

The RT-PCR additional value in detecting periodontal pathogens in periodontitis patients

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ABSTRACT

Objectives. The aim of this study was to evaluate the prevalence of selected bacterial species from periodontal pockets samples in a group of young adult periodontitis patients and explore the additional value of the Real-Time Polymerase Chain Reaction (RT-PCR) assay.

Material and Methods. Patients with periodontitis diagnosis and good general health were included in the study after a complete periodontal examination and following the inclusion/exclusion criteria. Samples from the deepest periodontal pockets were collected, and RT-PCR assay was performed in a private laboratory in order to identify eleven bacterial strains associated with periodontitis. The patient's report provided the bacterial pathogen load quantified according to the detection threshold and classified as negative, weakly positive, positive, or intensely positive.

Results. The RT-PCR assay detected periodontal pathogens, and their prevalence could be calculated based on the test report. The red complex bacteria were detected in all patients with the highest counts. Alongside the orange complex bacteria were also detected in periodontitis patients. *A. actinomycetemcomitans* remained undetected in our study.

Conclusions. The RT-PCR technique proved to be a useful and easy approach for identifying and quantifying subgingival periodontal pathogens in periodontitis patients. RT-PCR testing may facilitate accurate diagnosis and prognosis as well as successful treatment.

Keywords: periodontitis, RT-PCR assay, periodontal pathogens, diagnostic

INTRODUCTION

Periodontitis represents an inflammatory, bio-film-induced, highly prevalent non-communicable infectious disease that affects around 50% of the population worldwide. According to the Global Burden of Disease study (2017), it is the sixth most common chronic human disease [1], with severe forms accounting for 11.2% of cases [2]. Several clinical signs are associated with periodontitis, such as clinical attachment loss, periodontal pocket formation,

gingival inflammation with bleeding on probing, increased tooth mobility and radiographic alveolar bone loss [3].

According to the latest case definition, released in 2018 by the European Federation of Periodontology and American Academy of Periodontology, an individual can be considered a periodontitis case if the subject presents "interdental clinical attachment loss (CAL) at ≥ 2 non-adjacent teeth, or buccal/oral CAL ≥ 3 mm with a pocket depth (PD) > 3 mm at ≥ 2 teeth", that can be solely ascribed to periodontal

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causes [3,4]. Following the identification of the periodontitis case, periodontitis staging is established using a specific algorithm. Initially, the algorithm computed the stage based on the most severe detected CAL, with a CAL of 1–2 mm indicating stage I (mild destruction), CAL of 3–4 mm stage II (moderate) and CAL \geq 5 mm stage III/IV (severe). In addition to the severity criteria, some complexity elements are also considered for periodontitis staging. Grading provides additional information regarding the progression rate, therapeutical responsiveness, and its potential interference with the patient's systemic health [5,6].

The normal oral microbial flora consists of more than 700 bacterial species, among which approximately 400 bacterial species colonize periodontal sites [7,8]. The subgingival microbiota is a highly organized bacterial community [8,9], where bacteria interact, creating a complex network of cooperation and synergism with other microbial community members [10,11]. Some of these bacteria, named key pathogens, have a greater influence over the general community than the biomass they occupy [12].

The initiation and progression of periodontitis are determined by the multifaceted interplay between an altered subgingival microbiota and the host's dysregulated local immune-inflammatory response. The local inflammation process induces a shift in the microbial composition, and periodontal pocket formation promotes the microbial shift by further supporting the development of pathogenic species [13,14].

