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Molecular Study of *Entamoeba gingivalis* and *Trichomonas tenax* Among Plaque Induced Gingivitis Patients in Babylon Province

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Abstract: The oral cavity of humans contain many microorganisms and some parasites , which were shown that two protozoans, *E. gingivalis* and *T. tenax* may be responsible for oral parasitic infection. and these organisms causes damage of teeth and gum, and have an important effect in causing these pathological symptoms This study was designed to detect these parasites in oral cavity of patients with plaque induced gingivitis and compare it with healthy persons by using RT(qPCR) technique .

Materials and Methods : The study involves 100 subjects between age groups (>15-65)years,50 patients who are taken from them clinically diagnosed with gingivitis (plaque-induced gingivitis) whose attending to specialized dental center in Al-Hilla city, and from 50 person who are (Healthy Control), were collected from each patients dental plaque and saliva with clean tubes samples ,some factors were taken such as pH saliva, age groups, sex, smoking status, and chronic diseases status of patients (blood pressure, heart disease etc.). and then transferred these saliva samples to specific refrigerator (deep freezing) for molecular testing in (Scientific private Company)in Al-Diwaniyah, Iraq .

Results: This results shown that the overall percentage rates of infection with *E. gingivalis* and *T. tenax* in dental plaque samples were higher than in saliva samples in patients with gingivitis (9(56.3%), 19(55.9%)) respectively. these two parasites are present in healthy individuals less than in patients with gingivitis . there were statistically significant correlation between (sex, age groups, pH saliva and chronic diseases) and presence of the gingivitis disease, In addition, this result shown *E. gingivalis* infection by use RT(qPCR) technique and melting curves analysis (44.0%)of total case group patients with gingivitis.

Conclusion: This study showed *E. gingivalis* more common in patients with gingivatis diseases is that considered a protozoan in oral cavity. There was relationship between the presence of these parasites and the type of samples and the factors as (pH saliva, age groups, sex, smoking status, and chronic diseases status of patients (blood pressure, heart disease etc.).

Introduction

The oral cavity of humans contain many microorganisms and some parasites such as *E. gingivalis* and *T. tenax* and these organism causes damage of teeth and gum and has several properties that make it an environment for many micro-organisms (1) .

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The incidence is high in cases of gum disease, tonsillitis, tooth decay, and the yellow layer accumulates around the teeth, and these parasites have an important effect in causing these pathological symptoms (2,3) **.**

Some authors believe that these commensal could be opportunistic, that these, capable of proliferating in a gingival environment modified by periodontal and gingivites disease (4) .

E. gingivalis and *T. tenax* are parasites species that infect humans, transmission is primarily through oral-oral touch, droplet spray or exchanging eating utensils, transmitted either personally (kissing) or indirectly by trophozoite contaminated food, The trophozoite stage (its infective and the diagnostic stage) (5,6,7,8).

Aims of the Present Study

The present study aimed to applied the following objectives:-

1. Detection of *E. gingivalis* and *T.tenax* by using PCR and RT-PCR technique among plaque-induced patients with gingivitis and periodontal disease patients and then compare with healthy oral individuals.
2. Study the relationship with some epidemiological criteria (Gender, Age groups, pH, smoking status and patients status if suffering from chronic diseases .

MATERIALS AND METHODS

Samples collection

The study involves 100 persons their age groups between (>15-65)years,50 patients who are taken from them clinically diagnosed with PIG (plaque-induced gingivitis) whose attending to specialized dental center in Al-Hilla city, and from 50 person who are (Healthy Control), were collected from each patients, dental plaque and saliva samples taken with clean tubes , some factors were registries such as pH saliva, age groups, sex, smoking status, and chronic diseases status of patients (blood pressure, heart disease etc.). and then then transformed these saliva samples to specific refrigerator (deep freezing) for molecular testing in (Scientific private Company) in Al-Diwaniyah province Iraq.

