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## Identification of Red Complex Pathogens Group from Chronic Periodontitis Patients in Mosul City

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### ABSTRACT

This study is concerned with the isolation of red complex pathogens, identifying them by a new molecular method as the first locally used and described procedure and characterizing these pathogens by their phenotypic features and biofilms. Gingival fluids were sampled from chronic periodontitis and inoculated into three types of culture media, Schaedler Anaerobe Agar, *Tannerella forsythia* (TF) agar, and Trypton Yeast extracts Gelatin Volatile fatty acids and Serum (TYGVS) agar. The different appearing colonies were purified and identified by Loop-Mediated Isothermal Amplification protocol (LAMP) for the detection of red complex species. Bacterial biofilm was estimated in term of mono- and polytypic biofilm by measuring the absorbance of a crystal violet- stained biofilm formed in a microtiter plate. Different forms of colonies appeared at the primary isolation. LAMP method was of a significant value for perfect rapid identification of the target species of extracted DNA or intact cells within half an hour. The three types of red complex pathogens were simultaneously detected in the same gingival fluid sample. They formed mono- and polymicrobial biofilms in a synergistic manner particularly the two intimates *P. gingivalis* and *T. denticola*. In conclusion, the updated LAMP molecular protocol was attractive method for the diagnosis of red complex pathogens which showed great morphological variations. *P. gingivalis* fortified the growth of the other two to establish polymicrobial biofilms in a synergistic manner.

**Keywords:** LAMP method, Molecular diagnosis, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, Polymicrobial periodontitis.

## INTRODUCTION

Inflammatory disease of the periodontal tissues that causes destructive changes in the bone and connective tissues of the tooth is referred to as Periodontitis (Popova *et al.*, 2013). It is definite by increasing in the depth of gingival sulcus which culminates with the apparent periodontal pocket, loss of attachment of periodontium and alveolar bone. Generally, the infection occurs as aggressive or chronic periodontitis (Tanner, 2015). Chronic periodontitis is a major public health problem in all societies occurs as a polymicrobial infection induced by anaerobic Gram-negative rods and spirochetes consistently resident together in subgingival plaque biofilms (Kotsilkov *et al.*, 2015). By DNA- DNA hybridization technique these genera were grouped into six groups from which the “red complex” group involving *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*; adopts a crucial role in the infectious process (Mohamad, 2010). In subgingival pockets, these bacteria have to withstand the host’s immune response and the different stress factors in the oral cavity, like shear-forces and high flow rates of saliva (Wessel *et al.*, 2016). To be protected from these harsh conditions, bacteria form a biofilm within which they increase in numbers to start persistent infections which may lead to tooth loss, bone distortion and can also spread out to extra-oral sites and cause systemic infections (Ammann *et al.*, 2012). For the sought of causative diagnosis, anaerobic culture methods are of a chief importance for comprehensive microbiological studies of periodontitis (Tanner, 2015), however, isolation and identification of periodontopathogens by traditional methods is a difficult work because of the presence of many different bacterial species in a periodontal pocket in that 30- 100 species may co- exist at a single site in the pocket (Hasan and Palmer, 2016) and many of these anaerobes require prolong period to grow in pre-reduced anaerobically sterilized (PRAS) media and many biochemical tests for well identification; so they are of anxious for characterization by conventional methods (Tanner, 2015). Rapid identification kits e.g. API systems and VITEK MS- bioMérieux system require high dense inoculum which is difficult with the slow growing periodontal bacteria, also many anaerobes are non-reactive in biochemical tests and there is no available data base for each type of periodontal pathogens (Nagy *et al.*, 2018; Garner *et al.*, 2014). Therefore, most laboratory identification methods shifted from classical biochemical methods to the most potent identifier one, 16SrRNA-targeted molecular technique that overcomes limitations of culture depended- identification methods (wade, 2013). Amplification of the highly conserved genes code for 16S rRNA (small subunit rRNA (SSU rRNA)) in procaryotes ensures high efficiency because the conserved sequences flank the genes of interest. In addition to, the stable region of SSU rRNA enables comparison of distantly related species and the variable region enables comparison of closely related species (Akram *et al.*, 2017). The new version of these methods with more attractive options as more sensitive and specific one is the isothermal amplification techniques which copy the target to large numbers under one temperature, thus it is extremely fast and do not require many supplies (Crescente *et al.*, 2018). The current study tried to present for a first time in Mosul City the updated molecular techniques for the diagnosis of red complex pathogens and describe their phenotypic features and biofilms.

## MATERIALS AND METHODOLOGY

### **Samples Collection:**

Gingival crevicular fluids (GCFs) were collected from 30 periodontal pockets with chronic infections having depth more than 4mm and positive occurrence of bleeding on probe (BOP). Absorbent paper points size #40 were deepened inside the pocket for 30 sec. for well absorbance and then transported in an Eppendorf tube containing 500 µl of phosphate- buffered saline until reaching the lab. Specialist dentist in the Teaching Hospital of College of Dentistry in University of Mosul helped in specimens' collection during the period 14/10/ 2018- 28/2/2019. The participants (12 male and 18 females with average age of 35-50 years) provided that they don't have systematic diseases, not take antibiotics 3 months before the test, all of them were nonsmokers, and pregnant females were excluded.

**Culture Media:**

Three types of culture media were prepared for the isolation of red complex periodontal pathogens:

**Schaedler Anaerobe Agar supplemented with Blood, Haemin and Vitamin K** was dissolved in gram per liter distilled water according to Oxiod Company: tryptone soy broth (10), special peptone (5), yeast extract (5), glucose (5), sodium chloride (4), cysteine- HCl (0.4), tris- HCl (0.75), haemin (0.01), and agar (13.5). pH was adjusted to  $7.4 \pm 0.02$ . After autoclave, vitamin K1 (2mg), and vancomycin (75mg) were added (Kotsilkov *et al.*, 2015).

