Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Feb 25; 10(A):661-667.
https://doi.org/10.3889/oamjms.2022.8713
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Microbiology*

Structural and Genetic Diversity of *Entamoeba gingivalis* Trophozoites Isolated from Diseased and Healthy Periodontal Sites

Marwa A. Elmallawany[1]*, Marwa Ahmed EL-Dardiry[2], Reham K. Nahnoush[1], Mohamed Akmal[3], Adam A. Afife[4], Mohamed S. Badr[5]

[1]Department of Medical Parasitology, Faculty of Medicine, Cairo University, Giza, Egypt; [2]Department of Medical Parasitology, Faculty of Medicine, Fayoum University, Fayoum, Egypt; [3]Department of Orthodontics, Faculty of Dentistry, Cairo University, Giza, Egypt; [4]College of Life Sciences, Faculty of Medicine, Leicester University, Leicester, United Kingdom; [5]Department of Molecular Biology, Medical Research Center, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Edited by: https://publons.com/researcher/391987/mirko-spiroski/
Citation: Elmallawany MA, EL-Dardiry MA, Nahnoush RK, Akmal M, Afife AA, Badr MS. Structural and Genetic Diversity of *Entamoeba gingivalis* Trophozoites Isolated from Diseased and Healthy Periodontal Sites. Open-Access Maced J Med Sci. 2022 Feb 25; 10(A):661-667. https://doi.org/10.3889/oamjms.2022.8713
Keyword: Entamoeba gingivalis; Genotypes; Real-time polymerase chain reaction; High-resolution melting; Mutants
***Correspondence:** Marwa A. Elmallawany, Department of Medical Parasitology, Faculty of Medicine, Cairo University, Egypt.
E-mail: mamamllawany@hotmail.com
Received: 30-Jan-2022
Revised: 14-Feb-2022
Accepted: 15-Feb-2022

Funding: This research did not receive any financial support
Competing Interest: The authors have declared that no competing interest exists

Abstract

BACKGROUND: At present, there is little documented about the variability aspects of Entamoeba gingivalis (E. gingivalis) in relation to periodontal diseases. This is perhaps due to several specialists rejecting the notion that E. gingivalis can cause periodontal disease.

AIM: The aim of the present study was to compare the morphological and genetic variability within trophozoites isolated from diseased (n = 26) and healthy periodontal sites (n = 14).

METHODS: Detailed microscopic analyses were performed, in addition to post real-time polymerase chain reaction 18S-SSU rRNA gene scanning technology, using reference synthetic genes to analyze melting curve features from different isolates.

RESULTS: All trophozoites isolated from diseased sites were significantly larger in size than those isolated from healthy sites. In addition, they were found in clusters, containing many leukophagocytosis and in a significantly higher number than those from healthy sites. Gene scanning revealed diversity within the isolates with a significantly higher number of mutant forms (18 out of 26) within the trophozoites isolated from diseased sites, 14 of them were of unknown origin. Four melting curves matched *E. gingivalis* H57 strain and the remaining eight were related to the wild strain (ATCC-30927). Isolates from healthy sites corresponded to the wild type (12 out of 14) with only two related to H57 strain.

CONCLUSION: The study confirmed morphological and genetic variability between different isolates; We still recommend further in-depth molecular studies to investigate the role of this oral protozoan in the pathogenicity of periodontal affection. The study highlighted the importance of real engagement of multidisciplinary diagnostic strategies, involving experts from variable medical fields to reach truthful scientific outcomes concerning the association of certain microorganism to particular diseases or disorders.

Introduction

Entamoeba gingivalis (E. gingivalis) is a protozoan that usually exists in the oral cavity in trophozoite form without a cyst stage, thus requiring special contact through different means to be transmitted. In general, scientific studies concerning variable aspects related to this oral protozoan are not only very limited but also controversial. This is perhaps due to the denial by some clinical specialists that it may be the cause of such oral diseases. The difficulties facing the direct diagnosis of this oral parasite, in addition to the complexity in maintaining the parasite *in vitro* possibly added a burden to the researchers in this field and certainly reflected on the published research concerning this infection [1], [2].