Genomic DNA Extraction

Genomic DNA was extracted from oral wash samples(saliva and dental plaque) by using gSYNCTM DNA Extraction Kit (Geneaid. Taiwan) done according to company instruction as following steps:

200µl oral wash samples were transferred to 1.5 ml micro-centrifuges tube and 200mg silica beads. 200µl GST Buffer and 20 µl proteinase K was added to each tubes and then homogenized vigorously by vortex mixer adaptor.The samples incubated at 60Cº for at least 30 minutes . 200µl of GSB Buffer was added and then shake vigorously for 10 seconds. The samples incubated at 60Cº for 10 minutes.200µl absolute ethanol was added and then shake vigorously for 10 seconds. The lysate was carefully transferred into spin column that fitted in a two ml collection tube, and then closed the tubes and centrifuged at 8000 rpm for one minute.

Throughout lysate were discarded in disposal bottle, and then 500µl Washing buffer 1 (W1) was added to each spin column, and centrifuged at 8000 rpm for one minute. Throughout Washing buffer 1 was discarded in disposal bottle, and then 500µl Washing buffer 2 (W2) was added to each spin column, and centrifuged at 8000 rpm for one minute. Throughout Washing buffer 2 was discarded in disposal bottle, and then the tubes were

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centrifuged once more at 12000 rpm for one minute to completely remove ethanol. After that, spin column that containing genomic DNA were transferred to sterile 1.5ml micro-centrifuge tube, and then added 100µl of elution buffer and left stand the tubes for five minutes at room temperature until the buffer is completely absorbed into the glass filter of spin Binding column tube. Finally, all tubes were centrifuged at 8000 rpm for one minute to elute DNA, and storage at -20C^{o} freezer.

The extracted DNA were checked by using Nano drop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) and concentration ng/µl.

Real-Time PCR

Real Time PCR was performed for direct detection of *E. gingivalis and T. tenax* from oral wash fluid based on18S ribosomal RNA gene specific primers qPCR SYBER Green dye and technique was carried out according to method described by (9).

Real-Time PCR Master Mix Preparation:

qPCR master mix was prepared by using (GoTaq® qPCR Master Mix Kit) and this master mix done according to company instructions as Table (1):

Table(1) Master Mix of Standard qPCR

PCR Master mix	Volume
DNA template 5-50ng	5µL
qPCR 18S ribosomal RNA Forward primer (20pmol)	1µL
qPCR 18S ribosomal RNA Reverse primer (20pmol)	1µL
qPCR master mix	10µL
Nuclease free water	3 µL
Total volume	20µL

After that, these PCR master mix component that mentioned in table above transferred into Exispin vortex centrifuge at 3000rpm for three minutes. Then placed in Real-time PCR Thermocycler (BioRad . USA).

Real-Time PCR Thermocycler conditions:

Real-Time PCR thermocycler conditions were done according to primer annealing temperature and qPCR kit instructions as in the following table (2):

Table(2):RT(PCR) Thermo Cycler Conditions :

Step	**Condition**	**Cycle**
Pre-Denaturation	95 C° 5 min	1
Denaturation	95 C° 20 sec	45
Annealing/Extension	60 C° 30 sec	
Detection (Scan)		

Real-Time PCR Data analysis:

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qPCR data analysis were automated calculated by the threshold cycle number (CT value) that presented the qPCR positive amplification.

Statistical Analysis

The statistical analysis performed for the result by Chi-square (X^2 - square) differences at propability ($P \leq 0.05$) (10)**.**

RESULTS

Table 3 shows the results of a real-time PCR test for saliva and dental plaques in gingivitis patients and healthy subjects, the result of RT assay (PCR) (44.0%) for *E. gingivalis* and *T.tenax* (12.0%) positive for *T.tenax*, compared to healthy persons 4 (0.8%), and the results showed that the incidence of both parasites in gingivitis patients were higher than the infection in healthy subjects. There is a statistically significant correlation between RT (PCR) assay and patients.