**Tannerella forsythia (TF) Agar** was prepared in gram per liter distilled water according to Dashper *et al.*, (2014): trypton soy broth (15), brain heart infusion (18.5), yeast extract (10), Cysteine- HCl (0.5), haemin (0.005), and agar (13.5). pH was adjusted to  $7.2 \pm 0.02$ . After autoclave vitamin k (0.0004 gr), serum (20 ml), gentamycin (50mg) were added.

**Trypton Yeast Extracts Gelatin Volatile Fatty Acids and Serum (TYGVS) Agar** was prepared in gram per liter distilled water according to Muhammad and Al- Rawi (2011): trypton (10), brain heart infusion (5), yeast extract (10), gelatin (10), ammonium sulfate (0.5), magnesium sulfate (0.1), dipotassium hydrogen phosphate (1.13), potassium dihydrogen phosphate (0.9), sodium chloride (1), glucose (1), cysteine- HCl (1), sodium pyruvate (0.25), sodium bicarbonate (2), sodium thioglycolate (0.5), ascorbic acid (0.001), agar (7), and volatile fatty acids mixture (0.482 ml). KOH was used to set pH at  $7.2 \pm 0.02$ . Thiamin pyrophosphate (0.0125 gr) serum (2 ml %) and rifamycin (4mg) were added after autoclave.

**Sample Processing:**

Samples were directly streaked on the three types of prepared media then incubated for 4-7 days under the demanded condition of  $37^{\circ}\text{C}$  in anaerobic Jar using the microaerophilic atmosphere generation system, CampyGen according the instructions of the supplier, Oxiod Ltd Company, Japan.

**Identification of the Bacterial Isolates:**

The miscellaneous colonies on the three-culture media at the primary isolation were sub cultured and characterized by phenotypic features, cultural and cellular morphology by gram stain depending on the rules of Nagy *et al.*, (2018) for the identification of anaerobic clinical isolates. Molecular amplification, LAMP technique was used to confirm bacterial species.

**Loop- Mediated Isothermal Amplification (LAMP) procedure:**

**Targets:** Bacterial cells were grown in broth media for 7 days, then were centrifuged using cooled cent. (10rpm for 15min.) and used as a target either as whole cells or extracted DNA. DNA was extracted from bacterial cells using Wizard® Genomic DNA Purification Kit (Promega /USA).

**Primers'** sets specified the 16SrRNA genes (Miyagawa *et al.*, 2008) for each species of red complex group are listed in (Table 1). Primer stock solution was prepared according to the manufacturer, Integrated DNA Technologies (IDT) Company. Primers mix solution was prepared at 10X stock mix solution according to BioLabs®<sub>Inc</sub> (New England) Company depending on this equation:  $C1V1 = C2V2$ .

**Table 1: Sets of primers targeted 16srRNA gene**

Red complex species	Primers	Nucleotides sequence
<i>P. gingivalis</i>	Forward inner primer (FIP)	5'-CACCACGAATTCGCGCTGCCTGAGCGCTCAACGTTTCAGCC-3'
	Backward inner primer (BIP)	5'-ATCACGAGGAACTCCGATTGCGCGCCTTCGTGCTTCAGTG-3'
	Forward outer primer (F3)	5'-GGTAAGTCAGCGGTGAAACC-3'
	Backward outer primer (B3)	5'-GCGTGGACTACCAGGGTAT-3'
	Loop primer (LB)	5'-GCAGCTTGCCATACTGCGA-3'
<i>T. denticola</i>	Forward inner primer (FIP)	5'-CATCCTGAAGCGGAGCCGTAGTACCGAATGTGCTCATTAC-3'
	Backward inner primer (BIP)	5'-GCTGGTTGGTGAGGTAAAGGCCATCTCAGTCCCAATGTGTCC-3'
	Forward outer primer (F3)	5'-CCCTGAAGATGGGGATAGCT-3'
	Backward outer primer (B3)	5'-TGCCTCCCGTAGGAGTTTG-3'
	Loop primer (LB)	5'-CACCAAGGCAACGATGGGTAT-3'
<i>T. forsythia</i>	Forward inner primer (FIP)	5'-CCATCCGCAACCAATAAATCTCTAATACCTCATAAAACAGG-3'
	Backward inner primer (BIP)	5'-TAAGCCATCGATGGTTAGGGCGTGTCTCAGTACCAGTGTG3'
	Forward outer primer (F3)	5'-GATAACCCGGCGAAAGTCCG-3'
	Backward outer primer (B3)	5'-TGCCTCCCGTAGGAGTCT-3'
	Loop primer (LB)	5'-GTTCTGAGAGGAAGGTCCCC-3'

**LAMP- specific WarmStart Colorimetric 2X Master Mix** was bought from BioLab<sub>INC</sub> (New England) Company

**Mixture of LAMP protocol** was prepared by following the orders of BioLab<sub>INC</sub> Company in (Table 2). The mixture was set at 65°C for 30 min. to track color turning from pink to yellow.

**Table 2: Additives for the mixture of LAMP protocol**

Substance	Tube of target	Tube of control
WarmStart Colorimetric LAMP 2X Master Mix	12.5 µl	12.5 µl
LAMP Primer Mix (10X)	2.5 µl	2.5 µl
Target DNA	1- 5 µl	–
dH <sub>2</sub> O	9- 5 µl	10 µl
Total Volume	25 µl	25 µl