Many investigators considered *E. gingivalis* as a commensal organism which is usually found within the oral cavity [3, 4]. Others deem it as a pathogenic protozoan as it is frequently observed in periodontal pockets and considered as an important cause of periodontal disease [5]. Conversely, some studies had reported the presence of *E. gingivalis* in healthy subjects as well as patients suffering from periodontal disease, either immunocompetent or immunocompromised [4], [6]. Thus, the association of periodontitis with this protozoan is still debatable, fluctuating from complete accusation to intense rejection of such an association [5].

Kikuta and his colleagues [7] were the first research team to successfully amplify the small subunit of ribosomal RNA gene (SSU rDNA), using laboratory cultured *E. gingivalis* and designing their own original specific primers. Subsequently, these specific primers were used by the same authors to amplify *the* specific DNA isolated from dental plaques of patients suffering from periodontal disease, performing both conventional and real-time quantitative polymerase chain reaction (qPCR). Surprisingly, the authors recorded much higher sensitivity of the later molecular technique than the conventional one which failed to detect 42% of cases

proven to be positive by both microscopy and qPCR. This information about conventional PCR seemed shocking and urges any researcher to carefully consider utilizing the traditional PCR in their scientific research [5].

Furthermore, little is recognized concerning the genetic diversity of such oral parasite in relation to its exact responsibility about the occurrence of periodontal disease or its damaging consequences. The evidence of *E. gingivalis* genetic variability and its unhealthy oral manifestations was recorded among a particular category of immunocompromised patients suffering from HIV/acquired immunodeficiency syndrome, applying conventional PCR, followed by sequencing [2]. A high genetic diversity has been discovered between two different *E. gingivalis* isolates, identified in different categories of human subjects. The authors named the strain isolated from the infected patients as ST2 kamaktli variant and documented its prominent genetic deviation as well as its virulence from the ST1 *E. gingivalis* strain, performing the same consecutive molecular techniques; PCR followed by sequencing. However, these authors did not relate the identified variants with any morphological features [8].

High-resolution melting (HRM) curve analysis was innovated in the University of Utah by Idaho Technology aiming at minimizing the cost of the molecular techniques plus discovering the genetic diversity in a particular organism. The technique facilitates not only real-time analysis but also rapid, sensitive, and easy detection of genetic polymorphisms, mutations plus epigenetic variations within the double-stranded DNA sample in a closed tube, immediately after PCR amplification. The technique necessitates a real-time PCR machine with exceptional thermal stability and sensitivity, plus a specific software analytic program [9]. The present study assessed the possibility of *E. gingivalis* genetic variability in relation to morphological characters within healthy subjects versus patients suffering from periodontal diseases, performing HRM technology following real-time amplification of the lower ribosomal subunit of the 18S region (18S-SSU rRNA).

Subjects, Patients, and Methods

Study design and sample collection

The present cross-sectional study was approved by the ethical committee of Faculty of Medicine, Cairo University. Subjects attending outpatient clinics of oral medicine at Faculty of Dentistry, Cairo University, and suffering from periodontal diseases were enrolled in this study and informed consent was obtained from all enrolled subjects. Patients who recently received antibiotics or periodontal therapy, 3 months before enrollment, pregnant and immunocompromised subjects were all excluded from the study. A full oral clinical examination was performed at six locations, involving all teeth and considering the specific periodontal parameters**;** gingival index, plaque index, and probing depth in addition to clinical attachment level [10]. The diseased cases were managed and followed up according to the current institutional protocol. Patients were subjected to a complete oral examination involving visibly carious, tooth number and degree of mobility, evident plaques, and gingival bleeding plus other features.

