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Conventional and Molecular Identification of *Entamoeba gingivalis* from Periodontitis Patients in Nineveh Governorate /Iraq

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Abstract

One hundred and twenty four patients, both male and females of different ages, visiting teaching hospital at the College of Dentistry /University of Mosul city / Iraq and from Private dentists clinics, who suffered from gingivitis disease in addition to 32 healthy people as controls, were subjected to examination by swabbing of gingival pockets for detection of *Entamoeba gingivalis*. Samples were diagnosed by conventional techniques of isolation, culturing and staining methods. The recovered positive isolates were subjected to molecular analysis. Amplification of DNA extracted from *Entamoeba gingivalis* of both diseased and healthy people using 18 rDNA revealed PCR products with 600pb size for both diseased and healthy people. Alignment of the DNA sequences of the isolates revealed sequences for two strains gp11 from the patients with gingivitis disease and gP45 strain from healthy people. This is first trial in Iraq in sequencing *Entamoeba gingivalis* isolates. Phylogenetic analysis of gp11 strain showed resemblance to that isolated in Japan, while gP45 strain to that isolated from Brazil.

Introduction

The oral cavity contains nearly half of what is present from the symbiotic bacteria in the human body, which is approximately 6 billion microbes, representing (30 - 50) genus in the human mouth (Deng *et al* 2017) As these microbes feed on food remnants between the teeth, which causes an unpleasant odor and change in the color of the teeth and leads to the occurrence of tooth decay. It causes damage to the supporting fibers and tissues. (Dyke, 2017). The gingival is a species the genus *Eentamoeb*, and its life cycle includes only one phase, the vegetative phase, where it lacks the cystic phase. The presence of the parasite in the gums, especially in the gingival pocket, has been shown to decrease the level of oxygen, which is important in the survival of the vegetative phase, the parasite transmit directly from one person to another by kissing, droplet spray or by using contaminated utensils (Bonner, 2014). The vegetative phase contains an internal cytoplasm called endoplasm and an external cytoplasm called ectoplasm and contains a nucleus with a central particle called the endosome. The internal cytoplasm is characterized by containing contractile vacuoles and food vacuoles that have the ability to digest white blood cells, a characteristic of the parasite, in addition to red blood cells and bacteria. The inner cytoplasm contains a nucleus with a centrosome surrounded by regular chromatin granules and the vegetative phase is characterized by its characteristic amoeboid movement (Eloufir,

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2014). The parasite is found in the oral cavity and is often observed in gum pockets, especially in people with gingivitis (Mark, 2018). Studies have also shown the presence of the parasite in dental calcifications (Hussian , 2017). The parasite has also been diagnosed in the uterine and cervical region (Clark and Diamonds, 1992). What distinguishes this parasite is that it is opportunistic and has the ability to reproduce in the gums, and it is present in people with immunosuppression. (Vundela *et a.,l* 2016).Recent studies were conducted that showed comparisons between immunodeficient or immunocompromised persons with gingivitis and non-infected persons, and it was found that all persons with immunodeficiency had gingival amoeba (Eki *et al*., 2016). The gingival amoeba has also been found on the surface of the teeth and gingival pockets and near the base of the tongue (Fahimeh, 2016), studies conducted on gingival amoeba showed a comparison between the presence of the parasite in healthy people and its presence in people with gum disease, reaching 0, 12%, respectively, using the direct examination method and the PCR method (Mahmoud *et al*., 2019). In order to maintain pure cultures of *E,gingivalis* after swabbing, the microorganism should be cultured in specific media like modified Trypticase –yeast extract- serum-gastric mucin (TYSGM-9)(Hussian,2018) or Dulbecco's Modified Eagle Medium (DMEM) (Mahdali, 2011), for further identification by molecular PCR and sequencing methods. The direct correlation between different *E,gingivalis* phenotypes and their abilities in producing gingivitis urged many scientists to study the genome sequence and phylogenicity of *E,gingivalis* as a fundamental issue in resolving and understanding this phenomenon between different isolated strains (Das and Ganguly, 2014;Weeddall and Hall,2011;Cui *et., al* 2019). So the aim of this study was to compare the prevalence of *E .gingivalis* in patients with periodontitis and those in healthy people in Mosul citizens / Iraq by conventional and molecular methods.

