THE BENEFIT OF MOLECULAR MICROBIOLOGICAL ASSAYS IN OPTIMAL MONITORING OF PERIODONTAL DISEASE. A RETROSPECTIVE STUDY

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THE BENEFIT OF MOLECULAR MICROBIOLOGICAL ASSAYS IN THE OPTIMAL MONITORING OF PERIODONTAL DISEASE. A RETROSPECTIVE STUDY (Abstract): Periodontal disease is among the most frequent pathologies worldwide, with multiple consequences to the quality of daily patient life. Aim: To assess the prevalence and the DNA load of etiologic agents in selected patients with periodontal disease. Materials and methods: 55 saliva samples from periodontal disease patients were analyzed by multiplex Real Time PCR, using specific primers for: Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola. The positive sampled were further analysed for the evaluation the antibiotic susceptibility to Clindamycin, Metronidazole, Amoxicillin, Doxycycline, Ciprofloxacin, and Clarithromycin. Results: The most frequent bacteria detected were Treponema denticola, in samples from 47 patients (85.5%), followed by Porphyromonas gingivalis, in 43 patients (78.2%) and Aggregatibacter actinomycetemcomitans, in 5 patients (9.1%). Besides these 3 periodontal pathogens, one patient was positive for Tannerella forsythia, Prevotella intermedia, Eubacterium nodatum and Capnocytophaga gingivalis. Conclusions: The microbiologic diagnosis using sensitive molecular assay is compulsory for modern and optimal periodontal diseases assessment. Keywords: PERIODONTAL PATHOGEN, CLINICAL GUIDELINE, REAL-TIME PCR, DIAGNOSIS, TOOTH SURVIVAL.

Periodontal disease is the most frequent chronic noncommunicable human disease worldwide, with important implications in the daily life of diagnosed patients, as tooth loss and disability lead to lower quality-of-life, has been associated with diabetes, cardiovascular diseases and with increased medical expenditure. The global burden of severe periodontitis has been substantial and increasing over the past three decades. In 2019, there were registered 1.1 billion prevalent cases of severe periodonti-
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tis globally (1).

Many groups of researchers elaborate guidelines, for optimization of disease prevention, detection, and efficient therapy. Periodontal specialists elaborated a UK version of the European Federation for Periodontology S3-level clinical practice guideline which include consensus-based clinical recommendations of direct relevance to the dental community in the UK and encompasses 62 clinical recommendations for the treatment of stage I-III periodontitis, based on a stepwise process mapped to the 2017 classification system (2).

The UK healthcare experience recognize the benefit of Metronidazole + Amoxicillin for periodontal infections, but at the same time, the authors mention the risk of side effects regarding antibiotics overuse. These can lead to an increase in antimicrobial resistance genes, dysbiosis, and impacts on the microbiome. Given the potential harm vs. benefit considerations on the use of antibiotics (stewardship issues), the evidence-based recommendation supports the use of locally administered sustained-release antibiotics as an adjunct to subgingival instrumentation in patients with periodontal disease (2).

A more recent guideline of The European Society of Endodontology was elaborated by Duncan HF et al., which have used a robust, rigorous, and transparent process including the analysis of relevant comparative research in 14 systematic reviews. The guideline describes a series of clinical recommendations the effectiveness of diagnosing apical periodontitis, to preserve teeth over a patient's lifetime, according to the best comparative evidence currently available (3).

The understanding of etiopathogenesis of periodontal diseases, motivated the European researchers to evaluate the etiology of periodontal pathogens in a study group of Romanian periodontal disease patients, and to follow up the efficiency of antibiotic therapy.

AIM: to assess the etiology of periodontal pathogens in a study group of Romanian periodontal disease patients, and to follow up the efficiency of antibiotic therapy.

MATERIALS AND METHODS

For the accurate determination of periodontal pathogens, in the period 12.03.2018 - 17.11.2022, we have tested 55 patients clinically diagnosed with periodontitis or peri-implantitis, using PET (periodontitis / peri-implantitis pathogens test). From each patient we collected samples of plaque from the periodontal pockets or affected implants of patients with periodontal disease (2).
periodontal disease: Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Prevotella intermedia, Peptostreptococcus micros, Fusobacterium nucleatum, Eubacterium nodatum and Capnocytophaga gingivalis. For positive samples, the antibiogram was performed for Clindamycin, Metronidazol, Amoxicillin, Doxycycline, Ciprofloxacin, and Clarithromycin, using a CFU (colonies forming units’ assay) based assay. We analyzed our data with SPSS 20.0 software package, for descriptive statistics.

The institutional approval for the study was not applicable considering the retrospective design of the study.

RESULTS

The mean age of the study group was 35.15 years old (limits 13 - 79), 38 (67.9%) being female. Repartition of patients per age group and gender are reported in first table.

TABLE I.

Demographic data: age group - gender crosstabulation

<table>
<thead>
<tr>
<th>Age_group</th>
<th>F</th>
<th>M</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 - 20</td>
<td>7</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>21 - 30</td>
<td>10</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>31 - 40</td>
<td>10</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>41 - 50</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>51 - 60</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>61 - 70</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>71 - 80</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>17</td>
<td>55</td>
</tr>
</tbody>
</table>

The Real Time PCR assay included a qualitative and quantitative assessment of the detected pathogens. The detected bacterial load varied between $10^1$ - $10^9$. The highest bacterial load was detected for Porphyromonas gingivalis. The ratio of the different pathogens was also determined. This approach is driven by the fact that some pathogens pose a risk only when present in large numbers or when they coexist with other pathogens, while others can lead to disease progression even when present only in small amounts.