The subgingival biofilm associated with periodontitis comprises predominantly anaerobic bacterial species [14]. The development of periodontal disease is influenced by intricate relationships among particular pathogens, which are organized in specific bacterial complexes [7]. Periodontitis is strongly associated with red complex bacteria [7,15,16], but other bacterial species are also involved in the development and progression of periodontitis. Thus, *Aggregatibacter (Actinobacillus) actinomycetemcomitans (A. actinomycetemcomitans)*, *Porphyromonas gingivalis (P. gingivalis)*, *Tannerella forsythia (T. forsythia)*, are probably the most important species in the periodontitis onset [17]. The presence of species such as *Prevotella intermedia (P. intermedia)*, *Fusobacterium nucleatum (F. nucleatum)*, *Capnocytophaga sp.*, *Parvimonas micra (Micromonas micros) (P. micra)*, *Campylobacter rectus (C. rectus)*, *Treponema denticola (T. denticola)* in the periodontal pockets [17] are considered a marker of periodontitis development as well as an indicator of the periodontal inflammation progression [18,19]. *A. actinomycetemcomitans* has been closely linked to the etiology of severe molar-incisor periodontitis [20,21]. There is no unique microbial profile associ-

ated with periodontitis, as it may vary according to the patient's ethnicity [22–24], geographical location [24,25] or age [26].

The analysis of oral microorganisms is relevant for the assessment of patients' vulnerability towards periodontitis, diagnosing periodontitis, predicting the risk of disease, and periodontitis monitoring. However, several research groups revealed differences in the proportions and prevalence of subgingival species in periodontitis and periodontally healthy subjects in different countries, even when using the same microbiological assessment technique [22,27,28]. This could be explained due to differences in diet, genetics, disease susceptibility, and manifestation [16,29].

The identification and quantification of periodontal pathogenic bacteria are important in determining an accurate diagnosis and therapeutical approach as well as in predicting the disease progression rate [19]. The culture method has been considered the "gold standard" in identifying the major periodontal pathogens, assessing the type of oral colonization, and anticipating probable treatment outcomes [19]. This method requires experienced and trained personnel, as it is a laborious, time-consuming, and rather expensive [19]. Moreover, it also requires viable bacteria for testing. These drawbacks, together with technological advances in medical laboratory science, have led to the development of new methods for detecting and quantifying periodontal pathogens, such as enzymatic assays, DNA-DNA hybridization, immunoassays, and Polymerase Chain Reaction (PCR) assays [16]. Real-Time Polymerase Chain Reaction (RT-PCR) enables the quantitative identification of bacterial DNA achieved through the use of fluorescence, exhibiting high sensitivity and specificity. Also, it is rapid and easy to use for the detection of periodontal bacteria [16,30–32]. However, one of the major drawbacks of this microbiological assessment technique is its inability to screen large numbers of samples for large numbers of bacterial species [31].

Thus, the aim of this study was to evaluate the prevalence of selected bacterial species from periodontal pockets samples in a group of young adult periodontitis patients and explore the additional value of the RT-PCR assay.

MATERIAL AND METHODS

The present observational study was conducted at the Periodontology Department of Iuliu Hatieganu University of Medicine and Pharmacy/Ambulatory Care Unit of the County Emergency Hospital Cluj-Napoca, between February-May 2021 after receiving the approval of the Ethics Committees of both institutions (No. 80/1.02.2018, No. 10539/B/4.05.2018). The study complied with the guidelines provided by

the Helsinki Declaration on experiments involving human subjects. Prior to enrolment in the study, all participants provided their written informed consent.

Subjects were selected from patients referred to the Periodontology Department for periodontal consultation. The inclusion criteria were: patients with a diagnosis of periodontitis [3,6], with an age ranging from 18 to 45 years, in good general health. Patients with severe systemic conditions, pregnancy, previous periodontal or antibiotic therapy during the last six months, antimicrobial mouthwashes use during the last three months, and previous or current radiation or immunosuppressive therapy were excluded from the study.

A full-mouth examination, excluding third molars, was performed by a trained periodontist, in standard conditions using a UNC-15 periodontal probe (Hu-Friedy, Chicago, IL, USA). All periodontal parameters, probing depth (PD), gingival recession (GR), and clinical attachment loss (CAL) were evaluated at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual), following the standard clinical definitions [33,34]. After the initial periodontal examination, the periodontitis diagnosis, including staging and grading, was set according to the EFP/AAP 2018 classification and case definition [5,6].

