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# MOLECULAR DETECTION OF *ENTAMOEBA GINGIVALIS* USING POLYMERASE CHAIN REACTION

RAFLAA S. H. HUSSIAN

Department of Biology, College of Science, University of Babylon, Hillah, Iraq
Email: biobabil@yahoo.com

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## ABSTRACT

**Background and objective:** Periodontitis is still until now not understood, it's being recorded as public health problems. This disease is caused by bacteria and parasite. Two parasites found in mouth *Trichomonas tenax* and *Entamoeba gingivalis*. *Entamoeba gingivalis*; opportunistic parasite finding in mouth, has only trophozoite form. This parasite possibly has a connection with Periodontitis. The objective of the study: compares between multi – methods for *E. gingivalis* detection, including microscopic examination, culturing the parasite in a modifying Trypticase-Yeast Extract-Serum-Gastric Mucin-9 (TYSGM-9) medium and molecular detection of Polymerase Chain Reaction (PCR).

**Methods:** 100 Cotton swab samples from patients with Periodontitis were collected using three or two sterile cotton swabs for everyone. The 50 samples were diagnosed by microscopic examination and 50 samples culturing on modifying TYSGM-9 media and then using PCR to insure found Entamoeba gingivalis grew on media and The 50 samples were diagnosed by PCR using specific primers of the SSU rDNA gene (Small Subunit of the ribosomal DNA gene). The No. of sample under testing 100 or 150 .

**Result:** About 18 (36%) samples positive by microscope, and 20 (40%) samples positive by culture. Whereas the result of PCR positive for these 20samples of culture. So the PCR technique is the best method to detect *E. gingivalis* and modify TYSGM-9 media is perfect to grow this parasite. You mean all patients under study infections by parasite but no bacteria?

**Conclusions:** The modifying TYSGM-9 medium and 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 distinguished from other pathogens.

**Keywords:** *Entamoeba gingivalis*, modifying TYSGM-9 media, PCR and SSU rDNA gene

## INTRUDUCTION

Periodontitis has multiple clinical types. It was distributed widely among people (Burt 2005); Causes by bacteria and parasite such as *Entamoeba gingivalis and Trichomonas tenax* (Robert, et al., 2011). *Entamoeba gingivalis* is a parasitic protozoa of the oral cavity, it has trohpozoite only, which not create cyst (Gros 1849). This parasite is one of *Entamoeba* kind, which have a small nucleus and multiple vacuoles (Okada, et al., 2002). Periodontitis is most popular diseases all over the world. Inflammation of gum is characterized by pain, bone loss and bleeding of gum. In some cases, erosion of gum causes loss of tooth. So these signs are important for diagnosis of this dise-ase, so patient feel discomfort (Steele, et al., 2004). Habits of patients are related to frequent infection. For that we can detect *E. gingivalis* by using genetic methods (Kikuta, et al., 1996). Some studies helped to make sure the pre-sence of that parasite in periodntal pockets via molecular methods (Bonner, et al., 2014). The objective of the study compares between multi – methods for *E. gingivalis* detection, including microscopic examination, culturing the parasite in a modifying TYSGM-9 medium and molecular detection by Polymerase Chine Reaction (PCR).

## MATERIALS AND METHODS

**Sample collection:** The samples (100) were taken from patients with Periodontitis using two or three sterile cotton swabs for everyone. The 50 swabs used to detect *E. gingivalis* using microscope, whereas, the other swaps (50) have been cultured on modifying TYSGM-9 media.

**Microscopic examination:** The 50 swabs were tested under microscopic directly using wet mount swabs with normal saline (Jabuk, et al., 2015).

**Culture:** The 50 samples were culturing on modifying medium Trypticase-Yeast Extract-Serum-Gastric Mucin-9 **(**TYSGM-9) (this media prepare from: Casein digest peptone 2.0gm, Sodium chlo-ride 7.5 gm, Potassium phosphate dibasic 2.8 gm, Yeast extract (BBL) 1.0 gm, Potassium phosphate monobasic 0.4 gm and distilled water 970 ml) for the parasite growing, which incubated at 37˚C for 48-72 hours (Hussien 2017). After that the culture was examined with microscope to insure the prese-nce of *E. gingivalis* and then accomplished by molecular technique using PCR.