Materials and Methods.

Conventional methods for identification of *Entamoeba gingivalis*

In this study the population subjected for Conventional identification of *E. gingivalis* were included 124 patients with gingivitis (59 males and 65 females) attending teaching hospital (College of Dentistry / Mosul University) in addition to some outpatients from private dentist clinics in Nineveh Governorate / Iraq\. Thirty two healthy people (16 males and 16 females) were matched as a controls. All patients and healthy persons were physically examined for their gums color and texture, teeth calcifications and tooth displacements under the supervision of the specialist doctors during the period from 1/12/2019 until 1/12 / 2020. Samples from patients with deep pockets of previously established periodontics were taken.

Collected samples were delivered after preservation with normal saline solution to the department of Basic Dental Sciences Department / College of Dentistry and examined directly by making wet preparation on a clean glass slide, coveredto be examined under two lens power (10 X, 40X) for observation of amoeboid movement of *E. gingivalis* pseudopodium. (Hersh, 1985). Samples were also stained by Giemsa stain, according to the method of (Adam *et al.,* 1971).

Entamoeba gingivalis isolates were then cultivated using Modified Dulbecco's Eagle Medium (DMEM) (Genaxxon Bioscience,Germany) by planting in individual test tubes containing 4 ml

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of DMEM and incubated at 35 ° C., for monitoring *E. gingivalis* growth (Al-Nuaimi, 2017). The data analysed statistically by using Chi-sequare,with asignificant (P≦ 0.05) .

Molecular study

Extraction of DNA from *Etamoeba gingivalis*

Molecular DNA extraction from the positive *Etamoeba gingivale* samples were processed using kit provided by (Geneaid) by following the manufacturer's protocol. The elute DNA was preserved at -20C^{O} until further use.

The primers for the amplification of the 18s rDNA of *Etamoeba gingivale* sets, forward: 5′-AGGAATGAACGGAACGTACA-3′ and Reverse: 5′-CCATTTCCTTCTTCTATTGTTTCAC-3′. Amplification of 18 rDNA gene of *Etamoeba gingivale* was done by using 4 µL (100 nanogram) of template DNA and 1 µL (10 picompl) from each gene initiator added to the Master Mix contents. After that, reaction tubes were inserted into the Thermocycler device (Biolab, England) with a protocol shown in table (1), then separated by 2% agarose gel electrophoresis.

Table 1: PCR protocol

No.	Stage	Temperature	Time	Cycle number
1.	Initial denaturation	95	6 min.	1
2.	Denaturation	95	1.30 min.	35
3.	Annealing	52	1.30 min.	
4.	Extension	72	2 min.	
5.	Final extension	72	5 min.	1

Determination of the nucleotide sequence of the amplified pieces, based on the DNA sequencing technique

The sequence of nitrogenous bases was determined for the samples under study, as the products of the PCR reaction was read according to the 3130 Genetic Analyzer supplied by the Japanese company Hitachi. The gene sequences were matched with the gene sequences documented by the National Center Biotechnology Information (NCBI) and the results were analyzed using the BLAST program.

Phylogenic study: The aligned DNA sequences of the identified local isolates were used as reference for construction of the phylogenetic tree using (Dendrogram). The sequences of the identified local isolates were used for sequence of strains deposited in GenBank.

Results

The results of the current study by using direct method (wet preparation) and stained with Giemsa stain, showed that the rate of infection with *E. gingivalis* among patients with

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periodontitis attending the Teaching Hospital at the College of Dentistry / University of Mosul was 77.41% out of the total of 124 patients, while the rate of isolation in healthy people was 50% out of the total of 32 people as shown in (Table 2). There was no significant difference between the periodontitis patients and healthy persons at a significant level of ($p \leqq 0.05$).

