THE BACTERIUM *Fusobacterium sp.* MAY INTERFERE WITH CONCEPTION

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ABSTRACT

This study was aimed to investigate microbiological and molecular markers of periodontal problems and its association with conception in 101 Iraqi women, 50 primary infertile while 27 secondary infertile and 24 pregnant as control group. Major periodontal pathogen in the subgingival plaque and cervical vaginal swab specimens was detected, and molecular marker (16SrRNA) gene used for detection of *Fusobacterium* in our study. *Fusobacterium sp.* isolates from oral samples were examined biochemically and confirmed by molecular detection, they were also subjected to antibiotic sensitivity test and were shown to be resistant to β-lactamase family of antibiotics and were mostly resistant to Ciprofloxacin. Four *Fusobacterium sp* samples were identified in both Oral samples and vaginal-cervical specimens of the same four women from the primary infertile group. *Fusobacterium sp.* was significantly (P<0.01) more frequently detected in the Oral samples and vaginal-cervical specimens among primary infertile group (38.7%) than pregnant (9.1%) as so among secondary (30.0%) than pregnant (9.1%). Analysis identified a model of a significant predictive factor of presence of *Fusobacterium sp*.: primary infertile [Odd ratio (OR) = 6.3 at 95% CI = 1.33-25.1] and secondary infertile OR = 4.2 at 95% CI = 1.84-12.22. this can show that infertalty state can be more risked than pregnant women to have the *Fusobacterium sp* and be effected by it.

Keywords: peridontitis, , bacterial infection, oral bacteria. delay of pregnency.

بكتيريا *Fusobacterium sp*. واحتمالية تداخلها مع منع الحمل

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المستخلص

في هذه الدراسة,تم التحري عن المعلمات المايكروبية والجزيئية لمشاكل االتهابات اللثة وعلاقتها مع منع الحمل لدى 101 امراة عراقية,50 عقم اولي و27 عقم ثانوي بالاضافة الى 24 امراة حامل كمجموعة سيطرة. تم التحري عن العامل الممرض الرئيسي في كل من عينات قرح اللثة الثانوية ومسحات المهبل وعنق الرحم وذلك باستخدام الطرق الجزيئية وجين (16SrRNA) للتحري عن بكتيريا *Fusobacterium sp.* في هذه الدراسة. عزلات *Fusobacterium sp.* المعزولة من عينات الفم تم تشخيصها بالطرق البايوكيمائية وتم تاكيد التشخيص جزيئيا , كذلك اجري عليها فحص الحساسية ضد المضادات الحيوية واظهرت النتائج مقاومتها لمضادات عائلة البيتا لاكتاميز وخاصة مضاد Ciprofloxacin . اظهرت النتائج وجود اربعة عزلات *Fusobacterium sp.* في كل من عينات الفم ومسحات المهبل وعنق الرحم لنفس المراة لدى اربعة نساء من مجموعة العقم الاولي. أظهرت النتائج تواجدعزلات ال *Fusobacterium sp.* بتردد عالي في عينات الفم ومسحات المهبل وعنق الرحم لمجموعة العقم الاولي والثانوي من النساء (38.7%) و (30.0%) على التوالي مقارنة بمجموعة الحوامل (9.1%) , .حدد التحليل نموذجا لعامل تنبؤي لتواجد *Fusobacterium sp.* , ففي مجموعة العقم الاولي : (OR) = 6.3 at 95% CI = 1.33–25.1 اما في مجموعة العقم الثانوي :OR = 4.2 at 95% CI = 1.84–12.22 . مما يشير الى ان حالة العقم لدى النساء تكون اكثر تعرضا من النساء الحوامل الى خطر الاصابة بهذه البكتيريا.

الكلمات المفتاحية: التهاب اللثة,منع الحمل , الالتهاب البكتيري,*Fusobacterium sp*.

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INTRODUCTION

Fusobacterium, is a Gram negative bacterium, non-spore former, anaerobic species of the Fusobacteriaceae family that consists of nine genera including *Fusobacterium* and *Leptotrichia*. There are currently 14 species defined, Within the *Fusobacterium* genus some of which (including *F. nucleatum*) are identified as pathogens of human and animals that is gaining importance as a pathogen with a high number of associated diseases. (5). The *Fusobacterium* is a non-motile, rod-shaped, or bacilli bacterium. It shows a significant role in the progress of biofilm dental plaque as a bridge bacterium interaction with early and late colonizing bacteria in the oral cavity (3), as well as its role as a part of oral plaque, through its adhesive abilities which provide benefit as a link between early and late colonizers of this biofilm (27). It is one of the species found in the oral cavity, in both unhealthy and healthy humans. It is found in different kinds of periodontal diseases including the simple reversible kind of gingivitis and the progressive irreversible forms of periodontitis: chronic periodontitis and localized aggressive periodontitis (33). There are strong correlation between several of the cultivable bacteria for example *Prevotella intermedia*, and *Fusobacterium nucleatum* and periodontal disease has been mentioned (49). *Fusobacterium sp.* may cause with other oral species, e.g. *Tannerella forsythia, Porphyromonas gingivalis* and *Streptococci*, interaction in virulence is revealed which may result in increased bone loss, abscess, or death. (44). Previous studies have reported that *Fusobacterium sp.* was involved in wide variety of systemic diseases including gastrointestinal (GI) disorders, atherosclerosis (21), rheumatoid arthritis and respiratory tract infections (47). There has been an increasing interest in causes of infertility such as ovulation disorders (16), Fallopian tube pathology (35), ovarian insufficiency, endometriosis (46), and uterine or cervical abnormalities, as well as infection and inflammation that play a role and effect fertility in women by affecting the ovary, uterus, the embryo (26), and its relationship with periodontitis that share common risk factors such as age (25), obesity (12) and tobacco smoking (48), but the relation still needs more clarification and data of the effect of periodontitis on the success of becoming pregnant are restricted. However, research has shown that women with decreased clinical periodontal health and symptoms of gingival inflammation are more affected with *in vitro* fertilization problems (40, 4). One research has already shed light on the effect of microbiological markers of periodontal infection on conception (38).This study has been applied with a cohort of young women who failed to get successful conception and aimed to find a connection between the oral microorganisms and conception by using microbiological and molecular methods.

MATERIALS AND METHODS

Subjects:

Clinical specimens were collected from (101) of Iraqi women, who were grouped according to their conception status (primary infertility, secondary infertility and pregnant as control group), Subjects were enrolled from the High Institute for infertility Diagnosis and Assisted Reproductive Technologies/Al-Nahrain University, Baghdad-Iraq, from October 2018 to March 2019. Women ages ranged between (18-45) years, mean (29.47±2.5). Clinical diagnosis of Infertility and periodontitis was confirmed by consultation of clinical specialists.