Sample collection was done ensuing cautious drying of the selected sites, using a periodontal probe for healthy controls. In patients suffering from periodontitis, a sterile curette was used at the affected locations and samples were obtained from periodontal pockets greater than 4 mm. Following this, the specific mechanical management was completed by the specialist. Samples from each subject were divided into two. The first portion was diluted using poly vinyl alcohol solution at 25°C–28°C to be stained with iron and hematoxylin, according to the manufacturing instruction's manual (I and H, Dalynn Biologicals, catalogue No.SI70 and 71). Then, parasitological analysis was performed, using high power and oil immersion magnifications. *E. gingivalis* trophozoites were identified by their characteristic features, relying on its unique nucleus, pseudopodia, vacuoles, and leukophagocytosis inclusions [11]. The size of the parasitic stages was microscopically measured according to [12]. Observed trophozoites were measured using an ocular micrometer that had been calibrated against the stage micrometer in combination with the specific objective lens. For molecular technique, 100 μl of RNAlater ™ Qiagen® solution was added to the second portion of each sample which was placed in a sterile vial and then kept at –20°C until used for molecular analysis.

DNA extraction and amplification of Entamoeba gingivalis18S-SSU rRNA by quantitative polymerase chain reaction

In accordance with the manufacturer's instructions, DNA from each sample was extracted using QIAamp DNA Mini Kit (Qiagen®, Germany). The extracted DNA samples were quantified before molecular analysis using spectrophotometric analysis. Real-time PCR technique was performed qualitatively in this work intended for post-amplification HRM analysis, using the original primers designed in a previous study, for positive microscopically samples diagnosed by parasitologists [**7**]. The molecular technique was performed for post-amplification gene scanning, so no standard curve was created. The primers were purified by high-performance liquid chromatography and purchased from Metabion, Planegg, Germany. For amplification and HRM analysis, the LightCycler® 480 operator's manual was followed with a ready-to-use 2X conc., hot start reaction mix designed for amplification and detection of specific DNA sequence in the presence of the proper PCR primers. This step was followed by HRM

analysis to detect variants among different isolates, taking into consideration, the easy optimization of Mg^{2+} concentration supplied with the system ($MgCl_2$ stock solution) to avoid nonspecific byproducts. According to the *operator's* manual, 10 μl of the master mix, 2x conc. was used, 1.0 μl for a final conc. For each of the primers, 0.2 μm was added in the PCR reaction; forward primer; (5′-GAATAGGCGCATTTCGAACAGG-3′), reverse primer; (5′- TCCCACTAGTAAGGTACTTACTC -3′), 5 μl of the extracted genomic DNA and the reaction mixture was adjusted to a final volume of 20 μL using PCR grade water. The whole procedure was done in 75 minutes including 10 min pre-incubation and 15 min HRM. The condition parameters were as follows; one cycle pre-incubation, hot start 95°C for 7.5 min, 45 cycle amplification of 95°C for 1 min, followed by touchdown protocol covering a range of annealing temperatures from 65°C to 53°C, then 72°C for 30 s, followed by a 5 min extension at 72°C. All samples were run in duplicate and a negative control was included by replacing the template DNA by water PCR grade supplied within the kit.

Gene scanning and high-resolution melting curve analysis

The analysis was done using hot start technology and EvaGreen dye. Samples with variations in DNA sequence were differentiated by discrepancies in melting curve shape, compared to reference synthetic genes. The process began at 95°C for 5 min followed by 0.5°C decrease in temperature every 30 s to 47°C [13].

Internal control and reference genes

The three variable sequences of *E. gingivalis* genes were synthetized by gene synthesis technology to be used in this study as reference genes at Finan Company, Egypt [13], [14]. Genetic sequences corresponding to these strains were obtained from GenBank and identified by their accession number the original reference strain, ATCC-30927 (D28490) and the polymorphic *E. gingivalis* C (KF250433) and *E. gingivalis* H57 (KF250436) strains with single-nucleotide polymorphism (SNP). For logical as well as financial reasons, there was no need to use the other two *E. gingivalis* strains (*E. gingivalis* H14 and E) which were documented by the authors, being exactly the same sequence as H57. For the detection of *E. gingivalis* and its mutants, two types of original plasmid, "wild" (W) and "mutation" (M1 and M2) plasmids, were created using the TA cloning kit (Invitrogen Corp., San Diego, CA).

