E-ISSN: 2320-1215
P-ISSN: 2322-0112

Research and Reviews: Journal of Pharmacy and Pharmaceutical Sciences

Detection of Major Putative Periodontal Pathogens in Iranian Adult Patients Using Multiplex PCR and Culture

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Research Article

Received: 13/7/2015
Revised: 20/8/2015
Accepted: 28/8/2015

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key words:
Periodontitis, Multiplex PCR, Isolation

ABSTRACT

A therapeutic approach of periodontal diseases is based on the proper diagnosis of the root cause of the diseased condition. The present study was conducted to detect and characterize the periodontal pathogens associated with Iranian patients using culture and multiplex PCR techniques. 100 subgingival plaque samples were taken from 100 advanced adult periodontitis (AP) subjects. AP samples were obtained from the deepest periodontal pockets in each patient.

The samples were cultured immediately and assayed by multiplex PCR using gene specific primers. Total detection rate of periodontal pathogens was 64% by culture and 65% by multiplex PCR method. *Aggrigatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg)* and *Prevotella intermedia (Pi)* were present in 28%, 6%

E-ISSN: 2320-1215
P-ISSN: 2322-0112

and 3% of diseased sites, respectively. The combined presence of Aa, Pg and Pi was 27%. The bacterial isolation rates for Aa, Pg, and Pi in culture were 30%, 7% and 5% of diseased sites, respectively. The combined presence of Aa, Pg and Pi with multiplex PCR. Method was 23%. This study found that Aa is the most predominant bacterium detected by both culture and multiplex PCR. Although the detection rates were marginally similar in the two methods, the PCR technique is more preferential compared with the culture, and the data of the current study recommend the use of the molecular techniques for the detection of periodontal pathogens due to labor intensive and time consuming nature of culture technique. Also, molecular techniques may be more reliable than culture because of detection of specific target genes.

INTRODUCTION

Clinical Relevance

Periodontitis is an inflammatory gum disease with diverse incidence in different populations. In some communities oral bacteria are considered as major etiologic agents of the disease. The destruction of periodontal tissue is the consequence of both microbial activity and host immune response. This study found that the major putative periodontal pathogen is Aa with 30% (culture method) and 23% (multiplex PCR) prevalence. Although treatment of periodontitis is essential, oral hygiene can be considered as the most important parameter involved in preventing the outgrowth of potential periodontal pathogens and the consequent inflammation of the gums.

Periodontitis is one of the most prevalent infections of oral cavity with various incidence rates in different populations. The prevalence rate of periodontitis is attributed to various determinants including host immune response, chronic bacterial infection, socio-economic and hygienic conditions. In the early stages of periodontitis, the destruction of the supporting tissue of the tooth root occurs through the invasion of microorganisms. Progression of periodontitis results in the formation of periodontal pockets. More than 300 types of bacteria have been associated with periodontitis. The anaerobic and capnophilic bacteria play major role in the development of periodontitis[1,2]. The main composition of periodontal pathogens in the population of Iran has not been investigated in epidemiological studies. Considering the fact that the same pathogens found in the periodontal pockets of patients in the other parts of the world would be found in the Iranian patients, the major putative anaerobes (*P. gingivalis, P. intermedia*) and capnophilic periodontal pathogens (*A. actinomycetemcomitans*) have been considered specifically in this study. Although the main causative agents are still obscure, different studies have shown that there is a direct correlation between the number of periodontal pathogens (including *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia*) in the periodontal pocket and the severity and progression of periodontitis.

The treatment of periodontitis is basically aimed at the removal of damaged tissues followed by antibiotic therapy. The proper treatment or any preventive approach is based on the detection of the causative agents involved in the

E-ISSN: 2320-1215
P-ISSN: 2322-0112

diseased condition. Several factors may make the detection and quantification of periodontal pathogens rather cumbersome. Presence of uncultivable bacteria, lack of proper diagnostic techniques and difficulty reaching the sub-gingival tissues for sample collection are the major hurdles in bacterial isolation[3,4].