Samples collection

Oral samples: Sub gingival plaque samples were collected by a sterile paper point (Albion/UK) that was inserted into two deep pockets of the periodontal area for 30 econds and removed. Sampling sites were protected from saliva influx with sterile cotton pellets. The paper points were collected in a sterile Eppendorf tube for each with 1 ml of sterile Thioglycolate medium (Hi-media/India), and transported in a cooled box to the microbiology laboratory for further experiments (31).

Cervical-vaginal Swabs:

Gynecological Sterile Speculum (China) was used to collect the samples from women by sterile swabs (swabs were taken from vagina and upper cervix by specialist physician). Swabs were immediately suspended in 1 ml of sterilized phosphate buffered saline PBS (pH=7.4), and then stored at -20℃until use for further molecular experiments (45).

Isolation and identification of oral bacteria

Paper point specimens were mixed by micropipette and diluted in buffer phosphate saline (10^{-2}). An aliquot of 100 µL was spread on culture plates containing blood agar medium, which was supplemented with Human blood 50.0 ml/L, Hemin 5µg /ml (5 mg /L.), Vitamin K1 (1µg /ml) (1mg /L.) and Bacitracin 10.0 mg/L and Nalidixic Acid 15.0 mg/L antibiotics in1000.0 ml of Distilled Water)as selective agents for isolation of such fastidious, strictly anaerobic oral bacteria , Selective medium plates were incubated in a tightly packed anaerobic atmosphere jar at 37°C for (48-72 hr.). Microscopically examination was conducted on pure bacterial culture; they were stained with Gram stain to distinguish their response to the stain to examine their color, shapes and arrangements. Biochemical characteristics was achieved by biochemical tests to evaluate the following characteristics: production of H2S, Indole test catalase, urease and sugar fermentation (rhamnose, adonitol, salicin, arabinose, inositol, sorbitol, sucrose, mannitol, raffinose, and maltose) (19).

Antibiotic Sensitivity test

Antibiogram of isolated *Fusobacterium sp.* strains was detected by Disc diffusion method and were based on the susceptibility patterns for selected antibiotics which represented various classes of antimicrobial agents, as show in Table (1). According to Kirby-Baure method used to carry out antimicrobial susceptibility. The isolates were explicated as susceptible, intermediate or resistant to a particular antimicrobial agent by comparison with standard inhibition zones as mention according to CLSI, 2011 (14).

Preparation of inoculum: bacterial inoculum was prepared in normal saline to adjust its turbidity, from fresh culture that was incubated anaerobically for 18-24 hr.,3-5 colonies were taken by sterilized loop then suspended in 5 ml of normal saline to get a culture with 10^5 cell/ml (7).

Inoculation and applying antibiotic discs: A sterile cotton swab was used to transfer bacterial culture (swab immersed into inoculum tube) then carefully and evenly spread on Mueller Hinton agar plates and left for 10 min., and then the antimicrobial discs (Table 1), were placed on the agar medium by using sterile forceps and were compressed to ensure contact with the surface of agar. The plates that contained the antibiotic discs inverted and incubated at 37 ˚C for 24 hr. under anaerobic conditions. Inhibition zone for every antibiotic was measured by a metric ruler in millimeters (mm) CLSI, 2013 (22).

Table 1. Antimicrobial discs used in this study

No.	Antibiotics	Code	Disc (µg/disc)
1	Ampicillin	AM	25
2	Amoxicillin	AX	30
3	Penicillin	P	10
4	Imipenem	IPM	10
5	Cephalothin	KF	30
6	Cefotaxime	CTX	30
7	Cefixime	CFM	5
8	Amikacin	AK	10
9	Gentamycin	CN	10
10	Vancomycin	VA	30
11	Clarithromycin	CLR	15
12	Tetracycline	TE	30
13	Chloramphenic	C	30
14	Ciprofloxacin	CIP	10
15	Meropenem	MEM	10

Detection of oral bacteria by molecular techniques

DNA extraction:

Molecular techniques were obtained for both Oral bacterial isolates and Cervical-Vaginal swabs that were taken from women specimens. The DNA of pure bacterial colonies was extracted by Genomic DNA mini extraction kit (Geneaid /Korea), according to the manufacture protocol: pure bacterial colonies were collected from the plates with a sterile inoculation loop into 1 ~ 2 ml tube of buffer and pellet was collected by centrifugation for 1min at 16,000 rpm, and the supernatant was discarded. A volume of 180 µl of GT Buffer was added into sample tube and then resuspended by vortex or pipette. Microbial fluid was collected from the Cervical-Vaginal swabs suspended in 1ml of sterile Phosphate buffer saline. Microbial fluid was collected by centrifugation for 5 min at 16,000 rpm, and then the supernatant was discarded. Aliquot of200 µl of GT Buffer was added into the sample tube, and then resuspended by vortex or pipette. Proteinase K solution (20 µl) was added into all sample tubes, then mixed for 10 sec by vortex, after that incubated for at least

10 min at 60℃(tubes were inverted 3-4 times during incubation). GB Buffer (200 µl) was added into the sample tube, then mixed by vortex for 10 sec, incubated at 70℃for 10 min (tubes inverted every 3 min for complete lysis). After lysis completed, 200 µl of absolute ethanol was added into the sample lysate and mixed immediately by vortex (any precipitate appears had been broken with pipette). About 620 µl of mixture was transferred into GD column (in a 2 ml Collection Tube) without wetting the rim, the cap closed, and centrifuged at 16,000 rpm for 2 min. The filtrate was discarded and the GD column was placed in a new 2 ml Collection Tube. W1 Buffer (400 µl) was added to the spin column without wetting the rim, and centrifuged for 30 sec at 16000 rpm. The flow-through was discarded and reused the collection tube. A volume of 600 µl of Wash Buffer was added (ethanol was added) to the GD Column, and centrifuged for 30 sec at 16000 rpm. The flow-through was discarded and reused the Collection Tube and centrifuged again for additionally 3 min at 16000 rpm to dry the Column matrix. The spin Column was placed into a new 1.5 ml tube, and 50-100 µl of Pre-heated Elution Buffer directly added onto the membrane. Incubated for 3-5 min at room temperature to allow completely absorbed, and then centrifuged for 30 sec at 16000 rpm to elute the purified DNA. (6).

PCR detection: To amplify the 16SrRNA gene, the *Fusobacterium sp.* primers used in the study shown in (Table 2), PCR was achieved on DNA extracts from subgingival bactrial isolates and cervical-vaginal swab samples. Amplification reaction was done by a Thermal Cycler in a 25 µl reaction volume containing 5 µl of Taq PCR premix, 10 picomoles/µ (1 µl) of the forward primer, 10 picomoles/µ (1 µl) of reverse primer, 3 µl of genomic DNA, and 15 µl distilled water. The 16S rRNA PCR was performed for 3 min at 94℃ and 34 cycles, with each cycle consisting of denaturation at 94^{O}C for 30 sec, annealing at 49^{O}C for 45 sec, extension at 72^{O}C for 1 min, and final extension for 7 min. (32). The amplified products were analyzed by gel electrophoresis on a 1.5% w/v agarose gel in 1x TBE buffer for fifty minutes (80 volt) and visualized by staining with red safe stain by UV transillumination.

