Cantón R, Ruiz-Garbayosa P. *Eikenella corrodens* causing deep-seated infections. Six-year experience in a University Hospital in Madrid. *Enferm Infecc Microbiol Clin (Engl Ed)* 2020; 38: 76-8.
8. Hu L, Lin J, Li J, Cao Y, Lin L. Lung abscess secondary to lung cancer with *Eikenella corrodens* and *Streptococcus anginosus*: a case report. *BMC Infect Dis* 2020; 20: 1-5.
9. de Melo Oliveira MG, Abels S, Zbinden R, Bloemberg GV, Zbinden A. Accurate identification of fastidious Gram-negative rods: integration of both conventional phenotypic methods and 16S rRNA gene analysis. *BMC Microbiol* 2013; 13: 162.
10. Al-Hamdoni SA, Al-Rawi AM. Identification of red complex pathogens group from chronic periodontitis patients in Mosul City. *Rafidain J Sci* 2020; 29: 1-6.
11. Ebrahim-Saraie HS, Motamedifar M, Mansury D, et al. Association of *Fusobacterium* isolation from periodontal pockets with halitosis and the related risk factors in Shiraz, Iran. *Arch Clin Infect Dis* 2015; 10: e28544.
12. Slee AM, Tanzer JM. Selective medium for isolation of *Eikenella corrodens* from periodontal lesions. *J Clin Microbiol* 1978; 8: 459-62.
13. Rashid Mahmood A, Mansour Hussein N. Study of antibiotic resistant genes in *Pseudomonas aeruginosa* isolated from burns and wounds. *Arch Razi Inst* 2022; 77: 403-11.
14. Khaleel A, Faisal R, Altafi H. The efficiency of molecular methods compared to traditional methods in identifying bacteria from blood and cerebrospinal fluid samples. *Malays J Microbiol* 2023; 19: 115-22.
15. Khalid I, Nayyef NS, Merkhani MM. A Taxonomic study comparing the two types of medicinal leeches available in Iraq. *Res J Pharm Technol* 2022; 15: 1119-22.
16. Stoloff AL, Gillies ML. Infections with *Eikenella corrodens* in a general hospital: a report of 33 cases. *Rev Infect Dis* 1986; 8: 50-3.
17. Pichereau P, Audurier A. *Eikenella corrodens* infections. *Ann Med Interne (Paris)* 1987; 138: 56-60. (in French).
18. Zhou X, and Li Y. Subgingival Microbes. *Atlas of Oral Microbiology: From Healthy Microflora to Disease*. Springer, Singapore. 2020; 145-210.
19. Chen CC, Wilson ME. *Eikenella corrodens* in human oral and non-oral infections: a review. *J Periodontol* 1992; 63: 941-53.
20. Li L, Shi YB, Weng XB. *Eikenella corrodens* infections in human: reports of six cases and review of literatures. *J Clin Lab Anal* 2022; 36: e24230.
21. Mahmood Khudhur I. Investigating the ability of some bacterial species to produce slime layer. *Rafidain J Sci* 2014; 24: 36-49.
22. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: an evaluation of three different screening methods. *Indian J Med Microbiol* 2006; 24: 25-9.
23. Samie A, Nkgau TF. Biofilm production and antibiotic susceptibility profile of *Escherichia coli* isolates from HIV and AIDS patients in the Limpopo Province. *Afr J Biotechnol* 2012; 11: 8560-70.
24. Jesaitis AJ, Franklin MJ, Berglund D, et al. Compromised host defense on *Pseudomonas aeruginosa* biofilms: characterization of neutrophil and biofilm interactions. *J Immunol* 2003; 171: 4329-39.
25. Pedersen SS, Høiby N, Espersen F, Koch C. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* 1992; 47: 6-13.