Table 2: Prevalence of *Entamoeba gingivalis* in diseased and healthy persons.

Oral disease	Examined(N)	Infected(N)	Infected(%)	P value
Periodontitis	124	96	77.41	0.1898
Healthy	32	16	50	
Total	156	112	71.79	

$p \leqq 0.05$

The parasite appeared in an amoeboid shape with spherical violet nucleus with small central endosome, concentrated chromatin on the inner surface of the nuclear membrane and blue colored cytoplasm (Fig. 1).

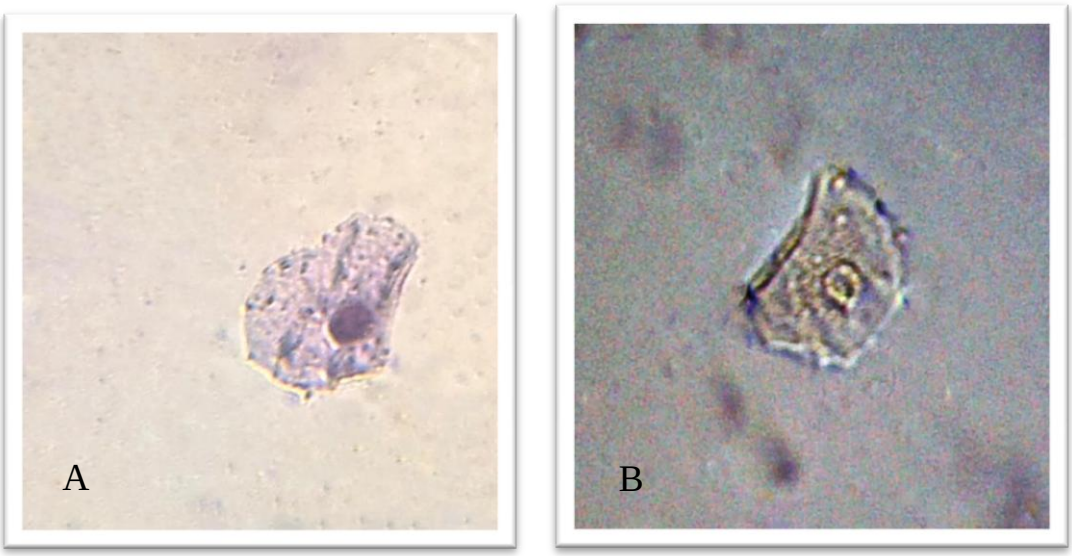

Figure 1: *Entamoeba gingivalis* stained by Giemsa (A), and not stained (B)

According to sex, the rate of periodontitis infection in males with *E. gingivalis* was 81.355% out of the total of 59 patients, while the rate in females was 73.48% out of the total of 65 patients (Table 3).

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Table 3: Prevalence of *E. gingivalis* in patients with periodontitis according to sex

Sex	Examination (N)	*E.gingivalis*		P. value
		Infected(N)	infected (%)	
Male	59	48	81.35%	0.7218
Female	65	48	73.84%	
Total	124	96	77.41%	

p≦ 0.05

In case of healthy people the prevalence of E. *gingivalis* was 50% in both males and females out of the total of 16 persons (8 males and 8 females) (Table 4)

Table 4: Prevalence of *Entamoeba gingivalis* in healthy people

Sex	Examination (N)	*E.gingivalis*		P. value
		Infected(N)	infected (%)	
Male	16	8	50	1.00
Female	16	8	50	
Total	32	16	50	

p≦ 0.05

The amplified product was electrophoretically separated in a submerged two percent agarose gel and visualized under ultraviolet light. The amplified product was sent to the 3130 Genetic Analyzer supplied by the Japanese company Hitachi. The gene sequences were matched with the gene sequences documented by the National Center Biotechnology Information (NCBI) and the results were analyzed using the BLAST program .

Extraction of DNA bundles from *E.gingivalis* samples that were cultivated on DMEM and transferred to the agar gel at a concentration of 2% is shown in Fig.2.