Table 2. The specific primer of gene 16S rRNA for the detection of *Fusobacterium Sp*

Primers	Sequence	Size product
Forward	5`-AGA GTT TGA TCC TGG CTC AG -3`	
Reverse	5`-GTC ATC GTG CAC ACA GAA TTG CTG-3`	360 b. p

Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). Estimation of Odd ratio and CI was also used in this study.

RESULTS AND DISCUSSION

Study population characters: The results presented in this study are based on a total of 101 Iraqi women, whom were divided into three main groups depending on the state of infertility or pregnancy:

1- Primary infertile: which included 50 women, ages (18-45), they failed to conceive for unknown causes, years of infertility was ranged between (2-13) years. 31 (62%) of them with periodontal problems

2- Secondary infertile: which included 27 women, ages (21-42) years, which experienced previous pregnancy and started failing to conceive for unknown causes, years of infertility after last pregnancy (2-12) years. 15 (55.56%) of them periodontal problems

3- Pregnant: which included 24 women, ages (21-45) with normal pregnancy 11(45.8%) who had periodontal problems. As shown in (Table 3).

Table 3. Distribution of Study groups subjects according to periodontal state with Mean ± SE of age and Years of Infertility

Subjects/parameters	Total No. (%)	Periodontal problem No. (%)	Mean ± SE Age	Mean ± SE Years of infertility
Primary infertility	50 (50%)	31 (62.00%)	27.20 ± 0.94	5.00 ± 0.40
Secondary infertility	27(27%)	15 (55.56%)	30.81 ± 1.26	5.52 ± 0.60
Pregnant	24(24%)	11 (45.83%)	30.41 ± 1.32	---
Total	101	57	---	---
P-value		0.0271 *	0.0365 *	0.467 NS

* (P<0.05).

The percentage of primary infertile group of women was (50%), which was significantly higher compared to both secondary infertile (27%) and pregnant (24%) groups, (Fig.1a) this was showed in other studies with similar percentage, that showed the percentage of primary and secondary infertility among women was 62.97% and 37.03%, respectively (9). The primary infertile group of women showed the highest percentage (62%) with periodontal problems compared to secondary (55.5%) and pregnant (45.8%) groups at ($p<0.05$), as shown in (Fig. 1b), this was agreed with studies of unknown causes of conception, this distribution was also compared with other studies that showed varied result, when chronic periodontitis was positively associated with increased time of conception (TTC) and the authors are recommending that women in child bearing age should be encouraged to have regular preventive dental checkups in order to maintain good oral and periodontal health (36)

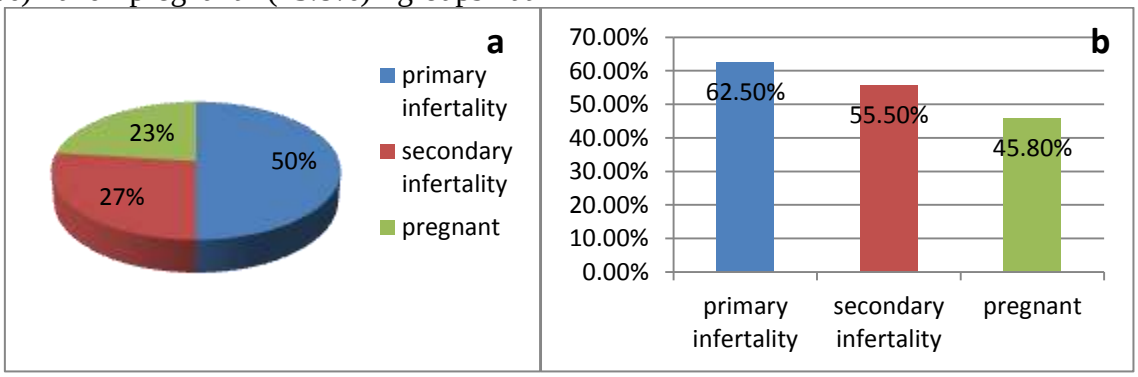

Figure 1. a) The percentage of women according to study groups (Primary, Secondary and Pregnant). b) The percentage of periodontal problems in study groups of (Primary, Secondary and Pregnant).

Periodontal diseases was known to be case of inflammation in the oral cavity which involves the teeth and surrounding gum (8). Studies have linked periodontitis with different diseases such as pre-eclampsia with low birth weight (41), diabetes - (42), Rheumatoid - Arthritis(31),increased cardiovascular diseases like Myocardial Infarction, - Atherosclerosis (43) and Alzheimer's Disease (37). Other studies also shed light on conception and abortions that exhibited the relationship between periodontitis and several pregnancy outcomes such as preterm birth, still birth and others (13).

Bacterial identification of periodontitis

Periodontitis may be caused by different agents, mostly known cause is bacterial agents, in which a wide range of bacterial species are related to periodontitis infections such as *Porphyromonas gingivalis* (30), *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* (1). Which are mostly anaerobic fastidious microorganisms that need special growth conditions to be isolated. Microbial examination of isolates from this study revealed different kinds of bacteria that varied in microscopic examination and showed different shapes and sizes of colonies, colors, on blood agar enrichment medium as well as for the microscopic examination that exhibited different bacterial shapes, arrangements and Gram positive as well as Gram negative bacteria. These isolates were sub-cultured for purification and a total of 21 (36.8%) different bacterial isolates were recovered of both Gram

negative and positive bacteria from the 57 paper point samples 16 (28.07%) yeasts and fungi were grown and 20 (35.08%) samples with no growth.

***Fusobacterium sp.* Isolates**

The *Fusobacterium sp.* bacterium was isolated from 4 oral samples of the primary infertile group. Microbial examination showed *Fusobacterium sp.* as Gram-negative, non-spore forming bacteria, slender or rod shaped bacilli usually with pointed ends. The bacterial colonies appeared on blood agar as small, convex, non-translucent and grey-creamy colonies and generally do not produce hemolytic reaction on blood agar these characteristics were also seen in the same of other studies (34) as shown in (Fig. 2a.b).

