Colorimetric LAMP Molecular Method for Immediate Detection of Three Periodontal Pathogens

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Abstract

Background and Purpose: For enhancing the efficiency of diagnostic tools and overcome drawbacks faced in diagnosis attempt, molecular methods have been developed aid accurate and rapid identification of bacterial species particularly those which are difficult to study with. The current study searched to find a more specific and rapid method serves researchers for the identification of periodontal pathogens and overcome disturbances encountered in study such anaerobic fastidious organisms.

Methods and Results: Three periodontal pathogens, *Porphyromonas gingivalis , Tannerella forsythia* and *Treponema denticola* were characterized and identified by phenotypic features and Loop- Mediated Isothermal Amplification (LAMP) techniques which involved 4 sets of primers targeted to 16SrRNA genes and loop primers and the Colorimetric Master Mix containing *Bst* DNA polymerase and Phenol Red for the detection of amplicon formation. There was a variance in phenotypic characters of the isolates of the same species. LAMP, as a novel molecular technique was of usefulness value in identifying the target weather as extracted DNA or whole cells in a high specific and very rapid manner within 30 min. by visual reading of the results.

Conclusion: It is strongly recommended the use of the novel LAMP method in researches work with periodontal pathogens as its advantages make it superior to other molecular techniques in respects of a higher specificity, rapidity, sensitivity and overcome DNA extraction step and special equipment in that it can be used at chair- side identification.

Background:

Aiming to increase accuracy and reduce the time required for diagnosis, molecular- based diagnostic techniques have been evolved as valuable tools, especially if the causative agent is difficult to be isolated due to fastidious nature as in the case of periodontal pathogens. PCR is the typical example of such techniques that is very sensitive, specific, rapid and simple for the detection of periodontal pathogens within few hours rather than days. Several PCR- derived methods have been developed for the purpose of facilitating detection and increasing sensitivity and specificity, e.g. nested PCR, PCR- RFLP, multiplex PCR and etc. [1, 2]. However, PCR methods require special reagents and devices (such as a thermocycler) and post amplification processing and are still time-consuming to accomplish at chair- side [3]; these drawbacks led to the emergence of the alternative isothermal amplification methods which are easier and not requiring thermal cycling as these methods amplify nucleic acids to large copies under isothermal conditions [4]. Several isothermal amplification protocols have been generated over the last 10 years, one of which loop-mediated isothermal amplification (LAMP). LAMP is a novel DNA amplification method which relies on the strand displacement activity of the Bst DNA polymerase under isothermal conditions during an auto-cycling reaction and within an hour, an excess amount of the amplicon will be produced which can be detected by the naked eye. It uses 4 primers which recognize 6 distinct sequences on the target. The two inner primers bind to both sense and anti-sense strand to initiate the polymerization by Bst DNA polymerase. The binding of the outer primers initiates strand displacement synthesis of a single

stranded DNA with stem-loop structure to be the template for the next cycle. The reaction is repeated several times to yield large amount of DNA with different length of stems. The reaction can be further accelerated by the incorporation of the loop primers [3, 5, 6, 7].

Results:

Primary Diagnosis of Bacterial Isolates:

Colonies from 30 GCF samples grown on each culture medium were selected depended on Moll (2016) [8]. The black colonies on Schaedler Anaerobe Agar, white or light yellow colonies on TF agar and white or creamy colonies on TYGVS agar were selected for subculturing. There was great variance in their cultural and cellular morphological characters, therefore, molecular techniques for diagnosis of these anaerobic fastidious periodontal bacteria was of crucial demand.

Molecular Diagnosis of Bacterial Isolates:

The current study depended on the powerful molecular technique, LAMP reaction for the identification of the three periodontal pathogens which amplifies the nucleic acid at the 16S rRNA gene, thus offering high sensitivity. Additionally, targeting 16S rRNA genes of a multicopy gene by 4 sets of primers, forward inner primer (FIP), backward inner primer (BIP) and two outer primers (F3 and B3) that recognize six distinct sequences in the 16S rRNA gene will further increases the specificity of the amplification with high speed by adding the loop primer (LB) [2]. Also, positive results were easily visualized by the naked eye within 30 min. by using the WarmStart Colorimetric LAMP 2X Master Mix as the color of the reaction solution changed from pink to yellow upon the presence of species- correspond amplicon (Fig. 1).