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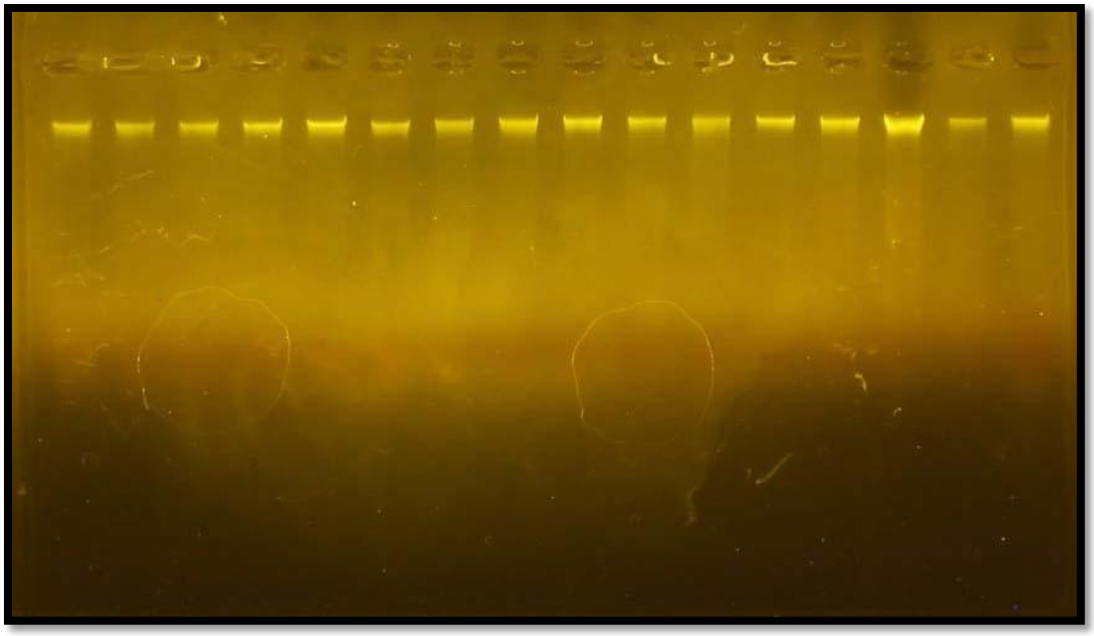

Figure 2: Extraction of DNA from samples of the *E.gingivalis*

PCR reaction of *E.gingivalis* parasite samples based on the primer of 18 rDNA show the reaction product of 600 bp (Fig. 3).