#### **Estimation of the mono- and poly microbial biofilm of red complex pathogens:**

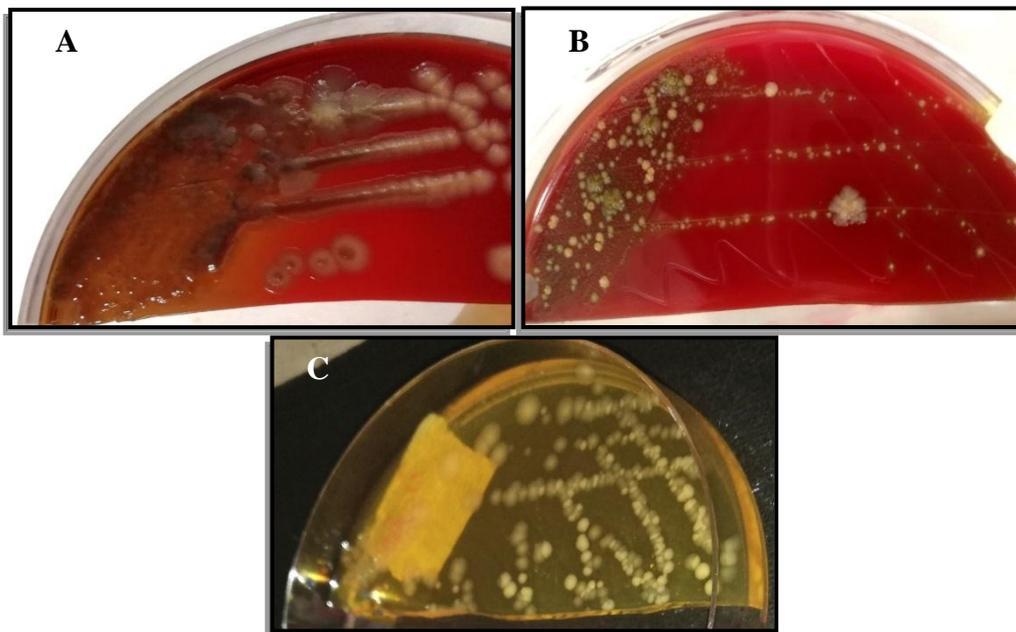
According to Yamada *et al.*, (2005), three days aged inoculums of each bacterial type was used to prepare a suspension in it correspond medium (free of antibiotics) equal to McFarland 0.5. In a 96- flat well plate, 200 µl of one bacterial type was added as a mono-microbial inoculum. In another wells 100 µl of two bacterial types or 66 µl of the three bacterial types were added as a

poly-microbial inoculum. The well plates were sealed and incubated anaerobically for 4 days. The contents of the wells were decanted and the wells were washed several times with distilled water to take out the non-adherent cells. 100  $\mu$ l of crystal violet stain (0.1%) was used to stain the adherent biofilm for 15 min., then after several washing with distilled water, the stained biofilm was extracted by a mixture of (80% ethanol + 20% acetone) and after 15 min. the absorbance was read at 630nm by a microplate reader.

## RESULTS AND DISCUSSION

### Primary Isolation of Red Complex Pathogens:

Our results showed that all the 30 samples from subgingival pockets gave bacterial growth on the three types of culture media with different morphologies at the primary isolation as evident in Figure 1. Many studies referred that about 30- 100 species may present at any single site of periodontal pocket which simultaneously can be grown and isolated (Paster *et al.*, 2006; Hasan and Palmer, 2016). *P. gingivalis* or other related species and *Prevotella sp.* are black pigmented on blood agar; as well as, *T. forsythia* and other *Bacterioids sp.* develop a tiny white colony on blood agar (Tanner, 2015). *T. denticola* must be accurately distinguished from other oral *Treponema sp.* which shows similar morphological features (Muhammad and Al-Rawi, 2011). Also, there were variations in their cellular morphology; and all were gram negative cells. Our observations were in accordance to the previous publication of Moll, (2016). To obtain pure culture of each type, each phenotypic form was subcultured for routine purification, and to point the desired types of red complex pathogens they were subjected to molecular diagnosis.

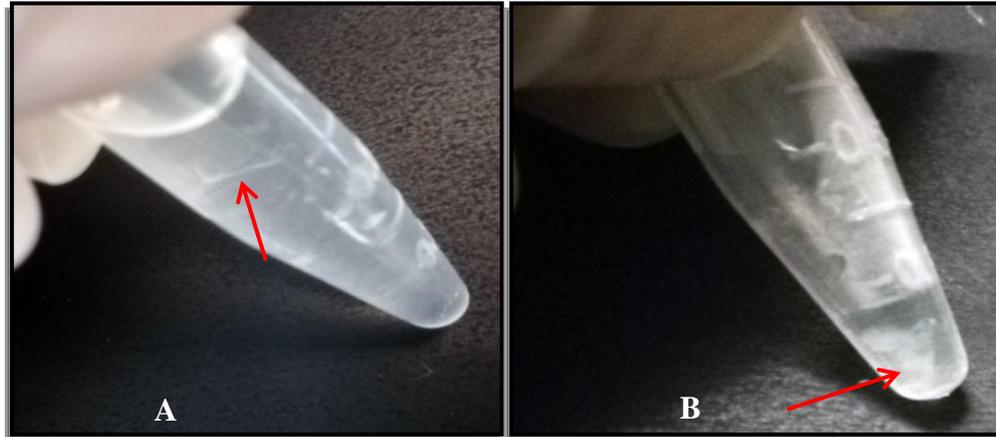


**Fig. 1: Different colonies at the primary isolation on (A): Schaedler Anaerobe Agar, (B): TF agar and (C): TYGVS agar.**