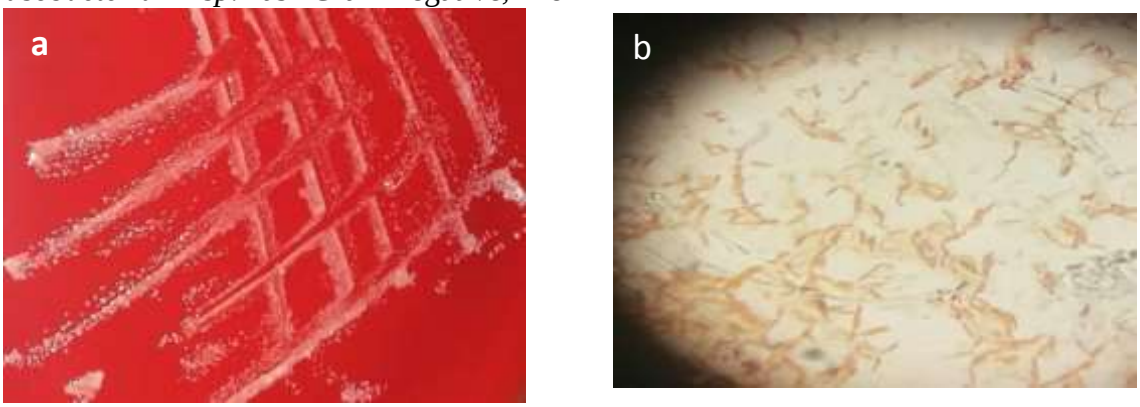

Figure 2a. *Fusobacterium* colonies cultured on Blood agar, (b) Micrograph of *Fusobacterium* isolated from subgingival plaque (by sterile paper point) by using Gram stain (100X). Cells appear as single, in pairs, or in short bacilli chains

Biochemical test

Fusobacterium sp. isolates showed negative result for urease test (11), while *Fusobacterium* gave a positive result for Indole test. Whereas the sugar fermentation test showed that *Fusobacterium sp.* isolates ferment some sugars by production of acids that convert the indicator chlorophenol red to yellow in one to three days of anaerobic incubation. The sugars (sucrose and maltose) were fermented by *Fusobacterium sp.* isolates, while six of carbohydrates (Rhamnose, salicin, arabinose, sorbitol, Mannitol and Raffinose) were not fermented by these isolates, these results agreed with that documented by Howe and coworkers (22).

Antimicrobial susceptibility of *Fusobacterium. sp* isolates

Disk diffusion method was used to detect the antibiotic sensitivity of *Fusobacterium.sp* against different kinds of antibiotics previously mentioned in (Table 1). *Fusobacterium* isolates showed resistance against many antibiotics (Ampicillin, Amoxicillin, Penicillin, Imipenem, Cephalothin, Cefixime and Amikacin), with significant differences at ($P<0.05$). whereas, these isolates showed susceptibility against (Cefotaxime (CTX), Gentamicin (CN), Vancomycin (VA), Clarithromycin (CLR), Tetracycline (TE), Chloramphenicol (C), Ciprofloxacin (CIP) and Meropenem (MEM).) as Shown in (Fig.3) and (Table 4).

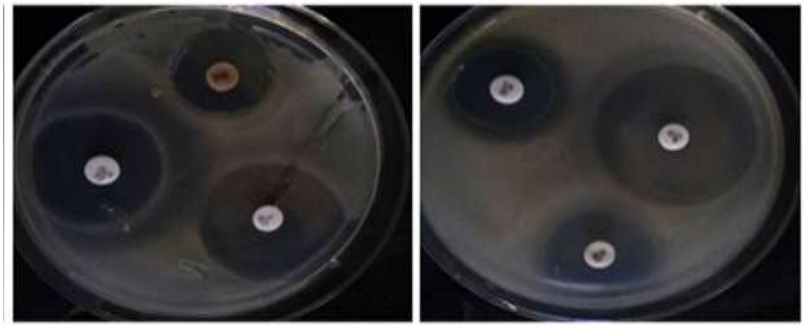

Figure 3. Antibiotic sensitivity of *Fusobacterium.sp* against Cefotaxime (CTX**), Gentamicin (CN), Vancomycin (VA), Chloramphenicol (C), Ciprofloxacin (CIP) and Meropenem (MEM)**

Table 4. Inhibition zone (mm) of a group of antibiotics against *Fusobacterium sp.*isolated strains

No	Antibiotics	Code	Disc (µg/disc)	Diameter of inhibition zones (mm)				Mean ± SE
				Strain 4	Strain 3	Strain 2	Strain 1	
1	Ampicillin	AM	25	R	R	R	R	-
2	Amoxicillin	AX	30	R	R	R	R	-
3	Penicillin	P	10	R	R	R	R	-
4	Imipenem	IPM	10	R	R	R	R	-
5	Cephlothin	KF	30	R	R	R	R	-
6	Cefotaxime	CTX	30	14	15	13	14	14.0 ±0.4
7	Cefixime	CFM	5	R	R	R	R	-
8	Amikacin	AK	10	R	R	R	R	-
9	Gentamycin	CN	10	23	22	23	20	22.0 ±0.7
10	Vancomycin	VA	30	16	19	18	20	18.3±1.1
11	Clarithromycin	CLR	15	26	26	24	25	25.1±0.47
12	Tetracycline	TE	30	13	16	19	20	17.0 ±1.58
13	Chloramphenicol	C	30	16	17	16	20	17.3±0.94
14	Ciprofloxacin	CIP	10	33	31	30	30	31.0 ±0.7
15	Meropenem	MEM	10	20	21	21	22	21.0 ±0.4
-	LSD value	-	-	-	-	-	-	3.061 *

*** (P<0.05); SE:Standard error; R: Resistant; mm: millimeter**

In this study *Fusobacterium* isolates showed resistance against β-lactams antibiotics. Many studies showed the ability of *Fusobacterium sp* to produce β-lactams and the first reports of penicillin resistance due to β-lactamase production by *Fusobacterium.sp* were published in the mid-1980s (50). The frequency of β-lactamase production by Fusobacteria seems to be increasing. In several studies observed surprisingly high frequencies of β -lactamase production by several anaerobic, gram-negative species in oral sites (28, 2). *Fusobacterium.sp* isolates showed resistance against Imipenem that is a semisynthetic thienamycin that has a wide spectrum of antibacterial activity against gram- negative and gram-positive aerobic and anaerobic bacteria including many multiresistant strains while *Fusobacterium.sp* showed sensitivity against Meropenem is similar to Imipenem but having greater activity against gram-negative bacteria (39), this variation in resistance of *Fusobacterium.sp* to antibiotics may be related to the presence or absence of plasmids that carry antibiotic resistant genes. Some studies have been reported that high resistance observed in several *Fusobacterium* strains which suggest the presence of antibiotic-resistance marker such as a plasmid or a chromosomal gene. Bacterial plasmids codifying for antimicrobial resistance have been recurrently observed (10). Sensitivity of *Fusobacterium.sp* isolates against other antibiotics may result from the differences in their chemical composition, antibiotic family and in concentration which lead to different inhibition zones. The highest inhibition zone was detected for the Ciprofloxacin (31.0 ±0.7). According to Sanford Guide to Antimicrobial Therapy (17), *Fusobacterium* can show sensitivity against Chloramphenicol, Meropenem and other antibiotics.