The positive results of LAMP reaction were seen when the target DNA of a certain isolate was added to a tube containing the primers specific to one species and hence, this target was identified to belong to that species related to that set of primers. The negative result (no color change of Phenol Red) was seen in the negative control tube (without target) and also in a tube when the target of the other two bacterial isolates was added to these primers sets. This confirms the high specificity of LAMP primers and the purity of the sample. Furthermore, the current study found that LAMP technique was also suitable for use with the whole intact bacterial cells without the need to extract the DNA as the same accurate results were obtained.

Phenotypic Description of Bacterial Types

The confirmed isolates by LAMP reaction were then depended for further description of their phenotypic characters. All the three types of bacteria were co- isolated from all specimens of chronic periodontitis. P. gingivalis isolates grew on Schaedler Anaerobe Agar as black pigmented circular, convex with entire- edge colonies after 4 days of incubation with beta-hemolysis (Fig. 2a and b). In subsequent sub-culturing, they may render brown or gray colonies (Fig. 2c and d). They were gram negative coccobacilli under light microscope (Fig. 3). Colonies of T. forsythia developed after 4 days on TF medium as white, small, non-

hemolytic colonies (Fig. 4a). After 7 days they enlarged and may gain yellow color (Fig. 4b). Their cells were gram negative bacilli under microscope (Fig. 5). T. denticola grew after 4 days to white- hazy or creamy colonies on TYGVS medium (Fig. 6a) and characterized by their spiral gram negative cells under the microscope (Fig. 7). Their colonies increased in diameter after 5 days due to their swarming habit in viscous medium (Fig. 6b).

Discussion

Isolation and perfect identification of periodontal bacteria has a recent focus due to the relationship of periodontitis not only with tooth loss but also with cardiovascular disease and atherosclerosis. Among the periodontal bacteria, the red complex group; P.gingivalis, T. forsythia, and T. denticola have been strongly implicated as chief pathogens of periodontitis, and the strong correlation between the presences of these bacteria and mixed periodontal infections was proved. These organisms are powerful contributors in the development of adult, chronic periodontitis and also oral malodor [9, 10]. Kasuga and others [11] pointed that the proportion of these organisms gives essential information on the severity of periodontitis. Therefore, great attention to investigate new powerful techniques that help one in the study of these organisms was paid. Confirmation of specificities is critical importance in the detection of oral bacteria because oral cavity is the habitat of over 700 species or closely related phylotypes. Therefore, the need to develop a rapid, sensitive and more specific detection system is of essential aspect in periodontitis for the task of causative diagnosis and healing improvement [1, 12]. Because of the anaerobic nature of these organisms, there were great variations in their culturing and cellular morphological properties when exposed to air during experiment processes which make it difficult to be sure of their true isolation and purification. Therefore, to aid in maintaining their cellular shape as much as possible, the stained smears were prepared from bacterial suspension in normal saline. Furthermore, they required a list of biochemical tests for identification and exact differentiation between the closely related species that become additional disturbance when identifying more than one species; also there is no available rapid identification systems specified for each species of periodontal bacteria, and the available one like Rapid ID32A which utilizes 32 tests are suitable for the identification of some anaerobic bacteria to the genus level. For these drawbacks the dependence on molecular techniques for identification is highly sought that greatly simplified the identification of such cases [13, 14, 15]. Although all popular 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 enables to get accurate results read by the naked eye within 30 min. of incubation under one temperature thus exterminates the need of a thermocycler apparatus to change temperature, post amplification processing, e.g. electrophoresis or a fluorescent- labeled probes and other supplements needed for PCR- based techniques.