### Identification of red complex pathogens:

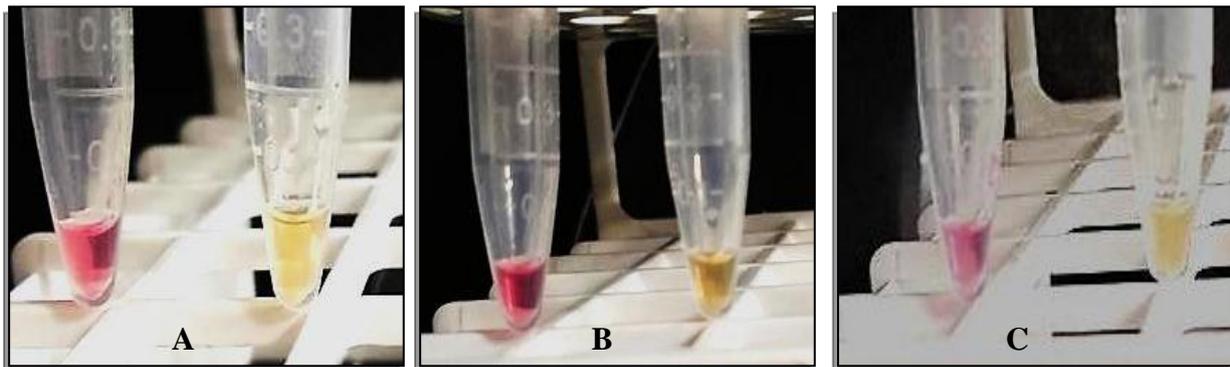
#### Molecular identification:

Fig. (2A) showed the extracted DNA as a white thread visualized by the naked eye, and Fig. (2B) showed the pellet of DNA mass.



**Fig. 2A: Thread of extracted DNA, B: the pellet of DNA**

The extracted DNA from different isolated colonies on the three types of culture media from 21 GCF samples was subjected to molecular diagnosis by LAMP method for the identification of isolates. Whole cells from the same isolates were also used as a target in another set of LAMP reaction. According to the protocol of this method, change in the color of reaction mixture from pink to yellow was assigned as positive for the presence of the species- specific amplicon; while no color change was assigned as negative result. The positive reaction arose with a target of extracted DNA or whole cells from one isolate which was assigned to belong to the species corresponding to the primers used in this mixture, while the negative reaction was found in the negative control tube with no target or when the target from other isolates was added to the same primers which confirmed the specificity of the procedure and purity of the isolate. These results are represented in Fig. (3).



**Fig. 3: LAMP reaction. Solution with pink color is the negative control; yellow color is positive amplification of the target DNA of (A): *P. gingivalis*, (B): *T. forsythia* and (C): *T. denticola*.**

In this technique, four primers amplified six distinct sequences in 16S rRNA gene to increase the sensitivity and specificity of the amplification which got faster with the loop primer (LB) (Crescente *et al.*, 2018). Also, the results were more rapidly obtained within 30 min. and easily read by the naked eye using the WarmStart Colorimetric LAMP 2X Master Mix. This pH- depended Master Mix, a product of New England BioLabs<sup>®</sup> Inc Company is an optimized designation of *Bst* 2.0 WarmStart DNA Polymerase in a special buffer solution supplemented with MgSO<sub>4</sub>, required cofactors and the visible pH-sensitive indicator, Phenol Red which turned from pink to yellow color if the pH of the reaction mixture diminished as a consequence of large amount of proton resulted from the extensive nucleic acid amplification activity of *Bst* 2.0 WarmStart DNA polymerase. This type of master mix promoted visual rapid and easy detection of LAMP result. *Bst*

2.0 DNA polymerase is the DNA polymerase I enzyme isolated from *Bacillus stearothermophilus* and genetically engineered for elevation isothermal amplification function with strong strand displacement activity under isothermal conditions with no need to denaturation step. It is referred to as WarmStart master mix because the enzyme is inactive at lower temperature but activated if the temperature of reaction solution exceeds 40°C (Web site of New England BioLab (UK) Ltd).

These two main supply of the recent invented master mix used in the current study, the enzyme and the indicator, made it possible to use simple device for incubation, and coloring detection of the amplicon by the naked eye within half an hour. The reaction proceeded uninterruptedly in a water bath at 65°C; hence, all samples were incubated at the same time to detect the amplicon of the three species simultaneously without the necessity for a thermocycler. In addition to, the coloring detection of the amplicon simplified the results' reading without the need for a post amplification processing. The results were also obtained when using intact cells as a target in the molecular diagnosis which added further attraction to this technique that overcomes the need to extract the DNA. It could be at least 1% of the cells were hurtled at temperature above 60°C, so the primers got encounter with their target (Miyagawa *et al.*, 2008).

In our previous study we proved the applicability of PCR for the detection of several oral treponemal species directly from gingival fluid samples of mixed extracted DNAs (Al- Hamdonii, 2012). Now, we noticed the applicability of LAMP without DNA extraction step. Other researchers published the applicability of the LAMP method with crude sample with no significant effect of the presence of non-target DNA from other sorts during sampling (Miyagawa *et al.*, 2008; Saharan *et al.*, 2014). Hence LAMP protocol is candidate for the quicker microbiological analysis of periodontitis at dental chair-sides directly from the sample as it didn't need several processing steps to detect the concern species involved in PCR protocols.

We directly used molecular method for the diagnosis of each bacterial type without involving the traditional biochemical tests. According to the previously published facts, these bacteria require a large list of biochemical tests for identification and exact differentiation between the closely related species and at least 3 days of incubation for appearing growth (Mohamad, 2010) that became additional disturbance when identifying more than one species by traditional methods; also there is no available rapid identification systems specified for each species of periodontal bacteria, and the available one like API kit or VITEK system are suitable for the identification of some anaerobic bacteria to the genus level and till now there is no available data base for each types of periodontal pathogens (Nagy *et al.*, 2018; Garner *et al.*, 2014). For these reasons we compensated these methods with direct identification by molecular techniques.

All common molecular techniques are of gold values for sensitive, rapid and specific identification. However, the current study puts LAMP method which utilizes WarmStart Colorimetric 2X Master Mix in the top of the list of procedures for diagnostic purposes as this method enabled to achieve high precise, specific, sensitive, rapid and simultaneous diagnosis of the three species by the naked eye within 30 min. of incubation under one temperature instead of programmed repeated cycles for more than one hour involved in protocols of PCR techniques in a separate procedure for each species, therefore, it exterminated many of needs such as a thermocycler apparatus to change temperature, post amplification processing, e.g. electrophoresis for more than one hour in a separate procedure to detect the amplicon of each species, or an expensive fluorescent- labeled probes and other supplements needed for PCR- based techniques.