As in **Table(4)** examination of recent specimen showed that a total of 34 saliva samples (50.0%) that were positive for *E. gigivalis*, *T.tenax* (5.88%) was identified from periodontitis patients . While it was found 16 dental plaque samples (31.3%) were positive for *E. gingivalis*, and (25.0%) of dental plaque samples were positive for *T. tenax* . showed result so there was a significant correlation between the presence of these two parasites and the sample type.

Table (5) showed higher *E. gingivalis* and *T. tenax* in males than in females,(46.6%,13.3%)(40.0%,10.0%) respectively, no statistically significant correlation between the gender of patients and presence of these protozoan parasites.

Regarding the relationship between patients age groups and presence of these protozoan, *E. gingivalis* was more common in patients age groups 36 to 45 years(70.0%), whereas *T. tenax* was more common in patients age groups 46 to 55 years(66.7%) as in **Table 6.** There were statistically significant correlation between the age groups of patients and presence of these protozoan .

Salivary pH ranged participants from 5 to 8.5, were found on both priorities in pH 7-7.5. Where *E. gingivalis* had the highest rate of *T. tenax* (44.0%, 12.0%) respectively as in **(Table7**), there were a statistically significant correlation between saliva pH and the presence of these parasites.

E. gingivalis was founed in higher rate in patients persons whose have chronic diseases (60.0%), while the parasite *T. tenax* was founed in higher rate in patients persons whose don't have any chronic diseases (12.5%) in patients gingivitis, There were a statistically significant correlation between patients' whose have chronic diseases and the presence of these parasites. as shown in **Table 8.**

The results of the current study showed that the incidence of *E. gingivalis* among smokers 6 (40.0%) is lower than that of non-smokers 16 (45.7%), and the incidence of *T.tenax* among smokers 3 (20.0%) is higher than that of non-smokers. 3 (8.57%),the overall infection rate for both parasites were higher in smokers 9 (60%), while the incidence rate were lower for non-smokers 19 (54.28%), as shown in **Table (9)**. The results of the statistical analysis showed that there were statistically significant differences between smokers and non-smokers according to the type of parasite .

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Table(3): Saliva and plaque real time PCR assay results among patients and healthy control persons for *E. gingivalis* and *T. tenax*

RT(PCR)	Examined No.	Infection No.				Controls No.			
		E. gingivalis	%	*T.tenax*	%	*E.gingivalis*	%	*T.tenax*	%
Negative	50	28	56.0	44	88.0	46	92.0	0	0
Positive	50	22	44.0	6	12.0	4	8.0	0	0
Total	100	50	100	50	100	50	100	50	0
E. gingivalis: $X^2 = (16.82)$. $P \leq 0.05$), (*T. tenax* : $X^2 = (28.88)$. $P \leq 0.05$)									

Table(4):The rate of infection with *E. gingivalis and T.tenax* of persons that suffering from problems with teeth and gums.

	Examined No.	*E. gingivalis*		*T.tenax*		total Infected No.	
		Infected No	%	Infected No	%	Infected No.	%
Saliva	34	17	50.0	2	5.88	19	55.9
Pluqe-induced	16	5	31.3	4	25.0	9	56.3
Total	50	22	44	6	12	28	56
$X^2 = (7.20)$ $P \leq 0.05$.							

Table (5): Distribution of *E. gingivalis* and *T. tenax* in gingivaties patients according to the sex

Gender	Examined No.	*T. tenax* Infected No.	(%)	*E.gingivalis* Infected No.	(%)	*T. tenax* and *E. gingivalis*	(%)
Males	30	4	13.3	14	46.6	18	60.0
Females	20	2	10.0	8	40.0	12	24.0
Total	50	6	12.0	22	44.0	28	56.0
t-test =(1.71) $P \leq 0.05$.							

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Table(6):Rates of infection of patients with *E.gingivalis* and *T.tenax* according with Ages Groups.