Quick results were promoted by using the pH- based WarmStart Colorimetric LAMP 2X Master Mix comprises an optimized formulation of Bst 2.0 WarmStart DNA Polymerase in a special low-buffer reaction solution containing $MgSO_4$ and all needed cofactors at optimized 2X concentrations and the

visible pH-sensitive dye, Phenol Red which changes color from bright pink to yellow when pH dropped as a consequence of proton production upon nucleic acid amplification that goes on from the extensive DNA polymerase activity; thus allowing visual rapid and easy detection of LAMP result. Bst 2.0 DNA polymerase is a Bacillus stearothermophilus DNA polymerase I, large fragment engineered for improved isothermal amplification function with strong strand displacement activity and improved amplification speed, yield, salt tolerance and thermostability. It is inactive at room temperature, and activated when the reaction is warmed above 40 °C [16]. These two components of Master Mix used in the current study helped the performance of the reaction in a water bath at 65 °C without the need to a thermocycler, and rapid detection of the amplicon within 30 min by the naked eye without the need of post amplification processing.

The high specificity of the designed primer sets of LAMP for each periodontal pathogen is correlated to the discrimination of the target sequence by six independent sequences in the initial stage and by four independent sequences during the later stages of the reaction. LAMP protocol also affords simplicity and quickness for uninterrupted amplification under isothermal settings within an hour and naked-eye scan. The previous trials of the crude template suggested the applicability of the LAMP method without the DNA extraction step as there was no significant influence of the co-presence of non-target DNA from other bacterial species. Also, the contamination from human cells during sampling step was not so critical as to influence the sensitivity. These findings put the LAMP methods as powerful tools for the microbiological analysis of periodontitis, especially in places such as private clinics, bedsides or dental chair-sides [17, 18, 19].

The possible use of intact bacterial cell in the amplification reaction was proposed by team of Miyagawa [2]; they estimated that this required cell number 100 times more than extracted DNA for amplification as it was hypothesized that at least 1% of bacterial cells would be hurt during the processing and incubation step at temperature above 60 °C. This novel method also possesses the potential in quantification assay of the amount of DNA by a kinetic analysis of the time-related changes in turbidity with the precipitation of magnesium pyrophosphate upon amplification [1, 17]. The sensitivity of this method was evaluated to be in the detection limits of 21 bacterial copies and 20–30 min from a serially diluted chromosomal DNA [1, 19]. The current study used one set of loop primers depending on the conclusion of others [2] who claimed that by using two LB primers, the method revealed high sensitivity and rapidity with detection limit of 1–2 copy genes/ tube; but in the case of nonspecific amplification, e.g. in the negative control tube it is considered to be contamination during the manipulation, aerosol from pipette etc. With one LB primer the chance of nonspecific amplification was reduced although slightly reduced the sensitivity.

Conclusion:

Because of the high perturbations encountered, the current study strongly recommended the use of the novel LAMP method in researches intend to work with periodontal pathogens and other fastidious microorganisms as all its advantages make it superior to other molecular techniques in respects of a higher specificity, rapidity, sensitivity that overcomes the DNA extraction step and special equipment.

Materials And Methods:

There is little if no local study other than our previous one [20, 13] works in the field of isolation of periodontal pathogens because of the difficulties encountered in the identification of such anaerobic fastidious organisms. Now our study aimed to show method that simplifies these difficulties to be the beginning on the road of its use in the identification purposes of researches.

Bacterial samples

Gingival Crevicular Fluid (GCF) samples were collected from periodontal pockets of chronic periodontitis having a pocket depth (PD) of ≥ 4 mm and positive Bleeding On Probe (BOP) using paper points (40 mm). The participants were checked for having no systemic diseases, not taking antibiotics prior sampling, non-smoking, no pregnant women. The paper points were deepened to the base of pocket for 30 sec. and then placed in an eppindorf tube containing 500 µl of PBS (pH7.2) as transport fluid. The specimens were taken under the supervision of a specialized dentist in the Teaching Hospital of Dental College in Mosul University.