The identification of periodontal pathogens was performed using the commercial kit Markerkeime Probenentnahmeneset bei Parodontitist (Institut für Medizinische Diagnostik - IMD Berlin MVZ, Berlin, Germany), according to the manufacturer's instructions. Samples for PCR test were collected from six of the deepest periodontal pockets, after supragingival plaque removal with professional brushes without using additional polishing paste. The selected

sites were isolated with cotton rolls, and measures to avoid contamination with saliva were taken. Sterile paper points provided in the kit were inserted into the periodontal pockets and left in situ for 20s until completely soaked. All paper points were then removed and placed immediately in an Eppendorf plastic tube. The tube was then closed, labelled with the patient's identification data, placed in the standard packaging together with the request form and sent to the laboratory for testing. The RT-PCR testing was performed in a private laboratory (Synevo, Cluj-Napoca, Romania) [35] according to the protocol and general instructions of the manufacturer.

The RT-PCR assessment was performed to identify the following eleven bacterial strains: *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, *T. denticola*, *P. micros*, *F. nucleatum*, *Eikenella corrodens* (*E. corrodens*), *C. rectus*, *Eubacterium nodatum* (*E. nodatum*), *Capnocytophaga spp.* The detection threshold of this test was 103 pathogens for *A. actinomycetemcomitans* and 104 for all other bacteria. The patient's report provided the bacterial pathogen load quantified depending on the detection threshold and classified as negative, weakly positive, positive, or intensely positive.

RESULTS

Eight generally healthy patients with an age ranging between 23 and 44 years were included in the present study. According to the EFP/AAP 2018 classification of periodontal diseases and conditions case definition, one patient was diagnosed with localised stage I periodontitis, one patient with localised stage III periodontitis, and the other cases were diagnosed with generalized stage III periodontitis. All patients were classified as grade C based on the

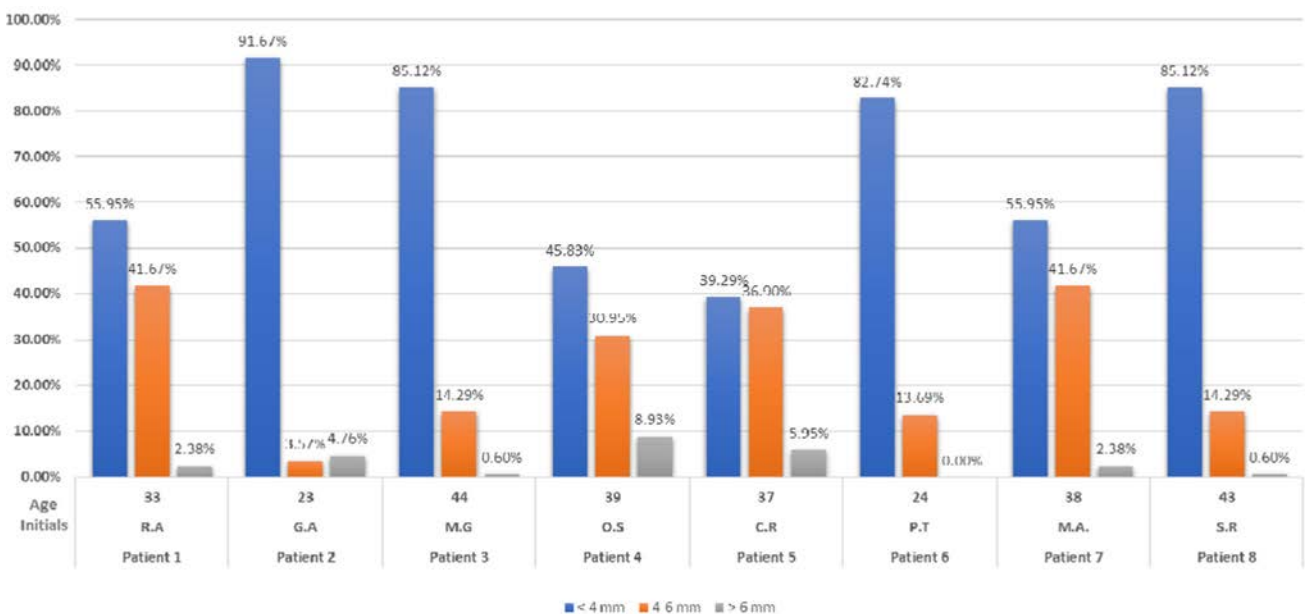


FIGURE 1. The proportion of periodontal pockets in the periodontitis patients

percentage of bone loss at the most affected tooth related to the patient's age [6].