Statistical analysis

SPSS statistics version 24 was used to analyze all data. Qualitative data were expressed as percentage and frequency. Numerical data were expressed as mean or range. Chi-square test was used to determine the relation between qualitative variables. $p < 0.05$ indicated statistically significant correlation while > 0.05 was not significant association [15], [16].

Results

The present study involved 40 samples which were proved positive for *E. gingivalis*, microscopically and subsequently molecularly, by real-time PCR. These 40 isolates were obtained from two categories of subjects; Group A; 26 patients suffering from chronic periodontal diseases (14 males and 12 females with mean age, 32.923 ± 5.214 years), plus 14 healthy volunteers (8 males and 6 females; with mean age, 33.429 ± 4.484 years). It is important to mention that these 40 positive samples were identified and enrolled in the study after examining samples from a total of 144 subjects.

With regard to the parasitological confirmation, motile *E. gingivalis* trophozoites were evident microscopically within all 40 positive specimens. The trophozoites were apparently smaller than that of *Entamoeba histolytica.* They were observed with the characteristic morphological features in the form of a single nucleus containing a tiny central karyosome with a rim of chromatin at the periphery and a delicately granular cytoplasm (Figure 1). It was noted that the inclusion bodies in the form of leukophagocytosis were clearly found within the cytoplasm of all samples taken from diseased cases. In addition, numerous trophozoites were found in bunches; resembling nests, within most of the samples isolated from diseased group (17 out of the 26). Fewer numbers of trophozoites were noticed in samples taken from healthy subjects. Interestingly, the size of trophozoites isolated from diseased cases (18.554 ± 1.375 μ) was significantly larger than of those taken from healthy subjects (12.679 ± 0.944 μ). Significantly more trophozoites were identified in patients with periodontal disease compared to healthy patients. 86.577 trophozoites ± 11.04 were recorded/10 microscopic fields within the examined samples from diseased cases, while only 5.357 trophozoites ± 1.905 were detected within samples of healthy subjects.

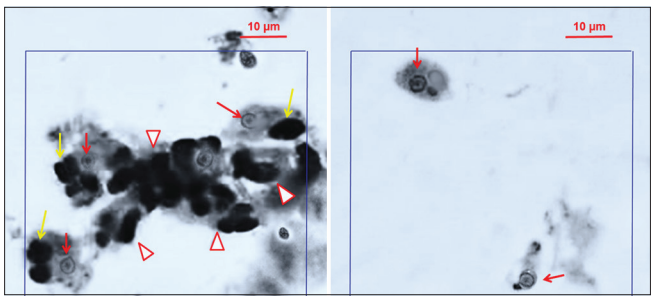

Figure 1: A mass of Entamoeba gingivalis trophozoites stained with Iron and haematoxylin stain with characteristic nucleus (Red arrow) with extensive collection of cytoplasmic dark inclusion bodies, leuckophagocytic vacuoles (yellow arrows). Many trophozoites appear missing the characteristic nuclei (arrow heads)

Table 1: Demographic data, oral health condition, morphological features, and genetic types of *Entamoeba gingivalis* related to diseased and healthy subjects