Culture media

Schaedler Anaerobe Agar with blood, haemin and vitamin K was prepared according to Oxiod Company, TF medium according to [21], TYGVS medium according to [1] and [20]. The GCF sample was inoculated into the three types of media and incubated in an anaerobic Jar using CampyGen, microaerophilic atmosphere generation system (Oxiod Ltd, Japan) for 5 days at 37 °C.

Identification the bacterial isolates

The colonies from each medium were identified and featured by phenotypic characters, cultural and microscopically with modified gram stain, and the molecular method, LAMP technique.

DNA extraction

Wizard® Genomic DNA Purification Kit (Promega /USA) was used to extract DNA from bacterial cells.

Primers for LAMP reaction

Five sets of species-specific primers (Table 1) targeted to 16SrRNA of each bacterial type [2] were purchased from ITD company. Primers stock solutions were prepared at 100 µM concentration in a volume of deionized water recommended by the manufacturer. LAMP primer mix solution was prepared as 10X stock mix solution for a reaction volume of 25 µl as recommended by $BioLab_{INC}$ (New England).

Table 1

species- specific primers for LAMP reaction

Bacterial type	Primer	Sequence
P. gingivalis	FIP	5'-CACCACGAATTCCGCCTGCCTGAGCGCTCAACGTTCAGCC-3'
	BIP	5'-ATCACGAGGAACTCCGATTGCGCGCCTTCGTGCTTCAGTG-3'
	F3	5'-GGTAAGTCAGCGGTGAAACC-3'
	B3	5'-GCGTGGACTACCAGGGTAT-3'
	LB	5'-GCAGCTTGCCATACTGCGA-3'
T. denticola	FIP	5'-CATCCTGAAGCGGAGCCGTAGTACCGAATGTGCTCATTTAC-3'
	BIP	5'-GCTGGTTGGTGAGGTAAAGGCCATCTCAGTCCCAATGTGTCC-3'
	F3	5'-CCCTGAAGATGGGGATAGCT-3'
	B3	5'-TGCCTCCCGTAGGAGTTTG-3'
	LB	5'-CACCAAGGCAACGATGGGTAT-3'
T. forsythia	FIP	5'-CCATCCGCAACCAATAAATCTCTAATACCTCATAAAACAGG-3'
	BIP	5'-TAAGCCATCGATGGTTAGGGCGTGTCTCAGTACCAGTGTG3'
	F3	5'-GATAACCCGGCGAAAGTCG-3'
	B3	5'-TGCCTCCCGTAGGAGTCT-3'
	LB	5'-GTTCTGAGAGGAAGGTCCCC-3'

WarmStart Colorimetric LAMP 2X Master Mix

Purchased from BioLabINC (New England).

LAMP reaction mixture

Prepared as ordered by BioLabINC instruction in Table 2. The reaction mix was incubated at 65◦C for 30 min. to trace the change of color from pink to yellow.

Table 2

constitutes of 25 µl volume LAMP reaction

Substance	DNA target	No- template control (NTC)
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_2O	9 µl	10 µl
Total Volume	25 µl	25 µl

Declarations

Ethical approval: All procedures performed in this study were in accordance with the ethical standards of Biological committee of Iraqi Universities.

Consent for publication: “Not applicable” in this section. There is no any information about the participants published in this article, which is an acquisition from the doctoral thesis.

Competing interests: "The authors declare that they have no competing interests" in this section

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Authors' contributions: Each author made substantial contributions to the work

Acknowledgements: "Not applicable" in this section.