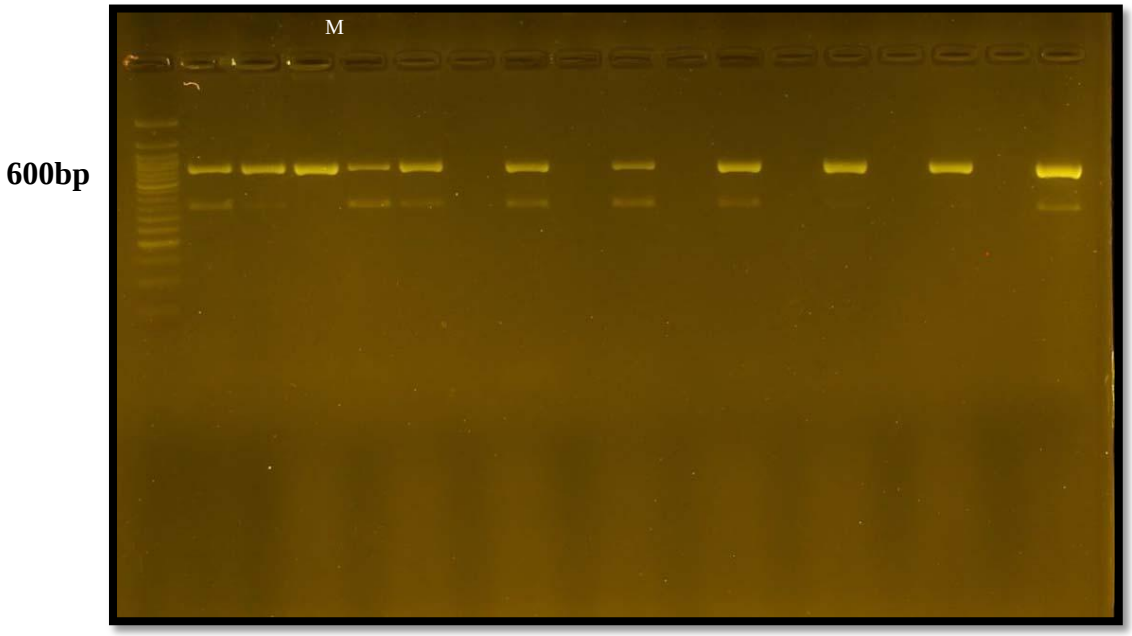

Figure 3: PCR reaction of *E.gingivalis* parasite samples based on the primer of 18 rDNA from periodontitis patients and the reaction product of 600bp

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PCR reaction of *E.gingivalis* parasite samples based on the initiator of 18 rDNA from healthy subjects and the reaction product of 600 bp shown in (Fig. 4).

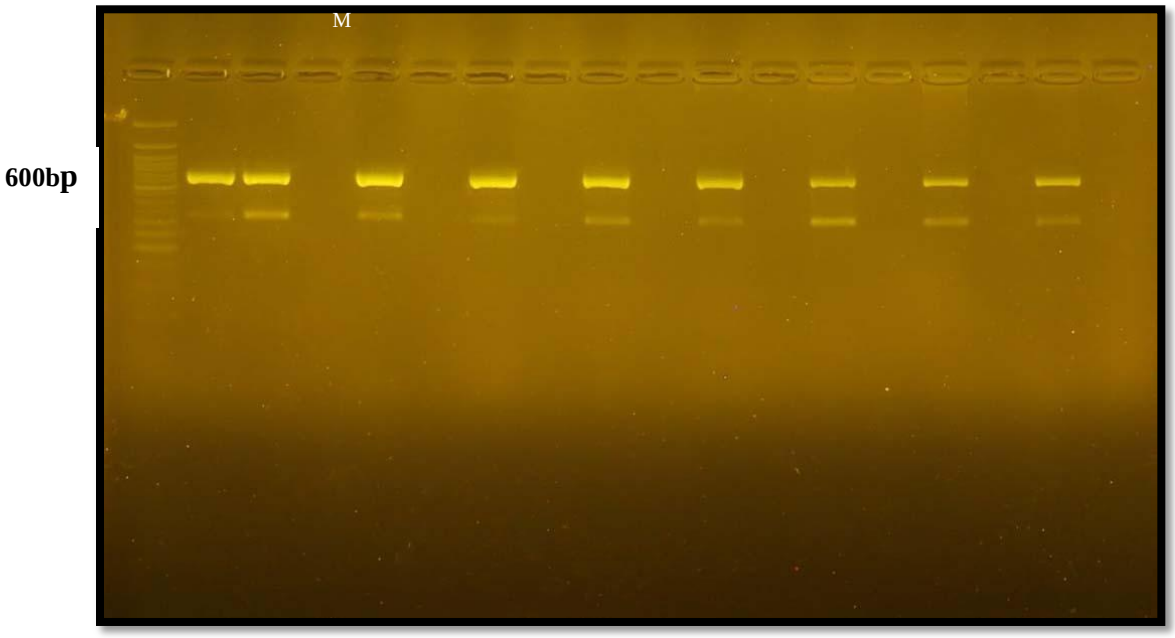

Figure 4: PCR reaction of amoeba parasite samples based on the initiator of 18 rDNA from healthy subjects and the reaction product of 600 bp

PCR reaction were sent for genetic sequences to GenBank and appeared in the patient samples *Entamoeba gingivalis* strain gP11 small subunit ribosomal RNA gene and internal Sequence ID : KX027297.1 Length : 2316 Number of Matches : 1 as shown in Fig. 6, it differs from what was found in healthy samples where it appeared as *Entamoeba gingivalis* strain gP45 small subunit ribosomal RNA gene and internal Sequence ID : KX027298.1 Length : 2287 Number of Matches : 1 shown in (Fig. 6).