Ages group Years	Examined No.	*E.gingivalis* Positive case	(%)	*T.tenax* Positive case	(%)
>15	2	1	50	0	0
16-25	18	7	38.9	0	0
26-35	14	4	28.57	2	14.28
36-45	10	7	70.0	2	20.0
46-55	3	2	66.66	2	66.66
56-65	2	1	50.0	0	0
Total	50	22	44.0	6	12.0
$(X^2=(10.72)\ P \leq 0.05), (X^2=(3.00)\ P \leq 0.05)$					

Table (7): Rates of Infection with *E. gingivalis and T.tenax* according to degree of pH of saliva.

	Examined No.	*E. gingivalis* Infected No.	(%)	*T.tenax* Infected No.	(%)
pH = 7	0	0	0	0	0
pH > 7	50	22	44.0	6	12.0
pH < 7	0	0	0	0	0
Total	50	22	44.0	6	12.0
E. gingivalis $(X^2 = (96.06).\ P \leq 0.05)$, *T.tenax* $(X^2 = (74.71).\ P \leq 0.05)$.					

Table(8): Rates of infection with *E. gingivalis and T.tenax* among chronic diseases according to persons whose don't have any chronic diseases.

chronic diseases	Examined No.	*E. gingivalis* Infected No.	(%)	*T.tenax* Infected No.	(%)
persons whose have chronic diseases	10	6	60.0	1	10.0
persons whose don't have any chronic diseases	40	16	40.0	5	12.5
Total	50	22	44.0	6	12.0

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***E. gingivalis* (X^2=(22.54), $P \leq 0.05$),*T.tenax* (X^2=(20.66),($P \leq 0.05$)**

Table (9): Rates of infection with *E. gingivalis* and *T.tenax* according to Smoking Status.

Smoking Status	Examined No.	*E. gingivalis* Infected No.	(%)	*T.tenax* Infected No.	(%)	*E. gingivalis* *T.tenax* Infected No.	(%)
Smoking	15	6	40.0	3	20.0	9	60.0
Non-Smoking	35	16	45.7	3	8.6	19	54.3
Total	50	22	44.0	6	12.0	28	56.0
X^2=(4.54). $P \leq 0.05$							