Variables	Diseased subjects (n = 26), n (%)	Healthy subjects (n = 14), n (%)	p
Demographic data			
Age (years), mean ± SD	32.923 ± 5.214	33.429 ± 4.484	0.598
Sex			
Male (n = 22)	14 (53.8)	8 (57.1)	0.842
Female (n = 18)	12 (46.2)	6 (42.9)	
Oral hygiene			
Poor (n = 27)	23 (88.5)	4 (28.6)	0.000*
Good (n = 13)	3 (11.5)	10 (71.4)	
Morphological features of *E. gingivalis*			
Number of trophozoites/10 field (mean ± SD)	86.577 ± 11.04	5.357 ± 1.905	0.000*
Size of trophozoites (μ) (mean ± SD)	18.554 ± 1.375	12.679 ± 0.944	0.000*
Leukophagocytosis			
Present	26 (100)	5 (35.7)	0.000*
Absent	0 (100)	9 (64.3)	
Bunches of trophozoites			
Present	17 (65.4)	0 (0)	0.000*
Absent	9 (34.6)	14 (100)	
Genotypes			
E. gingivalis strains			
Wild type	8 (30.8)	12 (85.7)	0.000*
H57	4 (15.4)	2 (14.3)	
C	0 (0)	0 (0)	
Mutant	14 (53.8)	0 (0)	

*Statistically significant, P<0.05. SD: Standard deviation, *E. gingivalis: Entamoeba gingivalis.*

Gene scanning revealed diversity within the isolates with a significant higher number of mutants (18 out of 26) within the trophozoites isolated from diseased sites, 14 of them were of unknown origin. Four melting curves matched E. gingivalis H57 strain, while the remaining eight were related to the wild strain (ATCC-30927). Twelve out of 14 isolates from healthy sites corresponded to the wild type with only two related to the H57 strain (Table 1 and Figure 2).

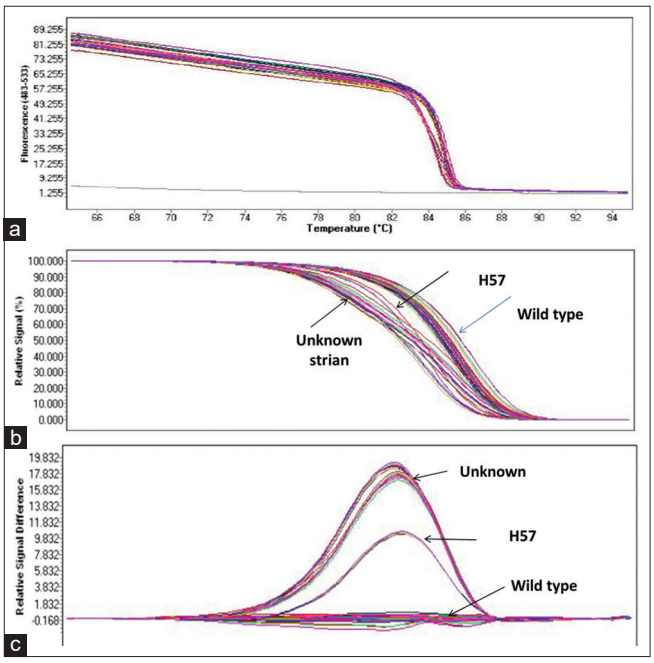

Figure 2: Figures represent HRM analysis generated by LightCycler 480 real-time PCR system and the mutation detection approaches A; Pre-melt (initial) and post-melt (final) fluorescence signals of all samples. B; normalized melt curve to relative values. C; Curve differences as magnified by subtracting each curve from the most abundant type

Discussion

Very few published scientific studies relied on the specific parasitological parameters to identify the oral protozoan microscopically, yet nearly all of them confirmed its high occurrence within progressive periodontal pockets and from 0 to 26% within healthy periodontal sites [17], [18], [19], [20], [21], [22], [23], [24]. Unfortunately, for unknown reasons, all these articles neglected the difference in morphological characteristics between the detected trophozoites among the diseased and healthy periodontal sites, except in a study which reported detailed information, yet not specifically in relation to the different conditions [24]. That is why this study aimed to investigate this structural issue in addition to the molecular investigation proposed to detect genetic variability within the isolates.