Untreated periodontal infections are believed to be potential risk factors that may lead to systemic diseases. There are several reports that link periodontitis to atherosclerosis/coronary heart disease and respiratory disorders[5-7]. Hence, the detection and identification of fastidious periodontal pathogens are of prime importance. We aimed to detect and characterize the periodontal pathogens in Iranian patients who were suffering from periodontitis using culture and multiplex PCR techniques in parallel.

MATERIALS & METHODS

Patients and samples

The patients including 62 females and 38 males (mean age of 49.5). They were referred to the periodontics clinic at the Faculty of Dentistry, University of Tehran Medical Sciences. Inclusion criteria were considered as follows: No history of antibiotic therapy for at least one month, depth of pocket equal to or more than 5 mm (one sample from each patient), and no history of systemic diseases. All demographic data were collected by filling out a detailed questionnaire.

For each subject, one subgingival site with the deepest periodontal pocket (≥5 mm probing depth, for 30 seconds) was sampled by the paper point method (Antaeos, Munich, Germany). The paper points were inserted into the sterile bijoubottle containing thioglycolate broth as a transport medium and delivered to the microbiology laboratory.

Bacterial culture conditions

The samples were streaked onto Brucella agar base (Merck, Darmstadt, Germany) containing 5% sheep blood, and several supplements including 5μg/ml hemin (Sigma-Aldrich, USA), 5% horse serum and vitamin K_1 (1μg/ml; Sigma-Aldrich, USA). Selective media for *A. actinomycetemcomitans, P. gingivalis*, and *P. intermedia* were prepared by adding the following antibiotics to the Brucella agar: 3.25mg/l baciteracin-vancomycin (SIGMA, USA) for Aa, 0.3mg/l colistin for Pg (SIGMA, USA), and 3.25mg/l vancomycin for Pi (SIGMA, USA). After 48 hours of incubation in anaerobic and capnophilic conditions at 37°C, the biochemical and identification tests such as Gram staining, oxidase, catalase, and indole production, fermentation of glucose, fructose, and lactose (Merck, Darmstadt, Germany) were carried out.

Molecular detection

DNA extraction was conducted according to the manufacturer's instructions (BOINEER, South Korea). Pure cultures of standard strains (*A. actinomycetemcomitans* ATCC 29523, *P. intermedia* ATCC 25611, and *P. gingivalis* ATCC 33277) were used as positive controls. The final volume of multiplex PCR reaction was 25 μl, containing 12 μl hot start master mix (QIAGEN, USA), 7μl distilled water, 1μl template DNA(1 μM) and 0.05 μl of each primer(0.50 μM). The primer sequences were designed as indicated in **Table 1**