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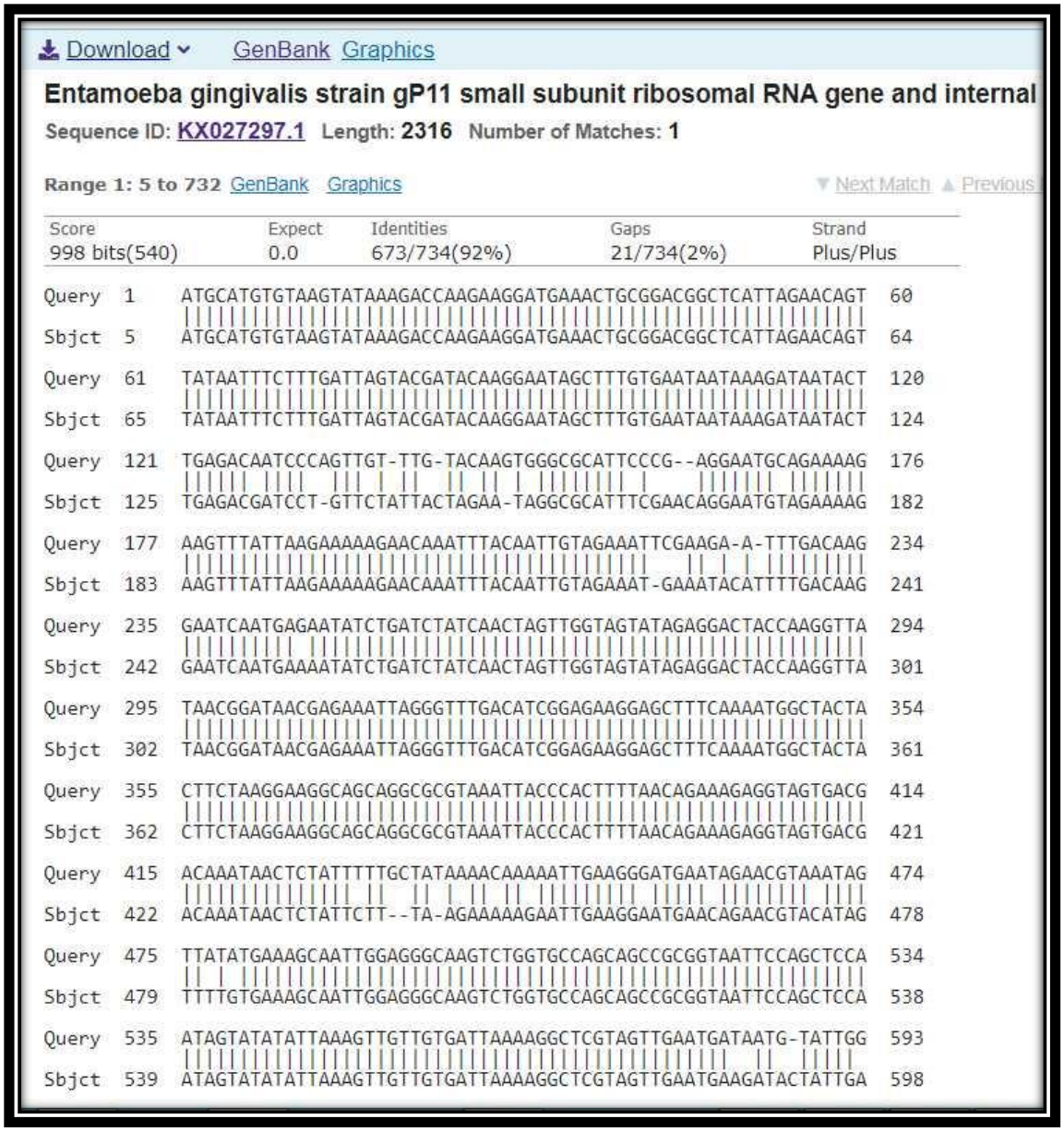

Figure 5: *Entamoeba gingivalis* strain gP11 in patients with periodontitis.