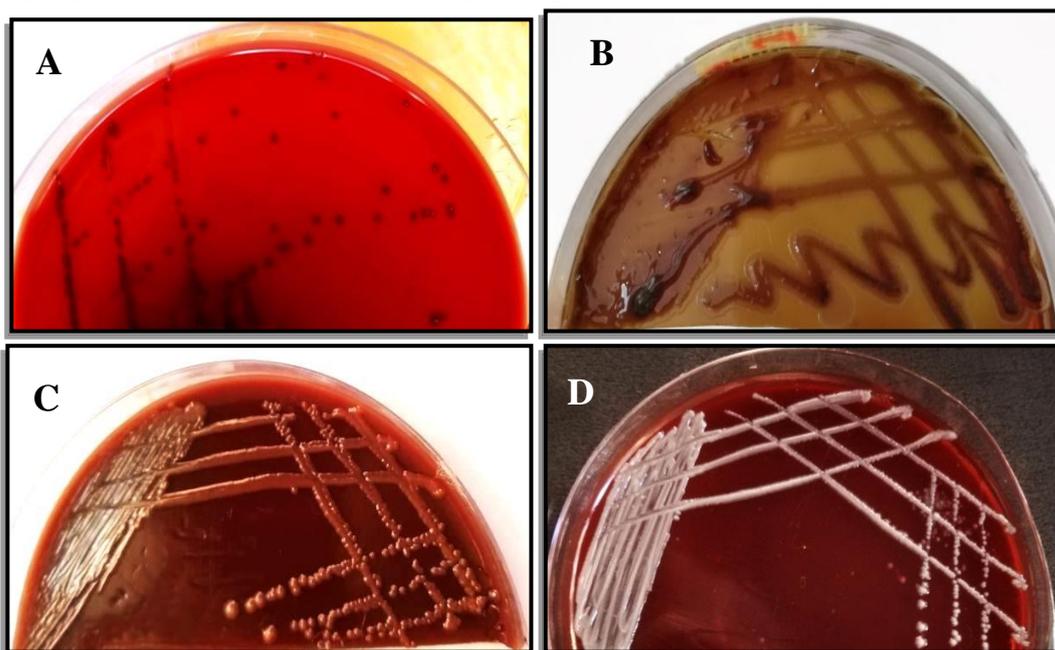
In our study, 21 gingival fluid samples were tested by the molecular technique and all of them were positive for the presence of the three types of bacteria, *P.gingivalis*, *T. forsythia*, and *T. denticola*, in the same specimen. The selected clinical cases were chronic periodontitis with pocket having >4 mm pocket depth (PD) and positive for the occurrence of bleeding on probe (BOP). These clinical indices increased the chance of isolation the three types of red complex group. In accordance to our data, the study of Tada *et al.*, (2012) recorded high percentages of simultaneous detection of *A. actinomycetemcomitance*, *P. gingivalis*, *T. denticola* and *T. forsythia* from chronic infections by molecular methods and the most prevalence was for the red complex pathogens.

Farias *et al.*, (2012) recorded that the most combination periodontopathogens were the types of red complex group from pockets > 4mm, whilst other causatives could be detected in pockets less than 4 mm.

The high percentage for the detection of these types was also documented by Ito *et al.*, (2010) who concluded the correlation of these types with mixed periodontitis in that the proportion of these organisms gave essential information on the severity of infection. Furthermore, the publication of Popova *et al.*, (2013) documented that among the six groups of subgingival bacteria, red complex group was the most responsible of chronic infection associated with the incidence of the clinical parameters of clinical attachment loss, increased depth of pockets and bleeding on probe which are due to their high proteolytic activities which facilitate their invasion in periodontal tissues. Hasan and Palmer (2016) revealed that the high proteolytic activities of these types enabled them to invade deeper in periodontal tissues to be protected from oxygen and establish their synergistic chronic pathogenicity.

### Phenotypic Description:

The confirmed isolates by LAMP reaction were then subjected to further description of their phenotypic characters. *P. gingivalis* isolates grew on Schaedler Anaerobe Agar after 4 days of incubation as circular, convex with entire- edge black pigmented colonies due to haemin accumulation and causing beta-hemolysis on blood agar as sources for iron it requires Fig. (4 A and B). The colonies were tightly attached to the medium with slimy appearance and sticky thread when picked up with loop due to capsule production, a character related to more virulent strains as a defense mechanism against human immunity. In subsequent sub-culturing, they may render brown or gray colonies (figure 4 C and D) with somewhat less capsule production; which may attribute to genetic exchange in response to the *in vitro* growth without exposure to immune defense. They were gram negative coccobacilli under light microscope Fig. (5). These phenotypic phenomena were explained depending on Brunner *et al.*, (2010).