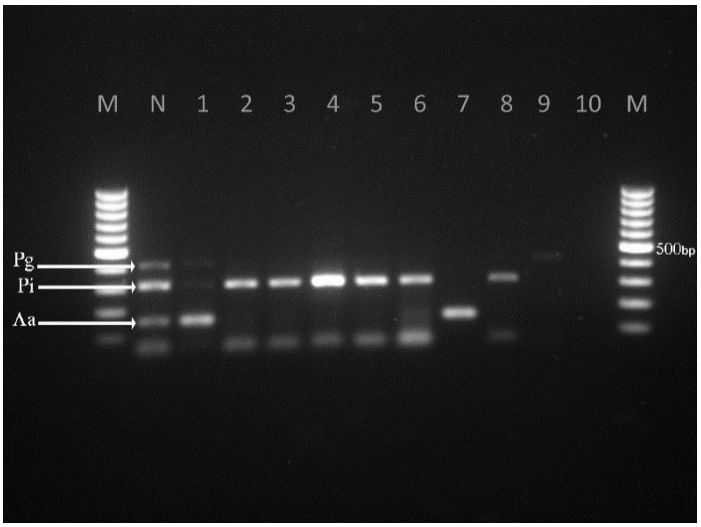
E-ISSN: 2320-1215
P-ISSN: 2322-0112

Table 1 PCR primer pairs used for Multiplex PCR

Target	Primer Pairs	Gene name	Base position (product size in bp)
Aggrigatibacter actinomycetemcomitans	F: 5'-CGCCGTTTTTATTGCTCATT-3' R: 5'-CGACATCGATGGTTTCAAGTT-3'	tbp A1	160
Porphyromonas gingivalis	F: 5'- GGAAATTGTTCGCGAGATGT-3' R: 5'- CATGGCCACCAGCAGGACGC-3'	tna A	431
Prevotella intermedia	F: 5'- CCAATCGTTTACCCTCAGGA-3' R: 5'-ACGGACATCGAATACCGACT-3'	Conserved hypothetical protein	317

Multiplex PCR protocol was as follows: 95°C for 5 min; 35 cycles of 94°C for 1 min, 62°C for 45 seconds, and 72°C for 30 seconds. Final extension was performed at 72°C for 5 min. The PCR products were run on 1.5% agarose gel containing ethidiumbromide to visualize the bands. **Figure 1**

Figure 1 Agarose gel electrophoresis of multiplex PCR products. M: DNA Marker (100 bp), N: Positive control, 1-9: multiplex PCR Samples, 10: Negative control



Statistical analysis

Statistical analysis in our study was performed with SPSS software version 16. When the P value was less than 0.05, statistical significance was defined.

E-ISSN: 2320-1215
P-ISSN: 2322-0112

RESULTS

The prevalence of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in patientswas determined by molecular and culture method.

Results of the bacterial cultures

62.9% of the female subjects and 63.2% of the male patients were positive for at least one of the target bacteria. 20% of the female subjects were infected by *P. gingivalis* and 16% were infected by *P. intermedia*, 38.5% with *A. actinomycetemcomitans* **Figure 2.** 20% of the male subjects were infected with *P. gingivalis* and 50% were infected by *P. intermedia* and 58.3% with *A. actinomycetemcomitans* **Figure 3.**The difference between male and female subjects was not statistically significant (*P*=0.57).

Figure 2. Frequency of isolated bacteria from female subjects using culture

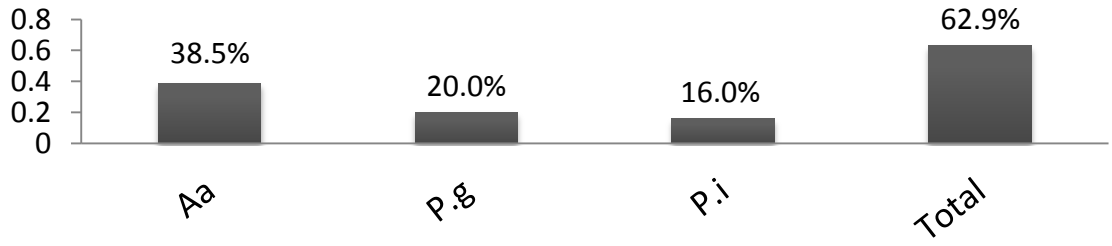
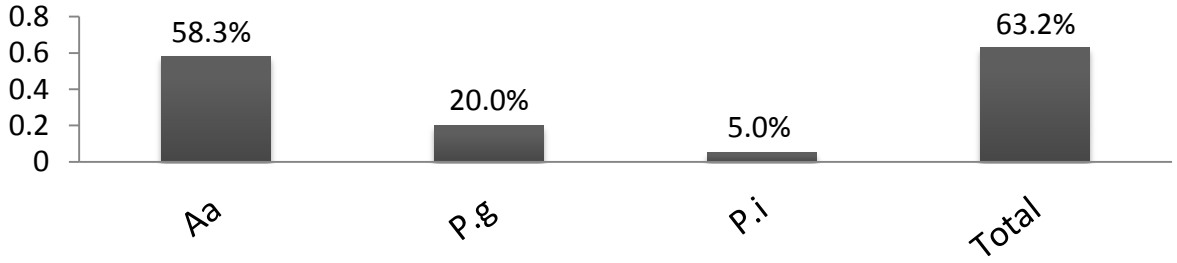