In the investigated group of patients, the periodontal assessment of the clinical parameters revealed an overall mean probing pocket depth of 2.9 (±SD 1.49) and a mean clinical attachment loss of 1.61 (±SD 1.45). Overall, the calculated mean number of moderate (4-6 mm) periodontal pockets was 41.4, and the mean number of deep pockets exceeding 6 mm was 5.4. The proportion of shallow (<4 mm), moderate (4–6 mm), and deep pockets (>6 mm) for each patient are provided in Figure 1.

The laboratory test findings of the samples collected from the deepest periodontal pockets and analysed with the RT-PCR technique for each patient are summarized in reports containing the results on the identification of the pathogens associated with

periodontitis, marked alongside the reference value (Figure 2).

Figure 3 presents the prevalence of detected bacteria in periodontitis patients based on RT-PCR analysis. The color code corresponds to the specific bacterial complexes that each pathogen belongs to.

A. actinomycetemcomitans was not detected in any of the analyzed samples. *P. gingivalis*, *T. forsythia*, *T. denticola* (red complex) and *F. nucleatum* (orange complex) were the most frequently detected bacterial species in all the patients, with the highest counts (91.67%). *C. rectus*, *P. intermedia* and *P. micros* (orange complex) showed a rather high prevalence in this group of periodontitis patients. Contrary, the frequency of detection of *E. nodatum* (orange complex) and *E. corrodens* (green complex) was the lowest, 20.83% and 25% respectively.

Denumire	Rezultat	UM	Interval de referinta
IMD Germania			
14 Identificarea germenilor asociati parodontitei			
Alte produse / PCR			
Actinobacillus actinomycetemcomitans	negativ		negativ
Porphyromonas gingivalis	intens pozitiv		negativ
Prevotella intermedia	intens pozitiv		negativ
Bacteroides forsythus (Tannerella forsythia)	intens pozitiv		negativ
Treponema denticola	intens pozitiv		negativ
Peptostreptococcus micros (Parvimonas micra)	intens pozitiv		negativ
Fusobacterium nucleatum/periodonticum	intens pozitiv		negativ
Eikenella corrodens	slab pozitiv		negativ
Campylobacter rectus	intens pozitiv		negativ
Eubacterium nodatum	pozitiv		negativ
Capnocytophaga spp	pozitiv		negativ
Comentariu			
Tratamentul antibiotic se face in functie de contextul clinic conform schemei atasate.			
Se recomanda control la 4 saptamani de la incheierea terapiei.			