Detection by molecular techniques

DNA extraction from oral and swab samples: The DNA was extracted from oral isolates of the oral samples and swab cervical-vaginal samples using Genomic DNA mini extraction kit. The bacterial isolates were chosen depending on the identification of bacteria by using laboratory procedures like microscopic examination and biochemical tests. The DNA quality and integrity were assessed through observing DNA bands by gel electrophoresis on 0.8% of agarose for 40 minutes. The bands looked sharp, single not dispersed, and no smear which might result DNA degradation .as shown in (Fig.4a,b)

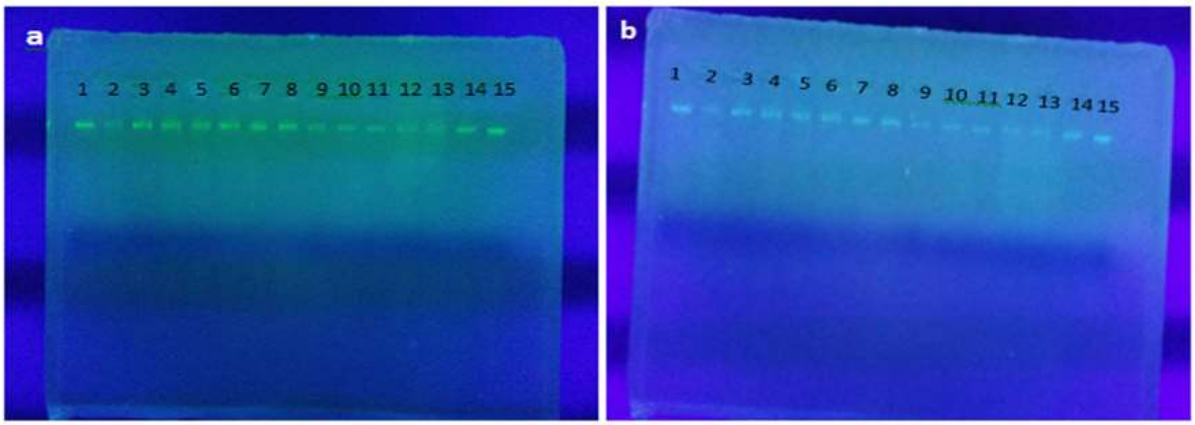

Figure 4. DNA bands extracted from a).oral isolates and b) Cervical-vaginal samples using Genomic DNA mini extraction kit (agarose 0.8% TBE buffer (1X), 5 V/cm for 40 min stained with red safe stain) visualized by U.V light

Polymerase chain reaction (PCR): Polymerase chain reaction technique was achieved for *Fusobacterium sp* isolates by using specific *Fusobacterium sp* primer targeting 16SrRNA. The amplified product size of PCR was 360 bp when compared to the DNA ladder. There are thirty one of Cervical-vaginal swabs and only four of oral sample that gave positive result for targeting the 16SrRNA gene, as shown in (Fig.5a,b). While reaming oral sample and swabs sample was negative and exhibited no band, which was evidence that it was not correlated to the *Fusobacterium* isolates. The same gene was used for *Fusobacterium* isolates detection was targeted by other studies (32).

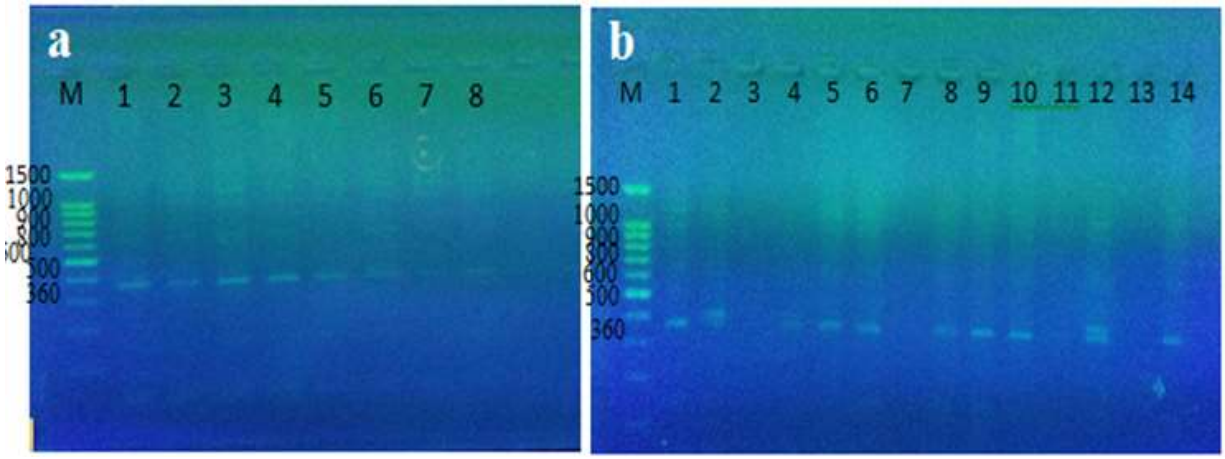

Figure 5. Gel of electrophoresis of PCR product of 16SrRNA gene of *F. nucleatum* isolates, size of the bands 360 bp. Electrophoresis was done on 1.5% agarose at 80 volt for 50 minute. DNA ladder (100),(a lane 1-4 referred to oral Fusobacterium Isolates, while the lane 5-8 referred to cervical-vaginal Fusobacterium isolates and all these isolates related to 4 specimens, (b) lane (1,2,4,5,6,8,9,12,and 14) are positive samples ,whereas lane (3,7,11,and 13) are negative samples. M is a marker, PCR amplified product visualized by U.V.

This is the first local study in Iraq using both microbiological and molecular methods to identify the presence of a common periodontal pathogen associated with conception. The chief result was the detection of *Fusobacterium sp.* in oral (subgingival plaque) and cervical-vaginal specimens, these bacterial species may raise the risk for ineffective conception among Iraqi women. There are relationship between periodontal pathogens and infertility that might share several common risk factors such as age, low socioeconomic standing (as education level) and obesity (38). As well as, there are conventional hazards and confusing factors, the finding of this study propose that *Fusobacterium sp*. can play a role in deferred conception or at least may be a sign of this association**.** According to PCR analysis result the percentage that gave positive result to *Fusobacterium sp* of swab samples in study groups with periodontal problems was (64.5%)

in primary infertility; (60.0%) secondary infertility compared to (6.4%) in pregnant, with significant difference at (P<0.01) as shown in (Fig. 7). Women with healthy oral state showed very less percentage of *Fusobacterium sp*, (21%) in primary infertility; (16.6%) secondary infertility, whereas the pregnant had no result, with significant difference at (P<0.01) as shown in (Fig. 6). These results show a high percentage of *Fusobacterium sp.* in infertile groups compared to pregnant group, Although previous studies state that *Fusobacterium sp.* is part of the normal flora of the human, the current studies show that it should always be treated as a pathogen, but the increased percentage of presence raise a question if it is really normal flora or is it bacterial transmission from the oral cavity. Prior studies that have noted the importance of link between periodontal microorganisms and adverse pregnancy outcomes emphases on preterm birth and premature rupture of membranes (PPROM) by documented intra-amniotic *Fusobacterium sp* in patients with preterm birth and in placenta and fetal tissues, comprising amniotic fluid, cord blood, fetal membranes (52) and neonatal gastric aspirates from pregnancies affected with PPROM as well as preterm birth with intact membranes (20). These studies support the result of our study in which the percentage of *Fusobacterium sp* in pregnant women (18.1%) who had periodontal problems, these pregnant women suffered of abortion and did not complete the pregnancy stages (Fig.6).