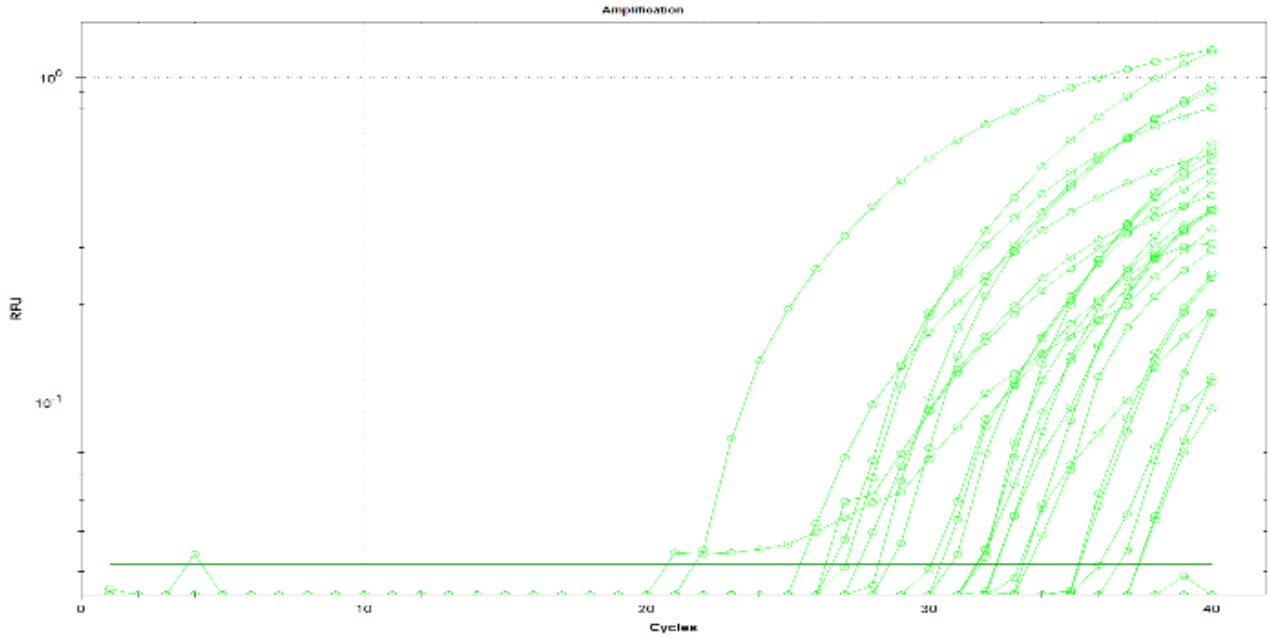

Figure (1): Real Time PCR amplification for *E. gingivalis* of 43 saliva and dental plaque samples in PIG disease case and control group.

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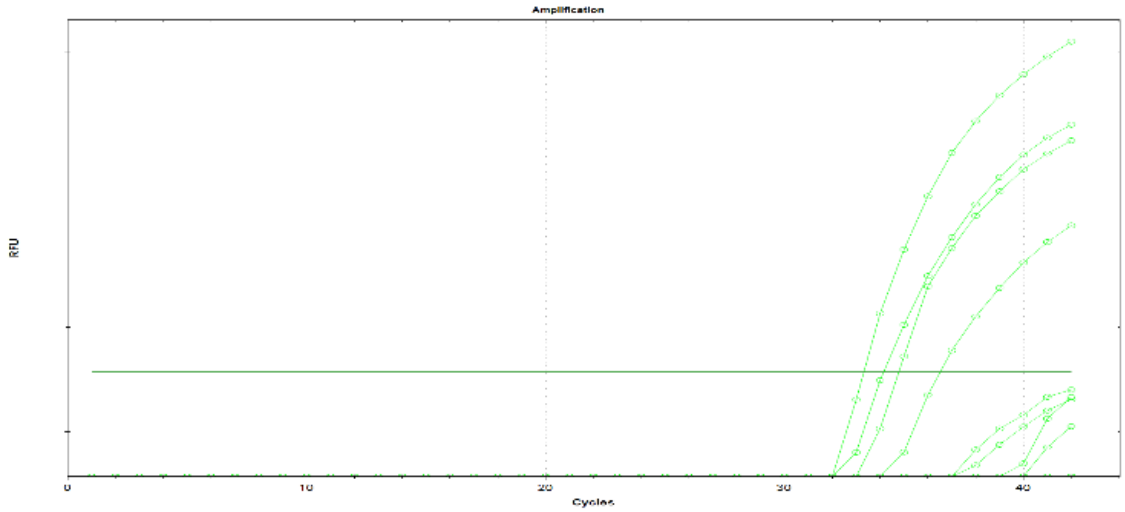

Figure (2): Real Time PCR amplification for *E. gingivalis* of 17 saliva and dental plaque samples in PIG disease case and control group.