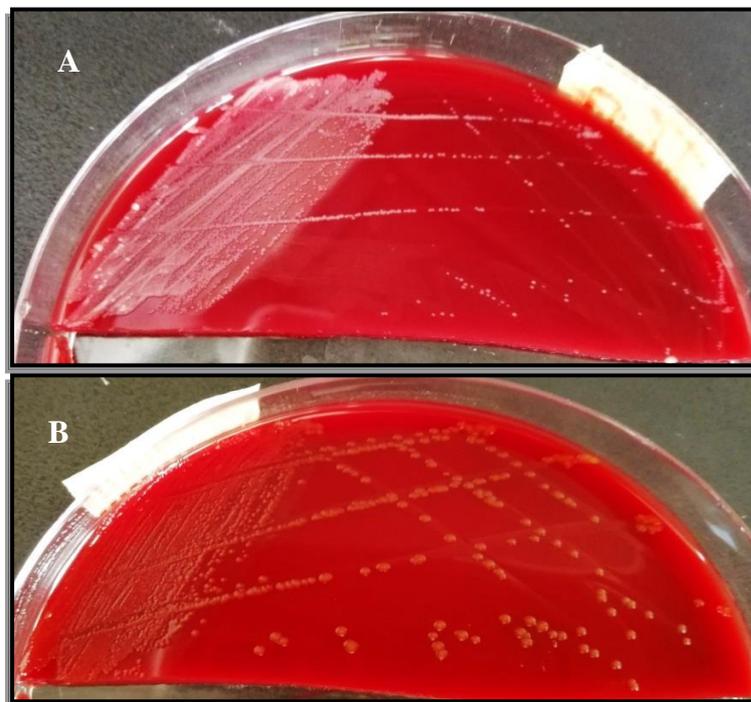


**Fig. 4: Colonies of *P.gingivalis* on Schaedler Anaerobe and Blood Agar pigmented as: (A) black, (B) with  $\beta$ -hemolysis, (C) brown or (D) gray.**

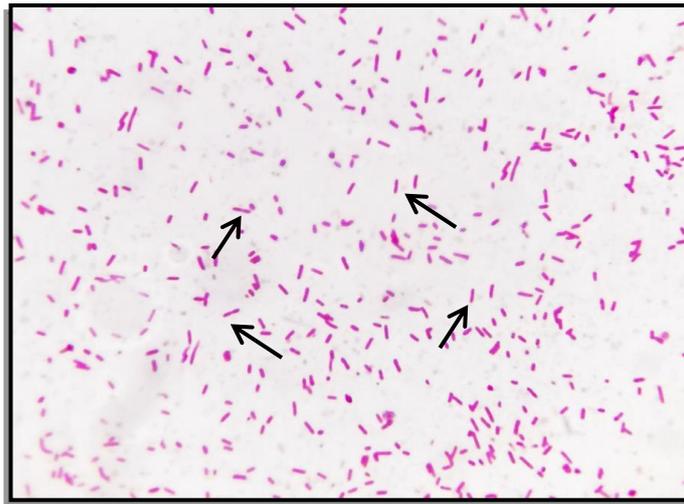


**Fig. 5: Gram negative coccobacilli cells of *P.gingivalis* (1000X) under light microscope**

Colonies of *T. forsythia* developed after 4 days on TF medium as very small white colonies that do not accumulate haemin or cause hemolysis Fig. (6 A). After 7 days they enlarged and may gain yellow color Fig. (6 B). Their cells were gram negative bacilli under microscope Fig. (7). These features were similar to the previously isolated *T. forsythia* in the study of Moll, (2016).

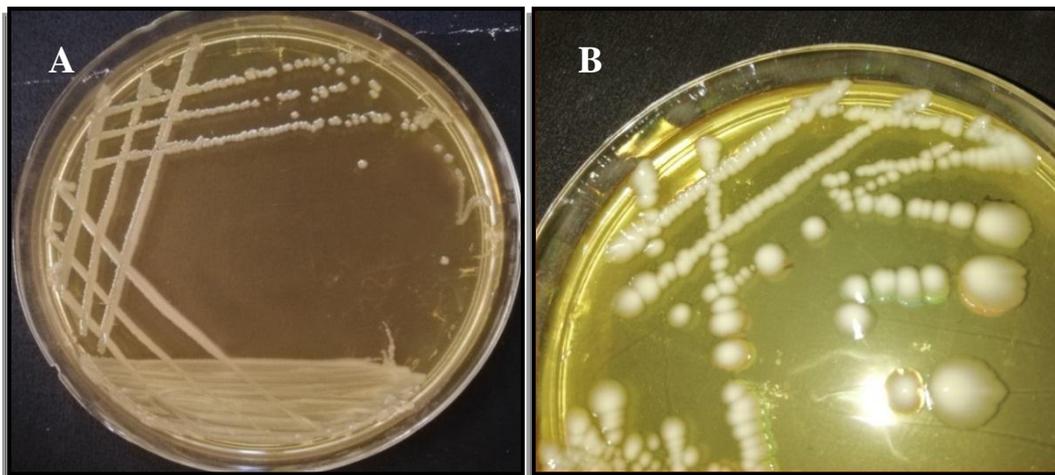


**Fig. 6: Colonies of *T.forsythia* on TF blood agar: (A) after 4 days and (B) 7 days**



**Fig. 7: Gram negative rod cells of *T.forsythia* (1000X) under light microscope**

*T. denticola* grew after 4 days to white- hazy or creamy colonies on TYGVS agar which increased in diameter after 5 days due to their free motility and diffusion in viscous medium Fig. (8 A and B). Their cells were coiled gram negative under the microscope Fig. (9). The coiled shapes in these bacteria are due to the winding of the periplasmic flagella around the cell cylinder. Our observations were explained according to Mohamad (2010).