Figure 3. Frequency of isolated bacteria from male subjects using culture



Results of multiplex PCR

63.9% of the female patients were positive for at least one of the target bacteria. 16% of them were infected with *P. gingivalis,* 7% were infected by *P. intermedia* and 20.5% with *A. actinomycetemcomitans* **Figure 4.** Among 38 male patients, 65.6% were positive for the presence of at least one of the periodontal pathogens, considering that 10% of them were infected with *P. gingivalis,* 6% were infected by *P.intermedia* and 40% with *A. actinomycetemcomitans* **Figure 5.**The difference between male and female was not statistically significant (*P*=0.51).

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P-ISSN: 2322-0112

Figure 4. Frequency of isolated bacteria from female subjects using PCR

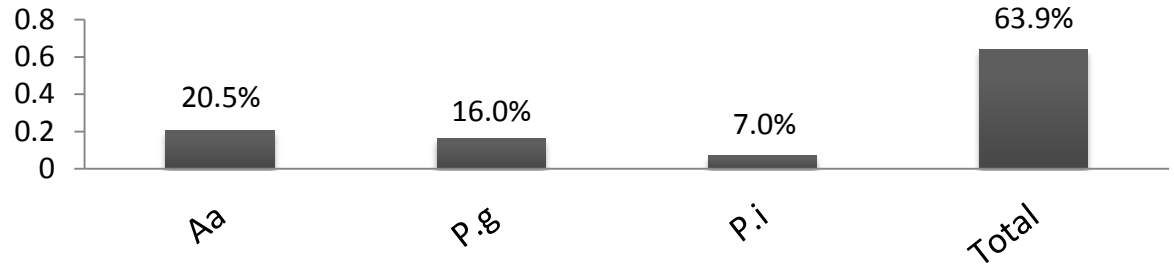
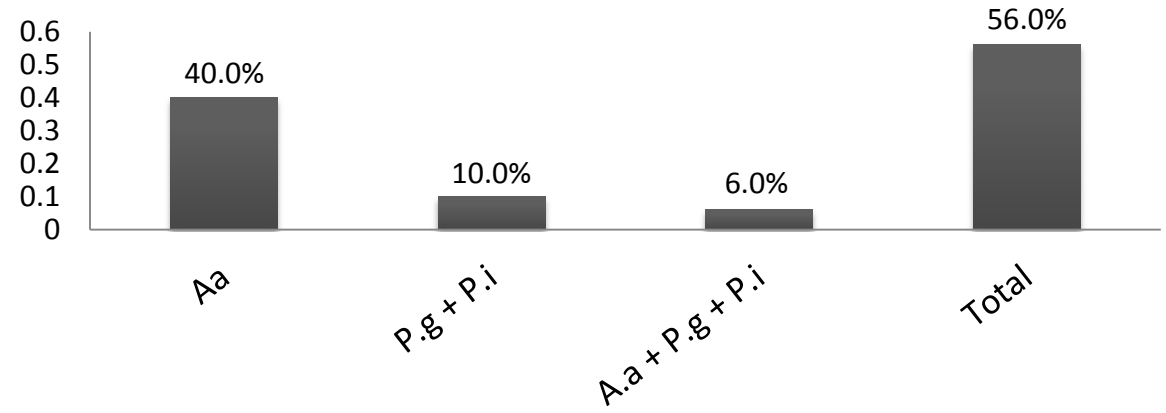