FIGURE 2. Patient’s periodontal pathogen identification report

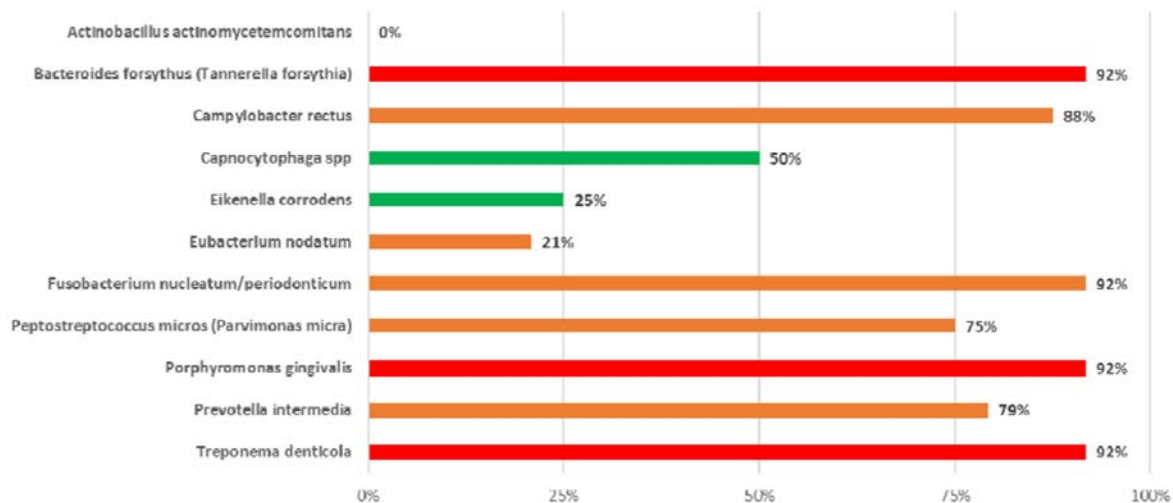


FIGURE 3. The percentage of RT-PCR positive samples for each of the detected bacterial strains in the periodontitis patients’ samples

Intensely positive results for red complex bacteria were found in the majority of patients, except for two subjects who had a smaller amount of these bacteria detected (positive). Figures 4 and 5 illustrate the detection level of the red and orange complex periodontopathogenic bacteria for each periodontitis case.

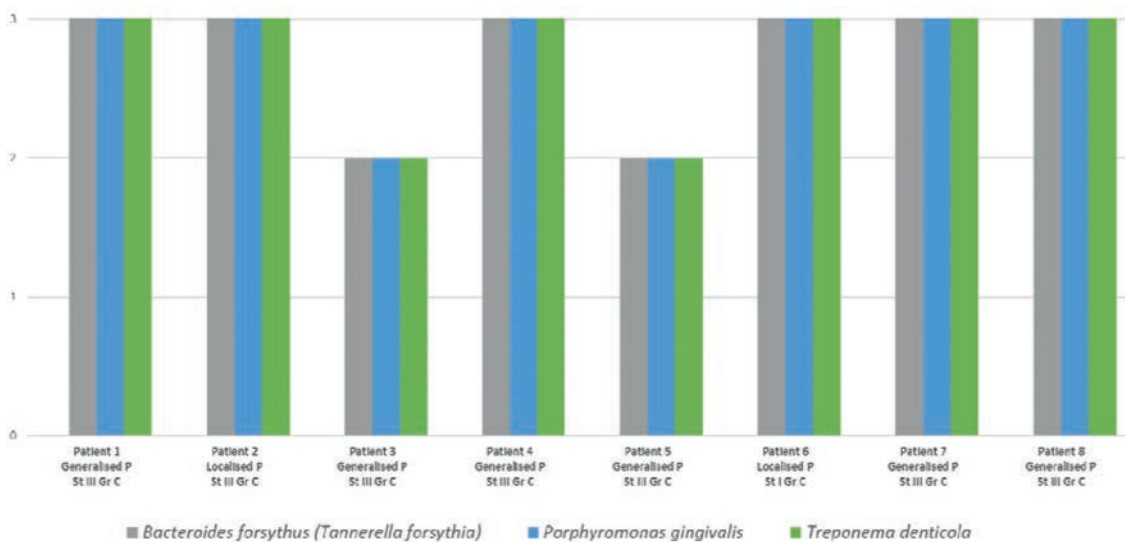
DISCUSSION

Periodontitis represents a very common, highly prevalent, chronic, multifactorial immune-mediated inflammatory disease associated with dysbiotic subgingival plaque containing various bacterial species with an increased number of anaerobic bacteria [19]. The subgingival microbiota contains a

mixture of bacterial species that are organized in non-homogenous biofilm. Several research studies reported the presence of periodontal pathogens in healthy periodontal sites, thereby highlighting the involvement of other factors in the disease onset [12,16,36].