**DNA extraction:** The DNA of the parasite was extracted using Stool or Tool DNA isolation-mini kit (Favroprep) according to the manual guide instructions. The DNA extracts were examined by agarose gel electrophoresis. DNA quantity and quality were determined using an ultraviolet-visi-ble

Spectrophotometer-NanoDrop instrument (OP-TIZ-EN POP - Korea). The concentration of DNA was measured at 260 nm, which ranged 50-100 ng/ ml and the DNA purity ranged between 1.7 and 2.0. The DNA extracts were stored at -20°C until PCR accomplished.

**PCR amplification and electrophoresis:** For *E. gingivalis* detection, the target DNA regions of the SSU rDNA gene were amplified using specific primers were mentioned in Table 1. The reaction mixtures were prepared using a PCR Master Mix (Promega GoTaq® Green Master Mix -USA) according to kit instructions. Each PCR reaction was carried out in a final volume of 20 µl under the conditions were displayed in Table 2. The reaction mixture composed of The using 12.5 µl of master mix from (promega company) but 5 µl mastermix from bionner company(making sure), 2µl of DNA sample (40ng), 1.5µl of each forward and reverse primer and 10µl free-nuclease distilled water. We used a TRIO Thermal Cycler (Biometra-Germany) for gDNA amplification. PCR products and 100bp DNA molecular ladder (Promega) were migrated and visualized in 1% (w/v) agarose gel electrophoresis in 1X of TAE buffer at 6 V/cm for 1 h. TAE (Tris Acetate- Ethylene-diamine-tetraacetic acid) buffer composed of 0.040 mol Tris-acetate and 0.001 mol EDTA were dissolved in 1 l deionized distilled water and the pH value was adjusted to 8.0±0.2. Gels were stained with ethidium bromide solution (5µg/ml) using horizontal electrophoresis (Cleaver Scientific, UK) and Gel Documentation System (UVsolo touch, Biometra-Germany).

**Table 1: Primers used in this study**

| Gene | Primer sequence | $T_m$ | Fragment size (bp) | Ref. |
|---|---|---|---|---|
| SSU rDNA | F: 5′-AGGAATGAACGGAACGTACA-3′ | 56.4 | 203 | 6, 7 |
| | R: 5′-CCATTTCCTTCTTCTATTGTTTCAC-3′ | 60.9 | | |

**Table 2: PCR protocol**

| Thermocycle | Temperature (°C) | Time (min) | Cycles |
|---|---|---|---|
| Initial denaturation | 94 | 3.5 | |
| denaturation | 94 | 1 | 40 |
| Annealing | 60 | 1 | |
| Extension | 72 | 1 | |
| Final extension | 72 | 1 | |
| Holding | 4 | | |

**Data Statistical analysis:** Data were analyzed using SPSS version 22 software Fisher's exact and T-Test with a significant $P<0.05$.

## RESULTS AND DISCUTION

Out of 50 samples tested by microscope, 18 samples were positive for *E. gingivalis*. Whereas, only 20/50 samples contained the parasite and had ability to grow in the modifying culture media (Fig.1). Out of 50 samples only 20 samples gave positive detection by PCR using specific primer of SSU rDNA gene (Small Subunit of the ribosomal DNA gene) as shown in figure (2); And the same samples gave the parasite cultures (20/20) which appeared positive molecular detection of *E. gingivalis* by PCR. These results indicate that the modi-fying TYSGM-9 medium and PCR detection were more specific for Derection of *E. gingivalis* than microscopic examination. In the other hand, the results of the parasites detected by microscopy primarily may be belong to the species *E. gingivalis* but not absolutely and that the presence of the parasite is correlated with periodontitis.

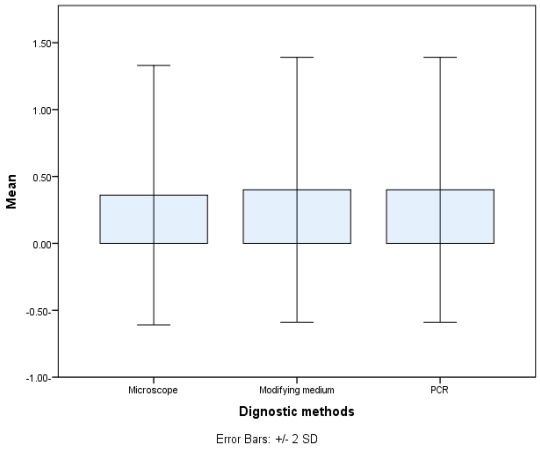


**Fig. 1:** Comparison between diagnostic methods of *Entamoeba gingivalis*.

The methods, including the direct examination by the light microscope, culturing in modifying TYS-GM-9 medium and PCR technique using specific primers of the SSU rDNA gene for *E. gingivalis* detection.