Figure 5. Frequency of isolated bacteria from male subjects using PCR



DISCUSSION

Although the main cause of periodontitis is still unknown, quantitative studies have shown that there is a direct correlation between the number of periodontal pathogens (including *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*) in the sub-gingival region and the severity and progression of chronic periodontitis. Clinical signs including gingival exudates, bleeding, pocket depth, connective tissue detachment, and the periodontal index are also associated with the quantity and diversity of the periodontal pathogens. Other underlying factors such as smoking, age, genetic disorders and chronic systemic diseases like diabetesmay play some roles in the progression of periodontitis[8]. It is a common belief that most people are affected by some sort of periodontal infection at least once in their life time. The incidence rate is higher in men compared with women and even higher in educated people versusnon-educated[9-12].

In the present study, we intended to detect and characterize the target periodontal pathogens in the Iranian patients. The average isolation rate of *A. actinomycetemcomitans* was 47.45% and 29.5% for the mixed infection with *P. gingivalis* and *P. intermedia.* Amel et al., showed that in a population of 232 individuals suffering from periodontitis, 7.3% carried A. actinomycetemcomitans, 4.2% P. gingivalis, 0.8% *Actinomyces israelii* and 1% *Actinomyces naeslundii* which is much less than the numbers found in this study. Based on racial differences and the type of sampling hygiene, isolation level will be significant.

E-ISSN: 2320-1215
P-ISSN: 2322-0112

In a study to determine the bacterial profile of the sub-gingival tissues of the chronic periodontitis patients in Chile, Colombia and Spain, Herrera et al. found that in 37 patients under study in Chile, 19.4% carried A. *actinomycetemcomitans,* 83.8% *P. gingivalis* and 19.4% *P. intermedia.* In 41 Colombian patients, 17.1% carried A. *actinomycetemcomitans,* 65.9% *P. gingivalis* and 72.5% *P. intermedia.* In 41 Spanish patients, 16.7% had A. *actinomycetemcomitans*, 77.8% P. gingivalis and 97.2% *P. intermedia*[14].

In our study *A. actinomycetemcomitans* had the highest rate of isolation in both culture and molecular method followed by *P. gingivalis* and *P. intermedia.* Our data corresponds with the data found in a study that 71% of the bacteria detected by multiplex PCR in a population of 204 Kuwaiti subjects (120 females and 120 males) were A. *actinomycetemcomitans*[15], whereas Esterla et al. found that *P. gingivalis* had a higher rate of isolation by multiplex PCR method from 22 periodontal patients[16]. Also,Tanner et al. performed a molecular study using multiplex PCR to isolate bacteria from gingiva and tongue of 221 periodontitis patients and found that *P. gingivalis* had the highest rate of isolation from the clinical samples. Egwariet [17] showed that in a population of 162 people under survey in Nigeria, 18.1% carried *P.gingivalis,* 9.6% *P. intermedia* and 6.4% *A. actinomycetemcomitans*[18].

In this study, no statistically significant differences was observed between male and female subjects in terms of recovering periodontal pathogens from sub-gingival samples and significant statistical differences were not observed between molecular and culture method. However, it is obvious that the molecular technique is more time-saving and accurate. In a study comparing bacterial culture and molecular method in patients with periodontitis D'Ercole et al. found that the sensitivity of the molecular approach was double as much as the traditional culture technique in 529 subjects [19].Daniluk et al. in a study to compare the detection of aerobic bacteria versus anaerobic bacteria in adult periodontitis patients found that the ratio of anaerobic bacteria to aerobic bacteria was 3 to 1[20].

CONCLUSION

This is the first report on the detection of periodontal pathogens by Multiplex PCR in Iran. Although the gold standard method for detecting the bacteria in clinical samples is still culture method, the molecular technique is highly considered as a time-saving and labor-saving approach for detecting bacteria.

ACKNOWLEDGMENTS

The authors would like to acknowledge Tehran University of Medical Science's academic support.

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