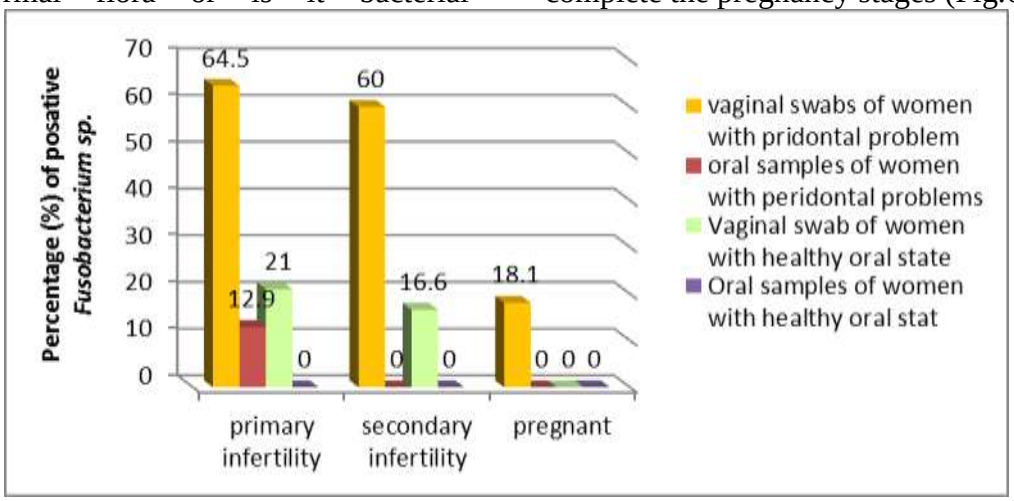

Figure 6. The percentage of *Fusobacterium sp.* in study groups of women with periodontal problems or with healthy oral state

Fusobacterium sp. was isolated from 4 women with periodontal disease. They were from primary infertility (*Fusobacterium sp* presented in the oral samples and swabs samples of the same women). The presence of this bacterium in oral and cervical-vaginal sites can suggest the association between this bacterium and delay of conception in group of Iraqi women .According to the total percentage of *Fusobacterium* sp. from both swab and oral samples, the percentage of *Fusobacterium sp.* in women with primary infertility (38.7%) showed significant difference compared to pregnant women (9.1%) at ($p < 0.01$), while in women with secondary infertility (30.0%) also showing significant differences compared to pregnant women (9.1%) at ($p < 0.01$). As showed in (Fig.7 a.b).

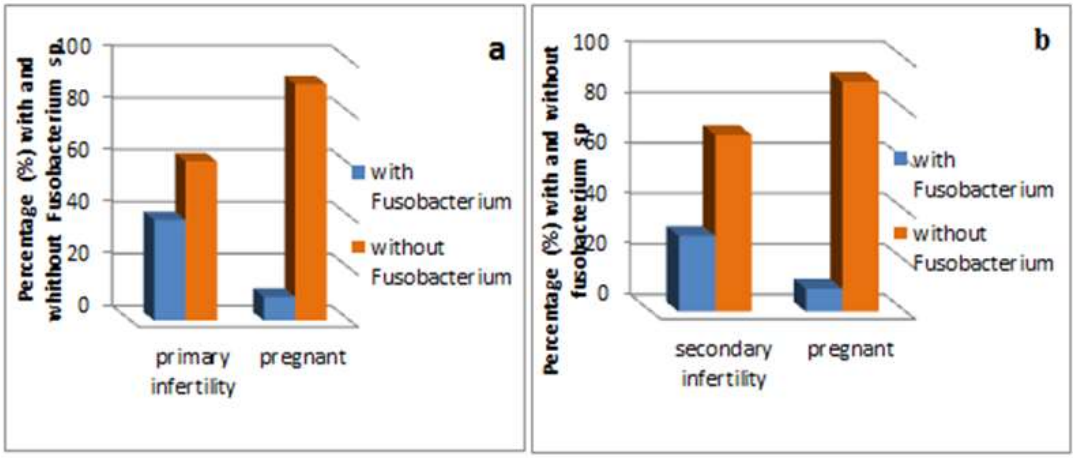

Figure 7. (a) The percentage of *Fusobacterium* and Non- *Fusobacterium* in women with primary infertility,(b) the percentage of *Fusobacterium* and non-*Fusobacterium* in women with secondary infertility, both compared to the pregnant group of women

Analysis identified a model of a significant predictive factor of presence of *Fusobacterium sp*.: primary infertile [Odd ratio (OR) = 6.3 at 95% CI = 1.33-25.1]; [Risk ratio (RR) = 4.8 at 95% CI =1.2-19.5], and secondary infertile OR = 4.2 at 95% CI = 1.84-12.22; RR = 3.4 at 95% CI =1.58-7.85. this can show that infertalty state can be more risked than pregnant women to have the *Fusobacterium sp* and be effected by it. The strains of *Fusobacterium sp* identified in amniotic fluid and placenta appear to match those from the maternal or the partner subgingival sites rather than the lower genital tract, the previous studies showed many evidences to support our result in the presence of *Fusobacterium sp* in the samples of pregnant women who suffered from abortion (19).Recently, many studies suggest that detection of the virulence mechanisms of *Fusobacterium sp* is a key to understanding its relationship with such an extensive spectrum of adverse pregnancy outcomes, *Fusobacterium sp* considered as an invasive bacteria, it can bind and invade both of epithelial and endothelial cells (51). In accordance with the present results, previous studies have demonstrated that *Fusobacterium sp* might translocate from the oral cavity of young girl's to their uterus when the immune system was weakened through the respiratory infection. In any case of the transmission route, the complications of pelvic inflammation disease (PID), including chronic pelvic pain, ectopic pregnancy, and tubal infertility with intra-abdominal scarring, highlight the potential importance of detecting *Fusobacterium sp* in females. Even though most of *Fusobacterium* infections are periodontal in nature, translocated infections can have long term effects for reproductive health (24).In this study detection of *Fusobacterium sp* by using specific primer in a group of Iraqi women and the results showed the presence of this bacterium in cases with primary and secondary infertility in addition to abortion but there are not presented in pregnant women, there is no data showing the relationship between the presence of *Fusobacterium sp.* and conception but this result provide further support for the hypothesis that several organisms like *Mycoplasma hominis*, *Bacteroides* spp., *Gardnerella vaginalis*, and *Fusobacterium nucleatum* usually display low virulence unless they reach the intrauterine environment (15). *Fusobacterium sp,* in usual is nonpathogenic oral anaerobic bacteria, that has been proposed to spread hematogenously to the placenta and change vascular endothelium permeability, potentially allowing for the colonization of other potentially pathogenic organisms, such as *Pseudomonas sp.* (23).