**Fig. 8: Colonies of *T.denticola* on TYGVS agar: (A) as white colonies (B) enlarged in size**

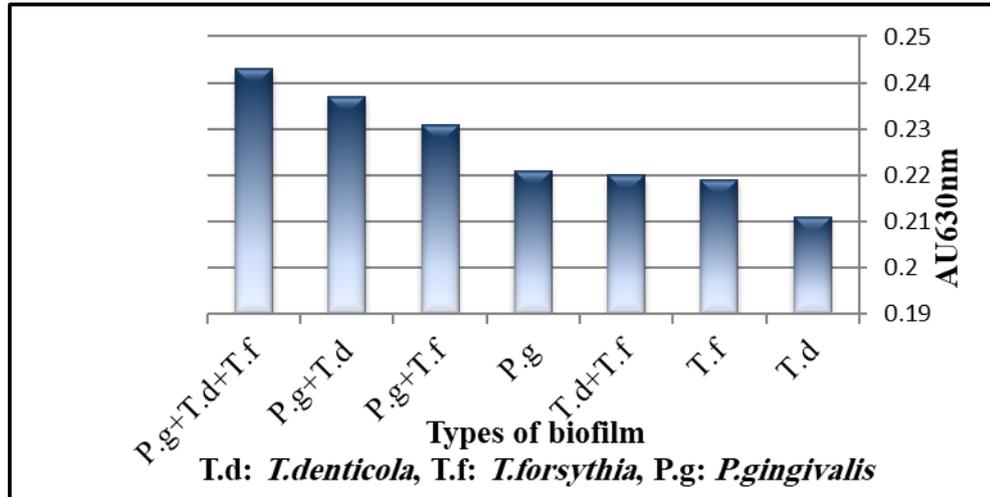


**Fig. 9: Coiled gram negative cells of *T.denticola* suspended in normal saline (1000X) under light microscope.**

From our current observations during laboratory experiments and those of previous reports the following conclusions can be documented: because oral tissues harbor over 700 species, most of which are closely related and communicate to establish infections (Bedran *et al.*, 2016), also, these bacteria response to each other by modulating gene expression of growth factors and cellular structural proteins (Zhu *et al.*, 2013) and change their gene expression in response to *in vitro* growth (Mendez *et al.*, 2019); therefore, isolation each one alone in culture media lead to changes in genes expression which caused great variations in their culturing and cellular morphological properties. Furthermore, because of their anaerobic nature, exposure to air during experiment processes highly affected their shapes that made it difficult to be sure of their true isolation and purification. Therefore, to retain their cellular shape as much as possible, the stained smears were prepared from bacterial suspension in normal saline (Muhammad and Al-Rawi, 2011). Dependence on the data outlined in the current study will be helpful guidelines to the wright pathway in the study and identification of red complex pathogens which must be supported by molecular methods.

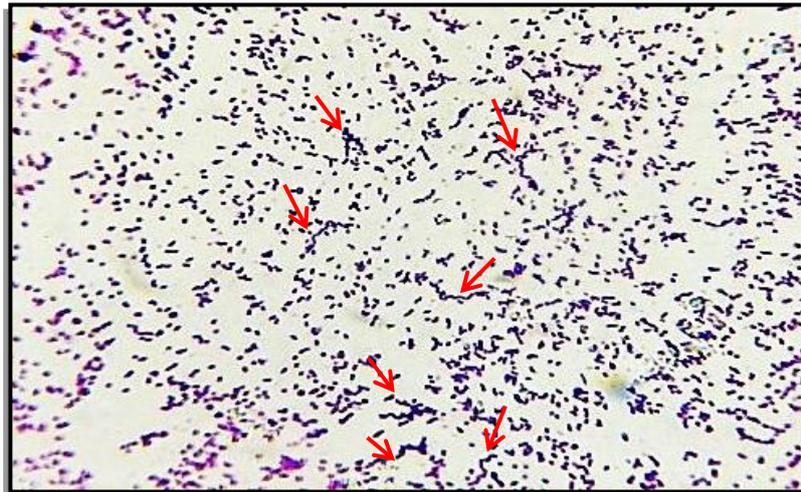
#### **Mono- and polymicrobial biofilm formation of red complex periodontal pathogens:**

The results of the current study about the formation of biofilm by the red complex pathogens either as mono or poly- species are represented in Histogram 1 as an absorbance reading of the crystal violet- stained biofilm. According to this histogram it is evident that the monotypic biofilm produced by *P. gingivalis* was the largest amount ( $OD_{630}= 0.221$ ) followed by biofilm of *T. forsythia* (0.219) and the less amount formed by *T. denticola* (0.211). When two types were grew together more amount of biofilm was produced rather than their monotypic biofilm, as it is clear that two- species biofilm formed by *T. denticola*+ *T. forsythia* ( $OD_{630}= 0.220$ ) was greater than the monotypic biofilm of both species, but however, this two species biofilm was less than the monotypic biofilm of *P.gingivalis*. The amount of two species biofilm involved *P. gingivalis* was greater especially that with *T. denticola* ( $OD_{630}=0.237$ ) then with *T. forsythia* ( $OD_{630}=0.231$ ). The most abundant one was the polymicrobial biofilm involved the three species with an  $OD_{630}= 0.243$ .



**Histogram 1: Mono- and polymicrobial biofilm formed by red complex pathogens**

According to our results, *P. gingivalis* was the most producer of biofilm than the two others as the amount of monotypic biofilm formation was larger than the two- type biofilm of (*T. denticola*+ *T. forsythia*) and when *P. gingivalis* involved in biofilm formation with each of two others, the amount of two- type biofilm was more increased especially with *T. denticola*. Contribution of the three types yielded much higher polymicrobial biofilm. These results revealed the synergistic, not antagonistic, response between these species when they grew together which was most evident between the two intimate types, *P. gingivalis* and *T. denticola* with the attractive notice that *T. denticola* could assume its typical spiral shape when grew together with *P. gingivalis* rather if grew alone and the cells of *P. gingivalis* were much higher than *T. denticola* as it is evident in Fig. (10).



**Fig. 10: Co-presence of *P.gingivalis* and *T.denticola* assists spiral coiled of the later for better viewed by 1000X of light microscope stained with crystal violet.**

Production of a homotypic biofilm by each type of oral bacteria and their contribution in a polymicrobial biofilm was considered as a prominent phenomenon during their life cycle (Wessel *et al.*, 2016). In accordance to our results, other researchers found that *P. gingivalis* acted as key stone pathogen that firstly adhered and produced larger amount of biofilm to promote the adherence of the less producer and poor colonizer *T. forsythia* and *T. denticola* which produced less amount of biofilm (Dashper *et al.*, 2014), and the synergistic cooperation between these three types was determined to involve nutrients exchange and facilitate adhesion which will be reflected by

increase their appearing growth and enhance biofilm formation, in such a way that promotes their cooperative pathogenicity in infection process (Yamada *et al.*, 2005).