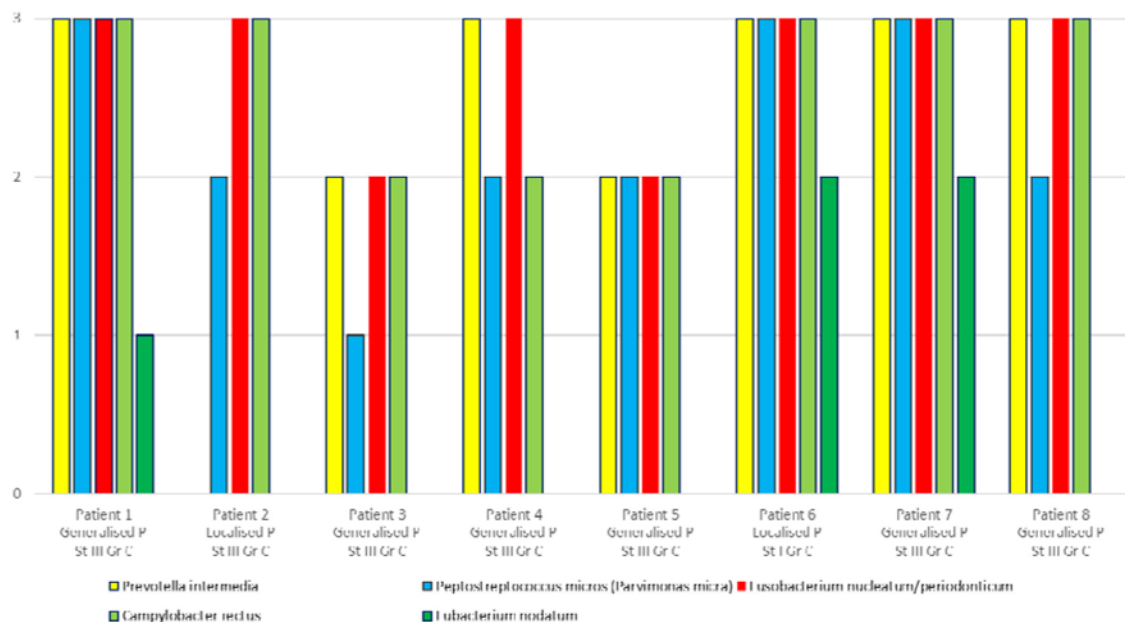
Methods based on molecular diagnostic tools, such as PCR assays, have been developed for the identification of several oral bacterial pathogens [18], as this method was found to be more effective than counting colony-forming units for oral pathogens detection, thereby overcoming the disadvantages of culturing procedures [37].

In the present study, RT-PCR assay was performed according to the well-established protocol, performed in a private laboratory to assess the pres-



Abbreviations: P = periodontitis; St = stage; Gr = Grade. X – axis: 0 - negative, 1 - weakly positive, 2 - positive, 3 - intensely positive

FIGURE 4. Red complex bacteria detection in periodontitis patients and level of detection



Abbreviations: P = periodontitis; St = stage; Gr = Grade. X – axis: 0 - negative, 1 - weakly positive, 2 - positive, 3 - intensely positive

FIGURE 5. Orange complex bacteria detection in periodontitis patients and level of detection

ence of periodontal bacteria in the subgingival environment of young adult periodontitis patients. This technique was used for the evaluation of the bacterial load of eleven pathogens in periodontitis patients aiming to demonstrate the added utility of this approach and make use of its sensitivity in everyday clinical practice. Some studies suggested that the RT-PCR assay can serve as a useful tool in aiding periodontal diagnosis and prognosis, as well as in planning a guided stepwise therapy approach, and post-therapeutical patients' monitoring over time [37-40].

Overall, in our investigated group of patients, there was an increased periodontopathogenic bacterial burden (Figure 3), which could also explain the presence of periodontitis mostly in severe forms despite the patients' young age. The RT-PCR assay results showed that the periodontitis associated microbiota of the study group contained increased levels of *P. gingivalis*, *T. denticola* and *T. forsythia* (91,67%) in all patients with severe generalized periodontitis. These bacteria belong to the red complex described by Socranski [36,41] and usually become dominant in the microbiota during the later stages of plaque development [16]. With respect to the increased prevalence of the red complex bacteria, our findings are in agreement with other studies, as the presence of these pathogens is usually associated with severe forms of periodontitis and periodontal sites expressing extensive and progressive tissue breakdown [42-45]. Interestingly, in this study, the same increased prevalence was also detected in one patient with localized stage I periodontitis which could be considered a marker for the rapid progression towards severe forms of the disease.