REFERENCES

1. Åberg, C. H.; P.Kelk and A. Johansson. 2015. *Aggregatibacter actinomycetem-comitans*: virulence of its leukotoxin and association with aggressive periodontitis. Virulence, 6(3), 188-195

2. Al-Haideri, H. H 2019. Gene expression of blaOXA-51-like and blaOXA-23 in response B-lactame antibioticin clinically isolated *Acinetobacter baumannii* and *Acinetobacter*

lowffii from urine samples. Iraqi Journal of Agricultural Science.,50(4):1120-1137

3. Ali,M.M.; A.H. Nerland; M. Al-Haroni and V.Bakken .2013. Characterization of extracellular polymeric matrix and treatment of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms with DNaseI and proteinase-K. J. Ora lMicrobiol. 5(1):21-23.

4. Ali,N.S. 2016. Biofortification and human health. Iraqi Journal of Agricultural Science., 47(7):144-147

5. Allen-Vercoe, E.; J.Strauss and K. Chadee.2011. *Fusobacterium nucleatum*: an emerging gut pathogen?. Gut microbes, *2*(5), 294-298

6. Alnasrawy,A.; Y. AL-khafaji; F.Altmimy and M.Alnasrawy.2015. Isolation, characterization and estimation of ellagic acid activity against *Porphyromonas gingivalis* isolated from adult periodontitis patients in Kerbala City. Karbala Journal of Pharmaceutical Sciences. 6: 16-29

7. Al-Quraishi, G.M.2018. The relationship between biofilm production and antibiotic sensitivity of (MRSA) *Staphylococcus aureus*. International Journal of Pharmacy and Biological Sciences, 3: 220-226

8. Baehni, P. and M.Tonetti. 2010. On behalf of group 1 of the European workshop on periodontology conclusions and consensus statements on periodontal health, policy and education in Europe: a call for periodontology on effective prevention of periodontal and peri-implant diseases. Action-consensus view 1. Consensus report of the 1st European workshop on periodontal education. European Journal of Dental Educucation 14 Supplementum, 1**:** 2-3

9. Benksim, A.; N.Elkhoudri; R.A.Addi; A.Baali and M.Cherkaoui.2018. Difference between primary and secondary infertility in Morocco: frequencies and associated factors. International Journal of Fertility and Sterility, 12(2) :142-152

10. Bennett, P. M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. British Journal of Pharmacology, 153(S1), S347-S357

11. Brink B. 2013. Urease tests protocol. American Society for Microbiology Microbe Library. B, VIII : 34

12. Broughton, D. E. and K.H.Moley. 2017. Obesity and female infertility: potential mediators of obesity's impact. Fertility and Sterility, 107**:** 840-847

13. Chanomethaporn, A.; A.Chayasadom; N.Wara-aswapati; K.Kongwattanakul; W.Suwannarong; K.Tangwanichgapong and W.Pitiphat. 2018. Association between periodontitis and spontaneous abortion: A case-control study. Journal of Periodontology., 90(4): 381-390

14. Clinical and Laboratory Standard Institute (CLSI).2011.Performance standards for antimicrobial susceptibility testing; 20th Informational Supplement. CLSI document M100-S21. CLSI, Wayne, PA: Clinical and Laboratory Standard Institute

15. Collado, M. C.; E.Isolauri; K.Laitinen and S.Salminen.2008. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. The American journal of Clinical Nutrition., 88(4):894-899

16. Conway, G.; D. Dewailly; E. Diamanti-Kandarakis,; H. F.Escobar-Morreale; S.Franks; A.Gambineri and M.Pfeifer. 2014. The polycystic ovary syndrome: a position statement from the European society of endocrinology. European Journal of Endocrinology, 171(4), P1-P29

17. David N.G.; M.George ; M.D.Eliopoulos; .F.Henry ; M.D.Chambers;S. Michael and M.D.Saag. 2014. Sanford Guide to Antimicrobial Therapy. Spiral Edition Sanford Guide. Antimicrobial Therapy. PP:44

18. De Souza Filho, J. A.; C.G.Diniz; N.B.Barbosa; M.C.De Freitas; M.S.Neves; R.N.Da Gama Mazzei and V.L.Da Silva. 2012. Morphological, biochemical, physiological and molecular aspects of the response of *Fusobacterium nucleatum* exposed to subinhibitory concentrations of antimicrobials. Anaerobe, 18(6):566-575

19. Gauthier, S.; A. Tétu; E.Himaya; M. Morand; F. Chandad; F. Rallu and E.Bujold.2011. The origin of *Fusobacterium nucleatum* involved in intra-amniotic infection and preterm birth. The journal of Maternal-fetal and Neonatal Medicine., 24(11):1329-1332

20. Gonzales-Marin, C.; D.A.Spratt and R.P.Allaker. 2013. Maternal oral origin of *Fusobacterium nucleatum* in adverse

pregnancy outcomes as determined using the 16S–23S rRNA gene intergenic transcribed spacer region. Journal of Medical Microbiology., 62(1):133-144

21. Han, Y. W. 2015. *Fusobacterium nucleatum*: a commensal-turned pathogen. Current Opinion in Microbiology., 23: 141-147

22. Howe, R.A. and J.M.Andrews. 2012. Standardized disc susceptibility testing method (version 11). Journal of Antimicrobial Chemotherapy., 67(12): 2783-2784

23. Hussein,N.N. 2019. Detection of the antibacterial activity of AgNPs biosynthesized by *Pseudomonas aeruginosa*. . Iraqi Journal of Agricultural Science. , 50(2):617-625

24. Iniw, S. A.White; J.Bates; P.K. Braverman and F.M.Biro.2012. The impact of health education counseling on rate of recurrent sexually transmitted infections in adolescents. Journal of Pediatric and Adolescent Gynecology, 25(6): 481-485

25. Johnson, J.A.; S.Tough and D.Sogc. 2012. Genetics committee. Journal Obstet Gynaecol Can, 34(1):80-93

26. Kavoussi, S. K.; B.T.West; G.W.Taylor and D.I.Lebovic. 2009. Periodontal disease and endometriosis: analysis of the national health and nutrition examination survey. Fertility and Sterility, 91(2), 335-342