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Figures

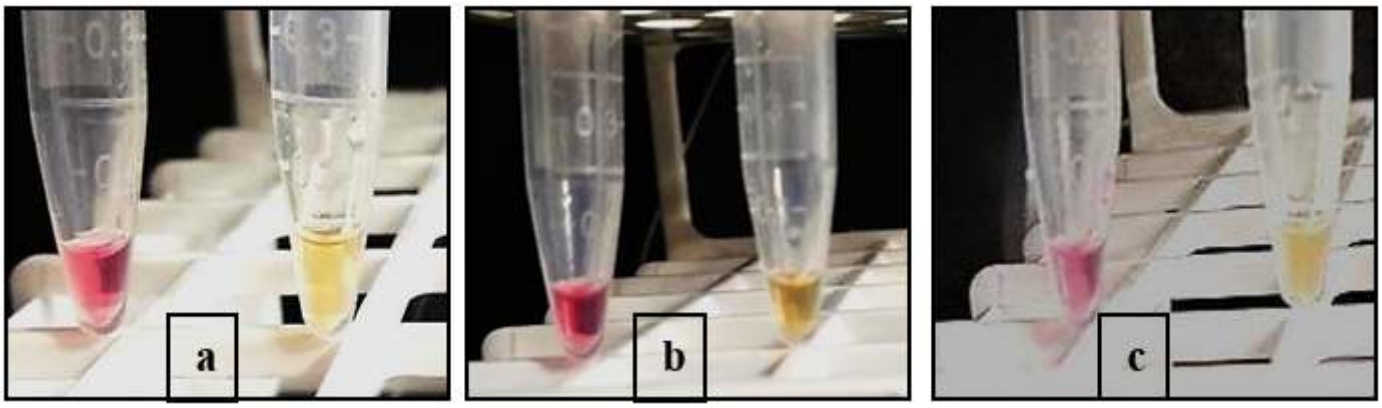

Figure 1

LAMP reaction; solution with pink color is the negative control; yellow color is positive amplification of the target DNA of P. gingivalis (a), T. forsythia (b) and T. denticola (c).

Figure 2

Colonies of P.gingivalis on Schaedler Anaerobe Blood Agar pigmented as black (a) with β-hemolysis (b), brown(c) or gray (d).

Figure 3

Gram negative coccobacilli cells of P.gingivalis suspended in normal saline (1000X) under light microscope.