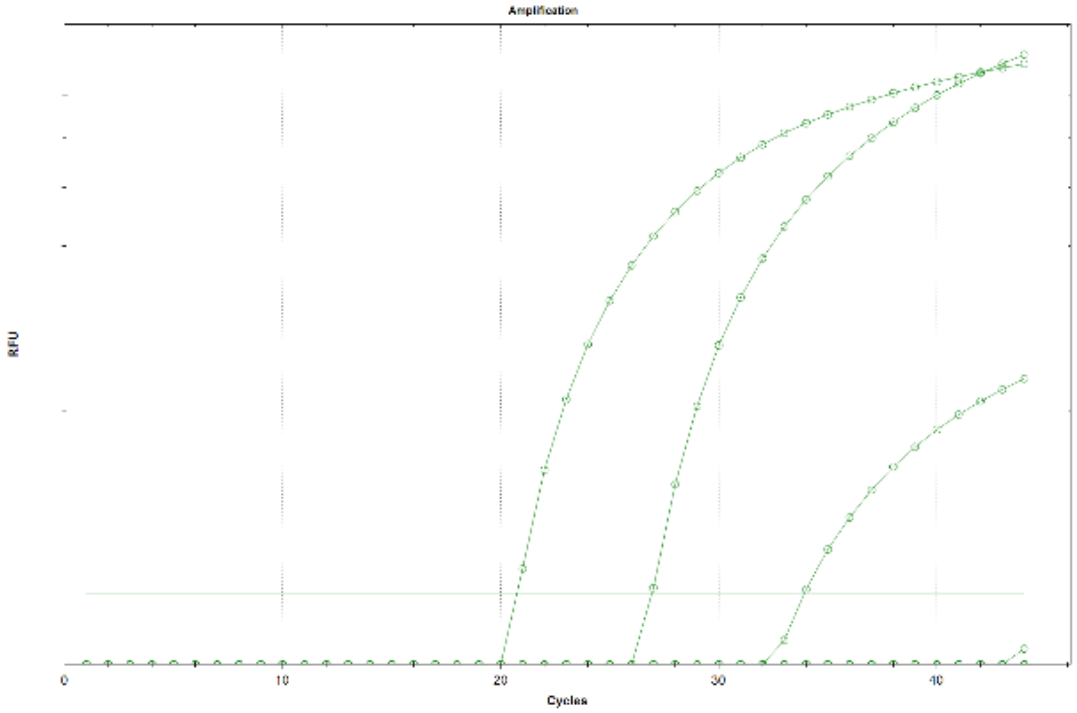

Figure (3): Real Time PCR amplification for *T.tenax* of 48 saliva and plaque samples in PIG and control group

Discussion

Gingivitis is a disease that occurs most commonly in the mouth after tooth decay, which disturbs more than 75% of the world's population (11, 12). Periodontitis is a reversible form of periodontal disease (13). There are a limited number of studies in specially in Babylon province, on the detection of *T.tenax* and *E. gingivatis* the use of qPCR technique in plaque-induced patients with gingivitis and healthy oral people. And also this is the new studies on gingivitis caused protozoa involved in the mouth.

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Real-time polymerase chain reaction (qPCR) technique was used in this study due to its increased sensitivity, specificity, reliability and ability to detect the carrying of specific amplified genes that were examined for these protozoa. A new real time QC RT PCR method, melting curve analysis provides immediate practical benefits in real-time PCR, obviating the need for gel electrophoresis by providing a reproducible signature of the amplified DNA sequence that may be used for typing PCR products(14).

E. gingivalis and *T. tenax* are commensal protozoa commonly found in human oral cavity, it is most probable that they are opportunists especially in the lesions of gingivitis (4). There are only few reports on the role of oral commensals in the pathogenesis of periodontitis and gingivitis despite the high incidence of certain protozoa, such as *E. gingivalis* and *T. tenax.*

Advances in molecular biology have facilitated analyses of the oral microbiome, When by using real-time PCR in the present study, presence *E. gingivalis* and *T. tenax* in saliva and dental plaque of patients with gingivitis. In the present study,the result revealed that the incidence of *E.gingivalis* was higher than the parasite *T.tenax* 22(44.0%) ,6(12.0%), respectively, this also in agreement with many studies, Among them, a study (15,16,17),and the rate of oral parasites higher than in control individuals, this also in agreement with many studies (18,19,20).The result of the present study corroborates the patients that implicated poor state of oral hygiene to enhanced prevalence of oral protozoa (21,22).

This study indicating that the incidence rate of *E. gingivalis* was 22(44.0%),this rate approximately for many studies (30,18,32,45).

Development of a real-time PCR assay for *E. gingivalis* was more sensitive than the conventional PCR assay for detection of this amoeba. This study found that is, the presence of *E. gingivalis* can induce gingivitis. The other researchers state that this protozoans may be opportunistic, since they are capable of proliferating in the microenvironment of the mucobuccal fold affected by periodontal disease (4) . Indicated the ability of *E. gingivalis* to cytolyze and ingest epithelial cell fragment of nuclear and red and white blood cells(28).