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Figure 6: *Entamoeba gingivalis* strain gP45 in healthy persons.

The results of phylogenetic study showed that *E.gingivalis* strain (gP11) which is isolated from patients is similar and matching the Japanies strain, while *E.gingivalis* strain (gP45)Which isolated from healthy is matching the Brazilian strain (Fig.7).

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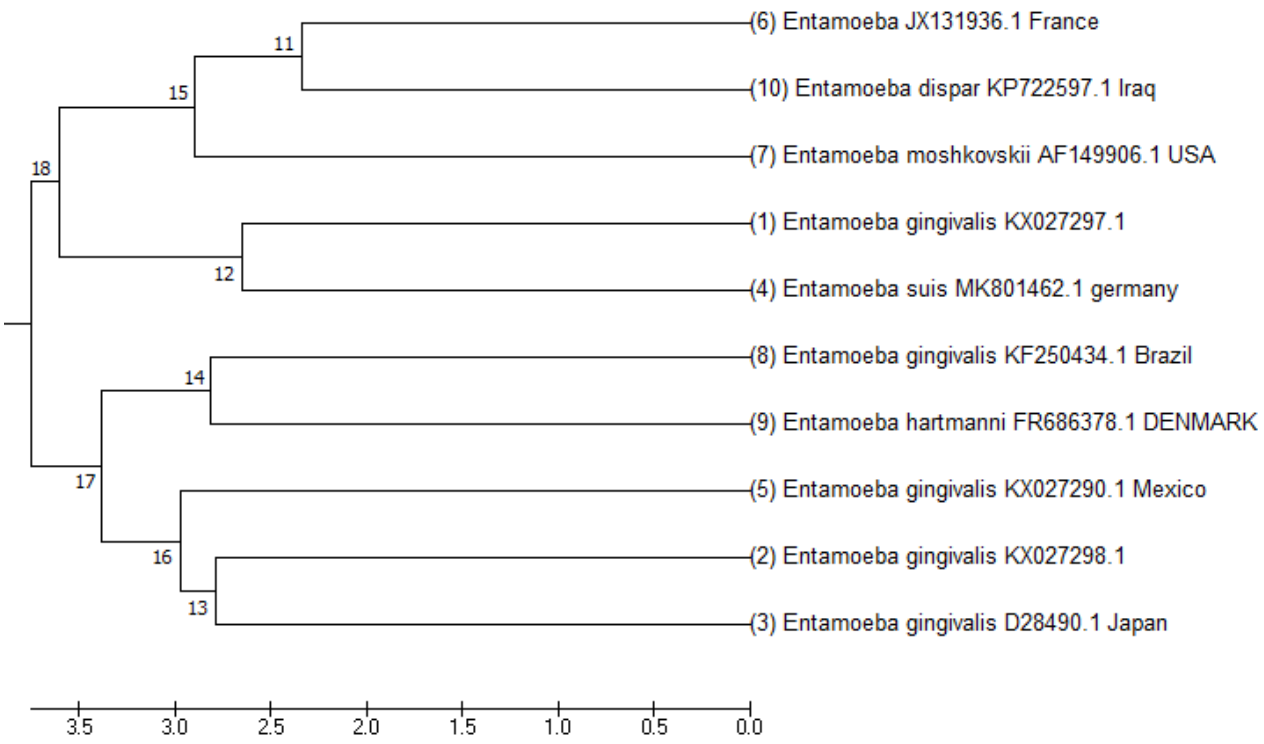

Figure (7:) Phylogenetic relationship of two local isolates of *E. gingivalis* and other entamoeba registered in the GenBank constructed to the Neighbor-Joining method..