The present study revealed significant morphological differences between *E. gingivalis* trophozoites within diseased and healthy sites including the size of parasitic stages, specific morphological variations in addition to the mean number of trophozoites between the two categories. Detailed morphological features reported in this study were sufficient to differentiate the parasitic stages within the diseased and healthy periodontal sites. This was in contrast to the findings reported by [8] in which morphological characteristics were ignored and excluded by the authors. They attributed this to the ineffectuality of microscopic examination to differentiate between amoebas. Similarly, other authors reported similar findings concerning morphology, in which no characteristic findings were reported [25], [26], [27], [28], [29].

García *et al.* [8] highlighted an example for such a problem, however, it was related to *E. histolytica* and *Entamoeba dispar.* They stated that both amoebas are morphologically similar, but their genetic and pathogenic differences allow them to be classified as independent, although closely related species [30]. However, many authors recorded a clear microscopic difference related to erythrophagocytic activity which was clearly observed in the former species, but never observed in the latter one. These unique microscopic findings are certainly in need of professional specialists, not to be misdiagnosed or misinterpreted. Thereby supporting the urgent need of real engagement of multidisciplinary diagnostic strategies, involving specialists from a variety of medical fields to reach truthful scientific outcomes concerning the association of certain microorganisms with particular diseases or disorders.

Morphologically in this study, in addition to the observed characteristic nuclei, leukophagocytosis vacuoles were found within all the trophozoites isolated from affected periodontal sites. This may be related to certain virulence factors, facilitating the invasive power

of *E. gingivalis* trophozoites, which is more or less similar to what is reported by [31], but in connection with erythrophagocytic ability of *Entamoeba histolytica*. This ability was not documented within the non-pathogenic *E. dispar* which has morphological features similar to *E. histolytica*. Phagocytosis of blood elements has been proposed as a pathogenicity indicator as well as prominent virulence marker for *Entamoeba* species [32], [33].

The significant difference regarding the larger size of *E. gingivalis* trophozoites identified within the diseased sites, possibly resulting from engulfing a number of white blood cells which may have cause cytoplasmic expansion. In fact, *E. gingivalis* is able to engulf one or more human cells at a time, mainly polymorphonuclear cells and neutrophils. These are the chief cells within the periodontal pockets, so engulfing these cells may exposes the first line of the innate immune mechanism to intense danger by consuming its powerful weapon [34]. Thus, *E. gingivalis* finds a perfect environment involving damaged tissue, bacteria, and fungi, permitting its safe establishment and colonization. This may explain the significantly higher number within samples isolated from the diseased cases, taking into consideration that all the enrolled subjects were immunocompetent. This intense colonization within immunocompetent subjects was previously reported in another work [24].

The existence of different parasitic subtypes with a higher proliferative ability and/or proteolytic activity was reported [8]. This explains why this study intended to molecularly scan the different isolates, performing post real-time PCR HRM to detect possible genetic variability between them. In this study, a significantly higher number of wild type was reported within healthy subjects. While a higher number of mutants were reported within diseased cases which were related to the known H57 strain of [2], plus unknown mutants revealed by HRM analysis (14 out of 26). Mutations observed in this work may have been triggered as a result of changes within the oral environment, which possibly turn the commensal amoebae into pathogenic one, creating a vicious circle that facilitates more periodontal damage without cellular immune controlling mechanism, resulting in acceleration of amoebic colonization and so on. This is in concordance with the study that documented the presence of *E. gingivalis* in some sulci that were not causing any symptoms as well as in diseased sites. However, certain conditions that change the surrounding environment can cause the pathogenic condition in oral cavity [35].

In general, molecular tools such as specific target gene amplification and sequencing certainly expand genetic information concerning *E. gingivalis*. This parasite was molecularly identified, using a long amplicon of 1400 bp. This work has invoked more debate concerning its molecular identity which was documented by various investigators [36]. *E. gingivalis* was molecularly detected only in diseased periodontal sites [6], [7]. While, the parasite was molecularly identified within 33.3% and 60% healthy subjects, respectively. The higher molecular results within the healthy subjects were explained to be due to the accurate control of PCR inhibitors and matrix degradation [34], [37].