Several studies demonstrated the efficacy of PCR in detecting *T. tenax* (9), and the prevalence of oral trichomoniasis in our study 6 (12.0%) was roughly consistent with many other published reports which ranged mostly between 12% - 32%(2,38,46,45,25). And also a study (16,43,15,53,52,17).

On the other hand, if the results of this study are compared with other studies, we find that the percentage of parasitic infection *T. tenax* is much lower than that reached by the study of (2,3,62) . Perhaps the reason for the fact that the incidence of parasitic infection is low to raise awareness of health and attention to oral hygiene and dental.

T.tenaxs role in periodontitis and gingivitis remains controversial. Although a relationship has been clearly demonstrated between the increased occurrence of this protozoan and the progression of this disease. (17). By combining a polyphasic approach that associates culture and q PCR, The development of molecular tools, including PCR and real-time PCR, to detect

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E. gingival and *T.tenax* in saliva and plaque samples has led to major advances in making an accurate diagnosis during recent years (23) . The results of the recent study showed that plaque accumulation is closely related to the prevalence of gingivitis among patients. In the last study, the results showed that the incidence of *E.gingivalis* and *T.tenax* (56.3%)in plaque than in saliva 19 (55.9%). The results of this study were consistent with the results of other studies, including a study(24,25,26). But another investigation showed that saliva was not an appropriate method for detecting parasites. (27). In the current study, by using real-time polymerase chain reaction (PCR), the results of this study showed that the prevalence of *E. gingivalis* was 5 (31.3%) less in plaque than in saliva 17 (50.0%).This also in agreement with many studies(8,20).

For many years, it was assumed that bacteria were the only microorganisms involved in the formation of dental plaque, (47)However, although largely comprised of bacteria, some fungal, mycoplasma and protozoan species are also found in dental plaque ,among these the flagellated protozoan, *T.tenax* (44).In study, the results showed that the incidence *T.tenax* 4(25.0%)in plaque than in saliva 2(5.88%). The results of this study were consistent with the results of other studies(32,49,48).

The current study showed a difference in the rate of parasite infection between male and female, as the total male infection rate was 18(60.0%), which is greater than the total percentage of female, which amounted to 8(50.0%), and this result was identical to a number of studies, including a study(20, 19,38,30) the incidence rate was higher for in males than for females . Also a study (8,29) showed that the overall prevalence of these two parasites was higher in Males than females.

In the present study the total incidence of males were 14 (46.6%) to *E. gingivalis* higher than the total percentage of females 8 (40.0%), and this result was identical to a number of studies, as study (31)(18) (32)(15). Other study (51) which they found that *E. gingivalis* in females lower than males, its due to females dental and mouth care is more respected.

While the total infection rate for females (10.0%) for *T.tenax* is less than the total for males (13.3%) and these results were identical to the results of several studies, including a study, (52) (48).

Perhaps the reason for this decrease in the incidence of both parasites among females is due to health awareness and the much care of the mouth and teeth , as women are concern with cleans their mouth more than males, and also to the high immunity of women against infection with many diseases, more than in Men (37).

If we took this study from another aspect, we would find it different and far from its results for many studies as study (21,43,37) and this is a characteristic contained in the results of scientific research as this is due to several reasons, including the time of taking the research samples, their type and conditions, and with the nature and level of the social cultural and economic factor of the society, including the study (50) which showed that the sex factor was not influential in the rate of infection with each parasite and that the

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differences in the infection rate between males and females were not statistically significant in all the studied cases. This was identical to the current study, where the results showed no statistically significant relationship in the infection rate between males and females.