The findings of the current study can be further discussed depending on the observations of former researchers who noticed that the duplication of *P. gingivalis* is earlier than *T. denticola* when they were co- grown in continuous culture because *P. gingivalis* reduced the energy- consuming metabolism and increased the biomass and they were bound to each other and developed a stable cells ratio of 6:1 respectively as was determined by real-time PCR (Tan *et al.*, 2014); therefore, *P. gingivalis* cells' number were greater than *T. denticola*. Their co-presence also activated the genes in *T. denticola* for dentilisin production (which mediate its adherence with *P. gingivalis*), glycine catabolism and genes for FlaB proteins (periplasmic flagella proteins) which judged the whole shape of cell cylinder (Kaplan *et al.*, 2009; Zhu *et al.*, 2013); therefore, *T. denticola* assumed its spiral shape when grew with *P. gingivalis*. In addition to, their co- presence showed activation the genes of *T. denticola* to produce succinate needed by *P. gingivalis* which in turn down regulated the genes for fatty acids synthesis and up- regulated the genes for thiamine pyrophosphate (TPP) and isobutyric acid production, both are required for *T. denticola* growth (Ammann *et al.*, 2012; Tan *et al.*, 2014). This syntrophic cooperation appeared to enhance growth and polymicrobial biofilm in an attractive way that these genes were not activated unless the two partners were being together (Dashper *et al.*, 2014).

Additional contribution of *T. denticola* in the abundance of biofilm was by its periplasmic flagella's distinctive motility in highly viscos media forming pores in the matrix of the biofilm thus increasing the follow of nutrients and remodeling the biofilm structure; hence enabling a higher biomass to be persistent (Popova *et al.*, 2013; Zhu *et al.*, 2013). From the other hand, *P. gingivalis* colonized firstly to help the colonization of the poor one, *T. denticola*; such biofilm adhered tightly in the subgingival plaque or in the microtitter plate and more withstood mechanical removal or washing during staining processes (Yamada *et al.*, 2005).

*T. forsythia* synergistic role in the subgingival red complex biofilm was recorded via the production of succinate used by *P. gingivalis* as precursors for phospholipids (Sharma, 2010); in turn, over growth of the highly proteolytic *P. gingivalis* lead to additional destruction of host proteins releasing peptides and amino acids nutrients for *T. forsythia* which by its own proteolytic enzymes also provides additional peptide and amino acids to enhance the overall growth of pathogens (Kuboniwa and Lamont, 2010). Add to this, *T. forsythia* was known to synthesize its own peptidoglycan subunits by a unique system that scavenges the by-products of peptidoglycan degradation of oral biofilm bacteria. By scavenging these by- products in periodontal tissues, which can act as activators of host innate pattern- recognition receptors, *T. forsythia* assists in evading the periodontal bacteria from the immune response (Boneca, 2005).

This helpful co-presence enriched the massive formation of polymicrobial biofilm and gives logic causes for the increased resistance in the polymicrobial presences. These mentioned data are also supported by the statistical analysis of the molecular quantitative estimation of bacteria in plaque samples done by Torrungruang *et al.*, (2015) who concluded that the severity of infection was related to the co- presence of red complex bacteria while if two of them were excluded, the presence of the later alone would not be significant.

## CONCLUSIONS

Several types of periodontal pathogens were simultaneously isolated from the same sample that required an accurate method for precise diagnosis. Molecular diagnostic protocols, especially LAMP, offered rapid accurate diagnosis of these pathogens with fewer steps. Red complex pathogens showed great morphological variations. *P. gingivalis* formed the much higher amount of biofilm and fortified the growth of the two others to establish polymicrobial biofilm in a cooperative synergistic manner.

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## تشخيص مجموعة ممرضات المعقد الاحمر من مرضى التهاب انسجة حول الاسنان المزمن في مدينة الموصل

### الملخص

عنيت الدراسة الحالية بعزل ممرضات المعقد الاحمر وتشخيصها بطريقة جزيئية حديثة كأول استخدام ووصف محلي لها وتوصيف هذه الممرضات بالصفات المظهرية والاعشوية الحيوية. تم اخذ عينة سائل اللثة من اصابات انسجة حول الاسنان المزمنة ولقحت على ثلاث انواع من الاوساط الزرعية، *Schaedler Anaerobe Agar*, *Tannerella forsythia* (TF) agar, and *Trypton Yeast extracts Gelatin Volatile fatty acids and Serum* (TYGVS) agar. تم تنقية الاشكال المختلفة من المستعمرات النامية وشخصت بطريقة *Loop-Mediated Isothermal Amplification protocol* (LAMP) للكشف عن انواع مجموعة المعقد الاحمر. تم تقدير الغشاء الحيوي البكتيري بمصطلح الغشاء الاحادي والمتعدد الانواع عن طريق قياس امتصاصية الغشاء الحيوي المتكون في الصفيحة العيارية والمصبوغ بالكريستال البنفسجية. ظهرت اشكال مختلفة من المستعمرات في العزل الاول. طريقة LAMP كانت ذو فائدة قيمة للتشخيص السريع للنوع الهدف من DNA المستخلص او الخلايا الكاملة خلال نصف ساعة. تم الكشف عن الانواع الثلاث من ممرضات المعقد الاحمر سوية في نفس عينة سائل اللثة. وأنها كونت غشاء حيوي احادي ومتعدد الميكروبات بنمط تآزري خاصة بين النوعين *P. gingivalis* و *T. denticola*. بروتوكول طريقة الجزيئي LAMP المتطورة كان مميز في تشخيص ممرضات المعقد الاحمر والتي ابدت تباين مظهري كبير. النوع *P. gingivalis* عزز نمو النوعين الاخرين لتكوين غشاء حيوي متعدد الميكروبات بنمط تآزري.

الكلمات الدالة: طريقة LAMP، لتشخيص الجزيئي، *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*، التهاب انسجة حول الاسنان متعدد المايكروبات.