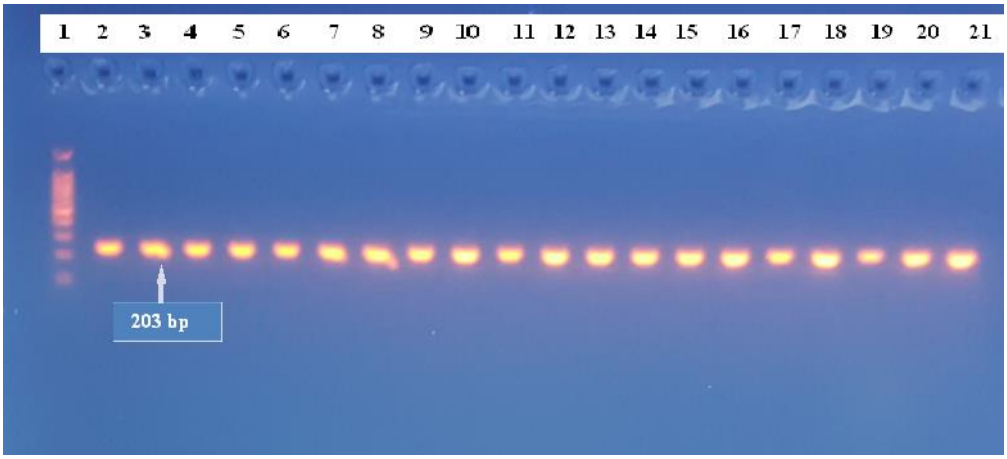


**Fig.2: Agarose gel electrophoresis of amplified band of SSU rDNA gene for *Entamoeba gingivalis* detection.**
Lane 1: Ladder 100-1000 bp; Lanes 2-21:Patients samples (*E. gingivalis* were isolated from patients and cultured in modifying TYSGM-9 medium. Electrophoresis conditions: 1% (w/v) agarose gel, 1X of TAE buffer and 6 V/cm for 1 h.

*E. gingivalis* found in oral cavity lives in gum, on the surface of the tooth and gingival pockets. The first way to diagnose this parasite using wet mount sample and tested by microscope(Jabuk, et al., 2015). The other way by culturing the parasite using different media, including modifying (MSF) culture, Wantland's egg medium, and (TTY-SB) to grow *E. gingivalis*. The comparison among them, the researchers found that these media were not suitable for culture or detection the parasite under different conditions (Gannon and Linke 1989). While, the other study used a modify Trypticase-Yeast Extract-Serum-Gastric Mucin to grow this parasite and showed it is a suitable medium for E. gingivalis growing (Hussien 2017). In recent study used modify (TYSGM-9) to grow parasite within 24h. and reach high growing at 72h. without using any solution for prasites growth. There are numerous genetic studies for Entamoeba genus, particularly to identify viru-lence factors of the pathogenic organism Entamoeba histolytica. In some studies, new amoebae similar to *E. histolytica*, recognized in samples from animals, and the molecular identification created the phylogenetic trees of the *Entamoeba* genus depending on their small subunit ribosomal DNA sequence (Stensvold, et al., 2011). Amoebae have the ability to colonize the human host are extensively within the genus, depending on the sequence of this gene; They reflected their differences in the nucleus number of their cyst form. The mouth-colonizing *E. gingivalis* has no identified cyst form, in spite of its vicinity to species producing mono- or tetra-nucleated cysts, depending on their small subunit ribosomal DNA (SSU rDNA) sequences (Bonner, et al., 2014). Recently, the researchers have been using PCR technique to detect *E. gingivalis* depending on genetic material(Bonner, Amard et al. 2014). That's useful to ensure it's the same parasite.

## CONCLUSION

The modifying medium Trypticase-Yeast Extract-Serum-Gastric Mucin-9 (TYSGM-9) and molecu-lar detection by PCR using specific primers of the SSU rDNA gene are the best methods for *E. gingi-valis* detection as ceasing to periodontal diseases and distinguished from other pathogens.

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