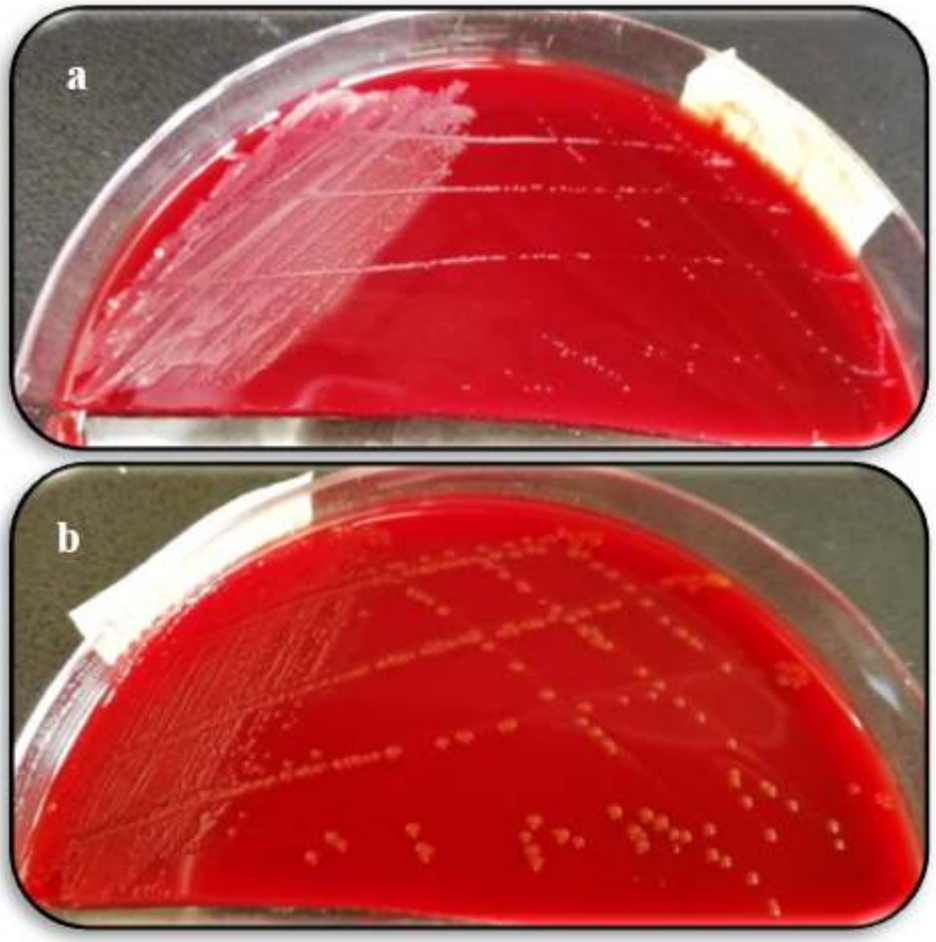

Figure 4

Colonies of T.forsythia on TF blood agar after 4 days (a) and 7 days (b)

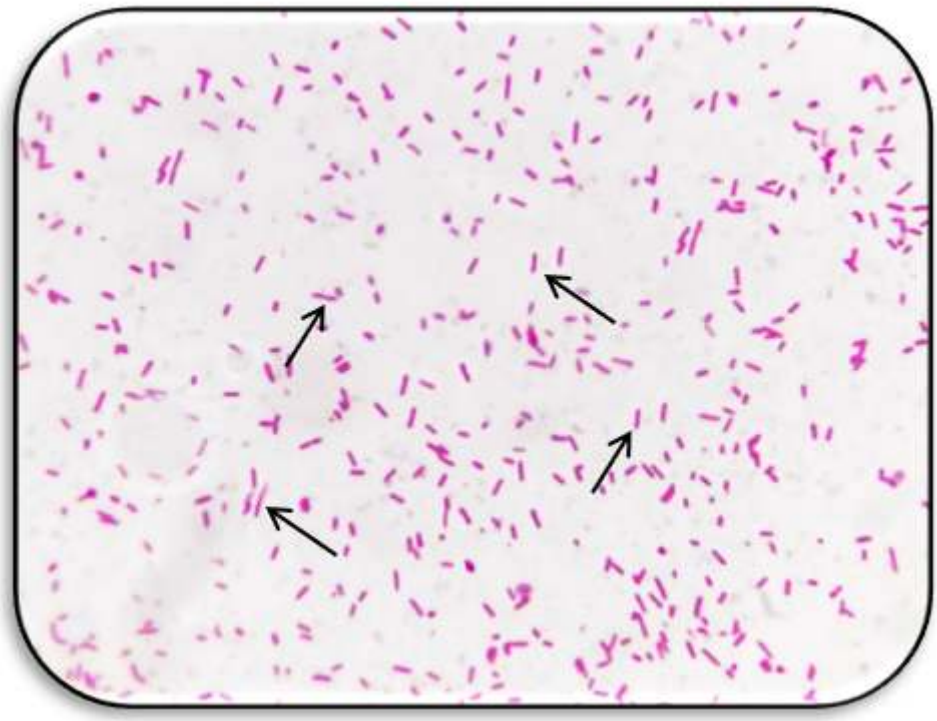

Figure 5

Gram negative rod cells of T.forsythia suspended in normal saline (1000X) under light microscope.