F. nucleatum is a common but unique microorganism in the oral environment, which in health conditions has a symbiotic relationship with the host [46]. However, *F. nucleatum* plays a key role in the biofilm development, facilitating the adherence of early and late colonisers [16,47] and interacting with other pathogens leading to severe oral diseases [48]. Our study findings show that *F. nucleatum* was highly prevalent and in high quantities in almost all tested samples (91,67% prevalence). *F. nucleatum* belongs to the orange complex, which is usually detected alongside the red complex bacteria, as shown in other studies [28,49,50].

P. intermedia, also a member of the orange complex, appeared to be strongly associated with periodontitis based on RT-PCR test results. It was detected intensely positive in the majority of our periodontitis patients. *P. intermedia* is less virulent and less proteolytic than *P. gingivalis* [51]. *C. rectus* is a gram-negative anaerobic rod that produces leukotoxin, with a commensalism interaction with *P. gingivalis*. This could explain the high prevalence of this bacterium detected in our study (87,50%).

The periodontitis patients' microbiological profiles could also have a direct impact on the treatment outcomes, as one study suggested that the specific features of the subgingival microbiome prior to periodontal therapy can be predictive of the treatment results [52]. Although individuals with refractory forms of periodontitis display a heterogeneous composition of the subgingival microbial composition, another study showed that the microbiota of refractory subjects was similar to that of untreated periodontitis patients. One particular profile, exhibiting high counts and proportions of red and orange complex bacteria, and a low prevalence of *Actinomyces spp.* was closely linked with refractory forms of periodontitis [53]. Thus, given the high proportion of red and orange complex members in our investigated group, tracking the changes in the proportion of selected periodontopathogenic bacteria by repeated PCR testing might be useful in tailoring the periodontal therapy approach for these individuals. Additionally, there is a need for more in-depth research into the identification and characterization of microbiological refractory and recurrent risk profiles.

A. actinomycetemcomitans, a Gram-negative, facultative anaerobic, highly prevalent bacterial pathogen in periodontitis, was strongly associated with incisor-molar pattern and grade C periodontitis, formerly known as aggressive forms of periodontitis, which usually develop in young, otherwise healthy individuals [54-56]. Interestingly, in our study *A. actinomycetemcomitans* remained completely undetected despite the high severity of periodontitis identified in the investigated individuals. Other studies observed a low prevalence [38,57] or the absence [58] of *A. actinomycetemcomitans* in the periodontal pockets. An explanation could be that *A. actinomycetemcomitans* may not be present in all the sites of the oral cavity in a periodontitis patient [21] and therefore remained undetected during the sampling of the periodontal pockets. To overcome such potential bias, sampling the subgingival plaque from more than just six periodontal pockets could be a way to detect *A. actinomycetemcomitans*, especially in cases of incisor-molar patterns and grade C forms of periodontitis in young patients. Alternatively, saliva samples might also be useful, since it contains oral epithelial cells, immune and inflammatory cells, and bacteria and is therefore considered a reliable source for periodontitis diagnosing and monitoring [59].

Our investigation correlated, in most of the cases, the presence of the periodontopathogenic bacteria with the severity of the clinical features (periodontal pocket depth and clinical attachment loss). All tested patients presented severe forms of periodontitis, and although the proportion of pockets with depth of 4-6 mm and beyond 6 mm was smaller

than the proportion of healthy sites, the RT-PCR test accurately revealed a vast bacterial load in moderate and deep periodontal pockets.

CONCLUSIONS

The RT-PCR technique proved to be a useful and easy approach for identifying and quantifying sub-

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