27. Kolenbrander, P.E.; R.J.Palmer ; A.H. Rickard ; N.S.Jakubovics ; N.I.Chalmers and P.I.Diaz. 2006. Bacterial interactions and successions during plaque development. Periodontol., 42:47-79

28. Könönen, E.; A.Kanervo; K.Salminen and H.Jousimies.1999. β-Lactamase production and antimicrobial susceptibility of oral heterogeneous *Fusobacterium nucleatum* populations in young children. Antimicrobial Agents and Chemotherapy., 43(5): 1270-1273

29. Koziel,J.;P.Mydel and J.Potempa. 2014. The link between periodontal disease and rheumatoid arthritis. Current Rheumatology Report., 16(408):1-7

30. Kuboniwa,M.;J.R.Houser;E.L.Hendrickson; Q.Wang; S.A.Alghamdi; A.Sakanaka and M.Whiteley. 2017. Metabolic crosstalk regulates *Porphyromonas gingivalis* colonization and virulence during oral polymicrobial infection. Nature Microbiology., *2*(11): 1493-1499

31. Lin, W.; W.Jiang; X.Hu; L.Gao; D.Ai; H.Pan; C.Niu; K.Yuan; X.Zhou and C.Xu. 2018. Ecological shifts of supragingival microbiota in association with pregnancy. Frontiers in Cellular and Infection Microbiology., 8**(**24):1-7

32. Liu, P.; Y.Liu; J.Wang; Y.Guo; Y.Zhang and S.Xiao.2014. Detection of *Fusobacterium nucleatum* and *fadA* adhesin gene in patients with orthodontic gingivitis and non-orthodontic periodontal inflammation. PLoS One., 9(1): e85280

33. Loozen, G.; O.Ozcelik ; N.Boon ; A.De Mol ; C.Schoen ; M.Quirynen and W.Teughels. 2014. Inter-bacterial correlations in subgingival biofilms: a large-scale survey. J Clin Periodontol., 41:1-10

34. Markopoulos, A. K. 2016. Oral Microbial Flora. A Handbook of Attention Deficit Hyperactivity Disorder (ADHD) in the Interdisciplinary Perspective.pp: 53

35. Mitchell, C. and M.Prabhu.2013. Pelvic inflammatory disease: current concepts in pathogenesis, diagnosis and treatment. Infectious Disease Clinics., 27**:** 793-809

36. Nwhator, S.O.; O.I.Opeodu ; P.O.Ayanbadejo;K.A.Umeizudike;J.A.Olamijulo; G.O.Alade and T.Sorsa. 2014. Could periodontitis affect time to conception?. Annals of Medical and Health Sciences Research., 4(5): 817-822

37. Olsen, I.; A.Martin; M.Taubman and K.Sim .2016. *Porphyromonas gingivalis* suppresses adaptive immunity in periodontitis, atherosclerosis, and alzheimer's disease. Journal of Oral Microbiology., 8(1): 1-13

38. Paju, S.; J.Oittinen; H.Haapala; S.Asikainen; J.Paavonen and P.J.Pussinen. 2017. *Porphyromonas gingivalis* may interfere with conception in Women. Journal of Oral Microbiology., 9(1):1330644

39. Papp-Wallace, K. M.; A.Endimiani; M.A.Taracila and R.A.Bonomo.2011. Carbapenems: past, present, and future. Antimicrobial Agents and Chemotherapy, 55(11), 4943-4960

40. Pavlatou, A.; A.Tsami; N.Vlahos; T.Mantzavinos and I.Vrotsos. 2013. The effect of *in Vitro* fertilization on gingival inflammation according to women's

periodontal status: clinical data. Journal of the International Academy of Periodontology.,15(2):36-42

41. Perez-Chaparro, P. ;P. Gracieux; G. Lafaurie; P.Donnio and M.M.Bonnaure.2008. Genotipic characterization of *Porphyromonas gingivalis* isolated from subgingival plaque and blood sample in positive subjects with periodontitis. J Clin Periodontol., 35(9):748-753.

42. Pınar G. and B.Nurcan. 2013. Diabetes mellitus and periodontitis: signs of a bidirectional relationship. EMJ Diabet, 1:30-36

43. Rydén, L.; K.Buhlin; E. Ekstrand ; U.de Faire;A.Gustafsson; J.Holmer; B.Kjellström and B.Lindahl. 2016. Periodontitis increases the risk of a first myocardial infarction: a report from the parokrank study. Circulation., 133:576–583

44. Settem R. P.; A. T. El-Hassan ; K.Honma ;G.P. Stafford and A. Sharma. 2012. *Fusobacterium nucleatum* and *Tannerella forsythia* induce synergistic alveolar bone loss in a mouse periodontitis Model. Infect Immun., 80:2436-2443

45. Shimoyama, Y.; Y.Ohara-Nemoto; M.Kimura; T.K.Nemoto; M.Tanaka and S.Kimura. 2017. Dominant prevalence of *Porphyromonas gingivalis* fimA types I and IV in healthy Japanese children. Journal of Dental Sciences., 12**:**213-219

46. Sourial,S.; N.Tempest and D.K. Hapangama. 2014. Theories on the pathogenesis of endometriosis. International Journal of Reproductive Medicine.,17(9):515

47. Strauss, J.; G.G.Kaplan; P.L.Beck; K.Rioux; R.Panaccione; R.DeVinney and E.Allen-Vercoe. 2011. Invasive potential of gut mucosa-derived *Fusobacterium nucleatum* positively correlates with IBD status of the host. Inflammatory Bowel Diseases.,17(9):1971-1978

48. Taylor,K.C.;C.M.Small; C. E. Dominguez; L.E.Murray; W.Tang; M.M.Wilson and M.Marcus. 2011. Alcohol, smoking, and caffeine in relation to fecund ability, with effect modification by NAT2. Annals of Epidemiology.,21(11): 864-872

49. Tsai, C. Y.; C.Y.Tang; T.S.Tan; K.H.Chen; K.H.Liao and M.L.Liou. 2018. Subgingival microbiota in individuals with severe chronic periodontitis. J. of Microbio. Immuno. Infec., 51(2):226-234

50. Tune´r, K.; L. Lindquist, and C. E. Nord.1985. Characterization of a new β-lactamase from *Fusobacterium nucleatum* by substrate profiles and chromatofocusing patterns. J. Antimicrob. Chemother.,16:23-30

51. Vander Haar, E. L.; J.So; C.Gyamfi-Bannerman and Y.W.Han. 2018. *Fusobacterium nucleatum* and adverse pregnancy outcomes: epidemiological and mechanistic evidence. Anaerobe.,50:55-59

52. Wang, X.; C.S.Buhimschi; S.Temoin; V.Bhandari; Y.W.Han and I.A.Buhimschi. 2013. Comparative microbial analysis of paired amniotic fluid and cord blood from pregnancies complicated by preterm birth and early-onset neonatal sepsis. PloS one., 8(2):e56131.