Genetic variability of *E. gingivalis* has been recognized by some authors who studied the genetic variability of 18S-SSU rDNA [2], [38]. Furthermore, genetically identical *E. gingivalis* species revealed different virulence factors as reflected by transcriptomic study [39]. This may explain the discrepancies in their molecular detection when compared with microscopy or clinical diagnosis as reported in this study. The presence of a kamaktli *E. gingivalis* variant which is located within a closely related clade to ST1 was documented but the authors confirmed its mutability and named it as ST2. However, they did not mention any link to pathogenesis or anything related to the periodontal manifestation [8]. Formerly, characterization of three samples of *E. gingivalis* by riboprinting the 18S rRNA region was done. Differential banding patterns observed after sample treatment with the restriction enzyme Rsa1 allowed the categorization of these three samples into two ribodemes: Ribodeme-1 (two oral isolates) and ribodeme- 2 (one uterine isolate). Dissimilar band patterns created by kamaktli variants [40]. The disparity from both ribodemal strains 1 and 2 was documented [8]. It was suggested that the unidentified *E. gingivalis* genetic type that was detected in immunocompromised patients is possibly related to kamaktli variant [2].

On the other hand, no cyst stage was found within our samples and all the observed parasitic stages were related to *E. gingivalis* trophozoite stage. This in fact was in concordance with most of the aforementioned documented reports. The report of García *et al.* [8] triggered a new debate about *E. gingivalis* when they suggested a possible ability of their kamaktli variant to produce a cyst stage which had never been suggested before, still leaving the morphology of such a variant in pending situation. Before this, some authors advised the investigators in this field to postpone any formal taxonomic categorization until clear morphological data are obtainable [28]. However, the kamaktli variant was categorized by the authors without any identifiable morphological characteristics, even the parasitic stage's level. Other authors discussed this issue and concluded that *E. histolytica* is the only well-documented human pathogen among all known amoebae and recommended the urgent need of an accurate and efficient detection methodology to correctly document other types of amoebae. Unfortunately, the debate concerning the role or the ability of some amoebae to initiate pathogenic effects within certain tissues (as in *E. gingivalis*) is still considered as a big obstacle facing the deeper understanding of its role in the pathogenicity of periodontal infection [6].

In this study, samples were collected from at least three different sites in each subject and confirmed positive after repeated microscopic examination by specialists. Only samples with positive microscopic confirmation were included, otherwise, all samples proven to be negative were excluded from the study. This may be due to the nature of our study which was not intentionally designed to compare variable diagnostic tools. The design was mainly targeting the detection of morphological and genetic diversity within different isolates. Therefore, parasitological confirmation, involving detailed morphological features, was a must. This possibly facilities specific figuring of isolates from different subject categories and possibly supported distinguishing each of them. Real-time PCR is an extremely sensitive molecular technique that operates within a closed system, thus avoiding the possible contamination during the post-polymerization process in conventional PCR. EvaGreen dye was used in our technique which is a third-generation dye developed to avoid PCR inhibition which may occur during the use of other dyes [13]. In the present study, another benefit was achieved by this molecular technique which was facilitation of simultaneous discovery of mutations using HRM software analysis and synthetic reference strains. Together, parasitological and molecular results supported the concept that *E. gingivalis* is one of the contributors to periodontal diseases, hence necessitating proper management to avoid the unnecessary destructive impact of this oral protozoal infection.

Conclusion

This study confirmed the morphological and genetic variability between different isolates and recommends further in-depth molecular studies to investigate the role of this oral protozoan in the pathogenicity of periodontal affection. This study also highlighted the importance of real engagement of multidisciplinary diagnostic strategies, involving the experts from a variety of medical fields to reach truthful scientific outcomes concerning the association of certain microorganism with particular diseases or disorders.

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