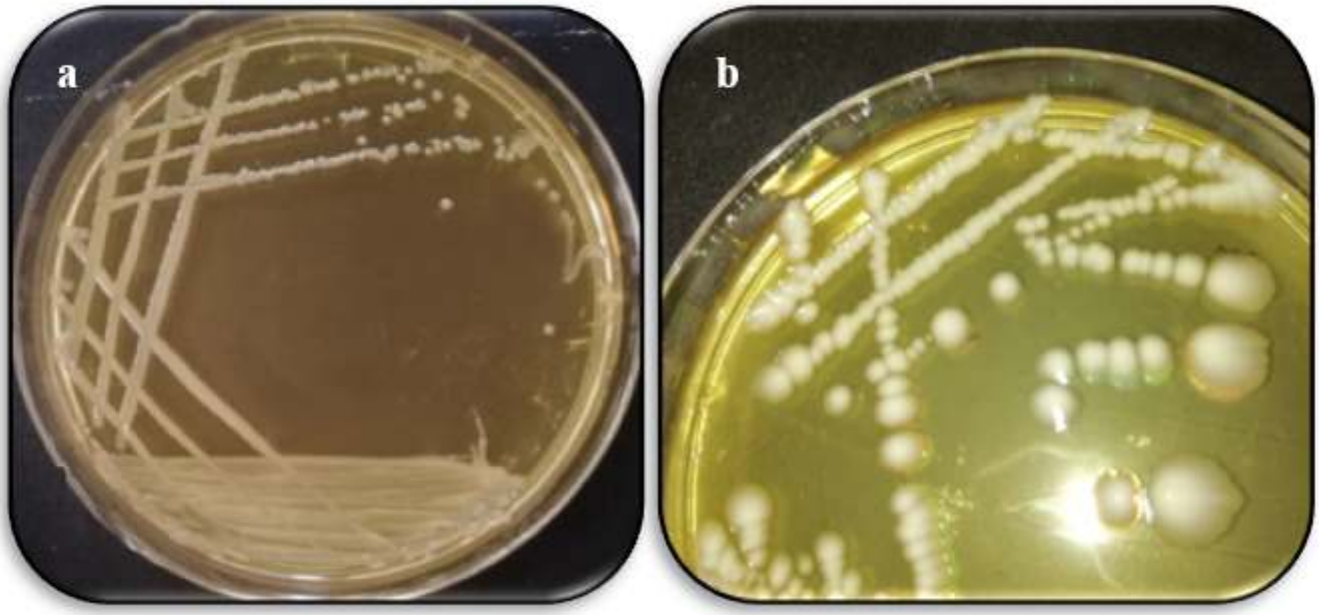

Figure 6

Colonies of T.denticola on TYGVS agar as white colonies (a) enlarged in size (b)

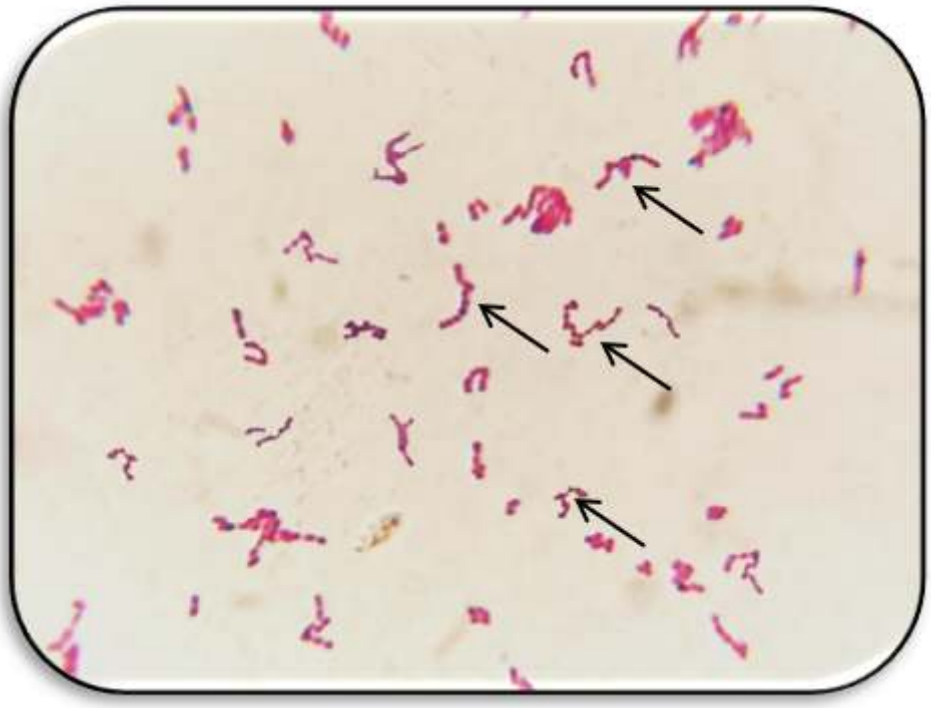

Figure 7

Spiral gram negative cells of T.denticola suspended in normal saline (1000X) under light microscope.