The results of the current study, the relationship between parasite infection with *E. gingivalis* and the age groups, where *E. gingivalis* found in higher rate in age group (36-45),(46-55) this finding support the idea that *E. gingivalis* may play an active role in the mouth disease,the both parasites were found in higher rate in age group(46-55),the parasite *T. tenax* was found in high rate in age group(46-55),*T.tenax* role in gingivitis remains controversial. Although a relationship has been clearly demonstrated between the increased occurrence of this protozoan and the progression of this disease. this results are in general agreement with other studies (51,17,45,49,21).Where and the lowest rate of infection with in the both parasite was in the age groups (<15),(16-25),(26-35)and (56-65)years , and the results of the current study were identical the study (43), some authors (1) .The oral protozoa *E. gingivalis* and *T. tenax* do not occur in small children and are rarely found in older ones. In adolescents their occurrence rate keeps increasing with age (46). while some were believed that oral protozoa were rarely found in children(3), perhaps the reason for this is that the children's teeth are of a newly developed structure, in addition to the fact that the child is few exposed to the factors that contribute to infection with such a parasite, the most important of which is the smoking factor, so infection with the parasite is few or rare get.In the case of elderly persons, the reason for their low infection rate may be attributed to the fact that their oral environment is not suitable for the growth of the parasite because most of them are taking antibiotics that would eliminate such parasites.

However, statistically significant correlation between age and presence of oral commensals a *E. gingivalis* . no statistically significant correlation between age and presence a *T.tenax* was observed .This apparent contradiction may be related to an absence of age group standardization.

Some studies indicate that age is an influencing factor in the infection of oral parasites in patients with periodontal disease. The prevalence of parasite infection increases with age as a direct correlation between age groups and oral parasites(38,39,40) .

Other factors that increase the risk of periodontitis and gingivitis disease are diabetes, pH, smoking status and accompanying of chronic diseases of patients such as (Diabetic mellitus, blood pressure, Asthma, Arthritis, antibiotic consumption etc.), in this study, there is statistically significance between these factors and the presence of these parasite, the reasons may be that the immunity which reduced in these patients, and the presence of these parasite are making facilitates. The present study recorded a relationship between the presence of E. *gingivalis* and *T. tenax* and these factors.

The smoking factor is one of the most important factors affecting the rates of infection with oral parasites and this is confirmed by many researchers, so the results of the current study were identical to them and showed that the effect of smoking factor on the rates of infection with oral parasites is clear. The total infection rate of both *E. gingivalis* and *T.tenax*

among smokers was (60.0%), which is higher than the incidence rate among non-smokers (54.3%).

These results were consistent with many studies that dealt with the effect of smoking on increasing the incidence of oral parasites and various oral diseases (33,50, 41,7,30,34,32).

This is due to the fact that smokers are often less concerned with oral and gum hygiene, with dental plaque and tooth decay being at a greater level than non-smokers, and their micro biota of higher level (35). Studies have shown that smokers are more likely to suffer from acute periodontal disease and damage to the periodontal ligaments, such as gum disease and tooth loss compared to non-smokers (36, 54,55,56,57,58,59,60,61, 62).

This study indicated that individuals having dental diseases and problems are more prone to oral protozoa colonization. Here, consideration can be given to the factor of hygiene and its impact on many illnesses, the parasites of the mouth is increased with less hygiene. Low immunity due to the immune system's inefficiency and lack of exposure to such parasites (42, 54,55,56,57,58,59,60,61, 62).

Conclusions:

Using Q(RT- PCR) technique and melting curves analysis was more sensitive and accurate with rapid results in diagnosis of *E. gingivalis* and *T.tenax* with traditional tools for diagnosis of parasites. The finding in this study showed prevalence of *E. gingivalis* higher with PIG disease patients than *T.tenax*. There were relationship between the presence of these parasites and the type of samples and the factors as (pH saliva, age groups, sex, smoking status, and chronic diseases status of patients (blood pressure, heart disease etc.). The current study found that the presence of *E .gingivalis* and *T. tenax* in samples of plaque by high compared with its presence in saliva samples (56.3), (55.9%) respectively.

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