Discussion

Comparing to the prevalence of *E.gingivalis* is close to 100% in advanced periodontal pockets, while that in healthy ranged from 0-26% (Linke et al., 1989), the prevalence of oral Entamobiasis (*E. gingivalis*) in this study in examined patients with periodontitis was (77.41%) versus (50%) in healthy control, and in males (81.35 %) comparing to females (73.84 %**).** Our results were close to those referred by (Mohammed *et., al* 2015), who found that the percentage of *E. gingival is* isolated in Baghdad /Iraq, was (76 %) being higher (46 %) in males vs. (30 %) in females through the examination of students at the Collage of Science /Al-Mustasyria University. Another similar results were obtained by Yaseen *et al.*, 2020 in active enrolment of participants at Jordan University Hospital, who found that PCR overall prevalence of *E. gingivalis* in gingivitis and periodontitis diseased patients was 71.7% compared to a lower (40.7%) detected by microscopic observation.

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The percentage of *E. gingivalis* isolation in India was also reported to be high with gingivitis of (88 %) and (76 %) with periodontitis, while in healthy they did not find more than 4% (Ramamurthy *et., al* 2018). The percentage of *E. gingivalis* isolated here in Nineveh governorate and in Baghdad/Iraq, were higher to large extent than those in Iran reported by (Sharife *et., al* 2020) in Adolescents with prevalence of (11.7%.) being (15.9%) in males (5.6%) in females, and by (Mahmoud et al 2019), who found that the percentage of *E. gingivalis* isolated in patients were (12 %) in males vs. (11.7 %) in females.

All the positive 96 samples (77.41%) collected in this study from patients with priodontitis, were successfully cultured on DMEM medium. Development of gene amplification by polymerase chain reaction and the sequencing of a gene of *E. gingivalis* , opened ways for the molecular identification of the parasite (Yamamoto et al., 1995) in this study, twenty of the morphologically – evaluated and cultured *E. gingivalis* samples were successfully amplified of the 18s rDNA of *Etamoeba gingivale* as at a rate of 64% (14/20) shown by targeted amplified product size of 600pb for isolates from patients with periodontitis and 45% (9/20) from healthy people. The results in this study were higher than those reported by (Hussian, 2017) who found that out of 50 samples, only 18 samples tested microscopically were positive, and 20 samples were able to grow in modified culture media, and concluded that the molecular detection by PCR using specific primers of the SSU rDNA gene are the best methods for *E. gingivalis* detection as ceasing to periodontal diseases and distinguish from other pathogens. The prevalence of *E. gingivalis* identification by conventional PCR, reported by (Bonner *et al*.,2014) was 80.6% (58/72) in periodontics cases and to less extent 33.3% (11/33) in healthy people. Moreover, (Cembranelli *et al*., 2013) , found that genetic variants of E.gingivalis have been identified . (Deng *et al*., 2017) achieved that in all *E. gingivalis* 18s rRNA sequence was detected in periodontal pockets and was less abundant in 40-60% in healthy sites (Deng *et al*., 2017).

A low detection limit (27%), was also obtained in the study conducted by Trim *et al*. (2011) in USA, by conventional PCR technique using QIAamp DNA mini kit for the small subunit ribosomal RNA gene (SSU rDNA) to detect the percentage in the periodontal pocket.

The phylogenetic results showed that the distance and closer among strains depending on each sequence. The environment factors play an important roles in changing the sequences. These

factors can insert or delete nucleotides from sequences to create new strain or new genes which is enable the strain to adapting to surrounding conditions.

These results went with the same trend with our results. The addition in this paper was gained by the phylogenic study that the strain of the Local healthy persons is *E. gingivalis* strain (gP45), and *E. gingivalis* strain (gP11) from patients with periodontitis, which mean that not all *E. gingivalis* strain are necessarily pathogenic and some of them are commensals, and that the differentiation should be based on phylogenetic study.

Conclusion:

Entamoeba gingivalis was detected in both healthy and diseased patients with periodontitis but with two different stains, gp11 from the patients with periodontitis disease and gP45 strain from healthy people.

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