Treponema denticola was detected in 47 patients (85.5%). The highest frequency was detected for the bacterial load of $10^4$ in saliva: 20 (36.4%). Porphyromonas gingivalis was detected in 43 patients (78.2%). The highest frequency was detected for the bacterial load of $10^5$ in saliva: 18 (32.7%). Aggregatibacter actinomycetemcomitans was detected in 5 patients (9.1%), each of them with different concentration. Prevotella intermedia was detected just in one patient (1.8%), with a load of $10^1$. Peptostreptococcus micros and Capnocytophaga gingivalis were detected just in one patient, (1.8%), each with a concentration of $10^1$. Fusobacterium nucleatum was not detected in any patients. Tannerella forsythia, Prevotella intermedia, Eubacterium nodatum and Capnocytophaga gingivalis were present just in one female patient, with a $10^4$ bacterial load for the first two species, and $10^3$ for the last two.

The highest prevalence for Porphyromonas gingivalis was detected for group age 31 - 40 (7/18, 38.9%), and the highest bacterial load ($10^6$) was detected in the 21 - 30 age group, 4/20, 20% (Figure 1). The highest prevalence for Treponema denticola was detected for group age 21 - 30 (19/20, 95%), and the highest bacterial load ($10^6$) was detected in the 21 - 30 age group, 1/20, 5% (Figure 2).

The prevalence for Aggregatibacter actinomycetemcomitans was equally detected for each age group (1, 29.1%), and the highest bacterial load was also in 31 - 40 age group: $10^6$, 1/14 (7.1%). We found no significant statistical association (tabs. II, III).
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Fig. 1. Crosstabulation between age group and *Porphyromonas gingivalis* bacterial load

TABLE II.

**Statistical analysis between age group and *P. gingivalis* load**

<table>
<thead>
<tr>
<th>Chi-Square Tests</th>
<th>Value</th>
<th>df</th>
<th>Asymp. Sig. (2-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-Square</td>
<td>27,166(^a)</td>
<td>30</td>
<td>0.615</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>20,957</td>
<td>30</td>
<td>0.468</td>
</tr>
<tr>
<td>N of Valid Cases</td>
<td>55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 41 cells (97.6%) have expected count less than 5. The minimum expected count is 5.

Fig. 2. Crosstabulation between age group and *Treponema denticola* bacterial load
TABLE II.

Statistical analysis between age group and *T.denticola* load

<table>
<thead>
<tr>
<th>Chi Square Tests</th>
<th>Value</th>
<th>df</th>
<th>Asymp. Sig. (2-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-Square</td>
<td>24,764^a</td>
<td>30</td>
<td>.736</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>27,958</td>
<td>30</td>
<td>.573</td>
</tr>
<tr>
<td>N of Valid Cases</td>
<td>55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. 40 cells (95.2%) have expected counts less than 5. The minimum expected count is .02.

Fig. 3. Example of a significant increase in the number of bacteria belonging to the red and orange complexes.

We found no statistical difference between age group and the most frequent bacteria identified in our samples.

For the patient with the above qPCR results (fig. 3), the suggested therapy according with antibiogram results, was Clindamycin, 2 x 600 mg/day, 7 days in association with Metronidazole 3 x 400 mg/day, 7 days.

**DISCUSSION**

In this study we have evaluated the prevalence of periodontal pathogens in 55 patients diagnosed with periodontal disease, using Real Time PCR. Our data are in accordance with those of other groups of researchers who used the same molecular assay.

The etiology of periodontal diseases has been evaluated by many researchers worldwide. Ergün E *et al.*, used Real Time PCR assay to evaluate the microbial content of periodontal abscesses, using 16S rRNA Universal primers. They detected *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum* in all 30 tested samples. Other bacterial species were detected with lower frequency: *Porphyromonas endodontalis*, *Tannerella forsythia*, *Actinomyces naeslundii* (5). Reddahi S *et al.*, from Morocco investigated periodontal pathogens in saliva from 53 patients with qPCR and they identified a significant difference in *P. gingivalis* and *A. actinomycetemcomitans JP2* clones in the saliva of periodontitis patient compared to the control group. The researchers consider testing of
etioligic agents of periodontal disease a good potential as diagnostic markers for periodontal disease (6). A more comprehensive study was realized by Chigasaki O. et al., in Japan, which tested almost 1000 periodontal disease patients with Real Time PCR. The authors found that P. gingivalis was strongly associated with an increase in the rate of sites of 4 mm or greater probing pocket depth, bleeding on probing, and 1.5 mm or more of bone loss on dental radiographs sites. For this study group, the amount of P. gingivalis was strongly associated with the severity of periodontal disease. Other bacteria from red-complex as T. denticola and/or T. forsythia showed heightened progression of periodontitis (7).

Eick S. et al., tested by RT-PCR Swiss adolescent subjects, and detected 10 % to be positive for P. gingivalis, 23% for A. actinomycetemcomitans and T. forsythia, and 47.5% for T. denticola (8).

Wang P et al., evaluated through the same assay, qPCR, periodontal pathogens in 25 patients with chronic periodontitis. Our study shows comparable results to theirs, Treponema denticola having the highest prevalence. The authors identified that human beta-defensins may play an important role by limiting an increase of bacterial load at the initial stage of periodontitis (9).

In a recent study from 2023, Castillo Y. et al. aimed to standardize a qPCR assay for periodontal pathogens, in 55 subgingival biofilm clinical samples, from different stages of periodontitis. The results obtained using qPCR were compared with next-generation sequencing (NGS) results, and the researchers considered that this qPCR test was standardized with efficiencies between 90% and 100%. Eubacterium saphenum, Eubacterium brachy, Desulfobulbus oralis, and Filifactor alocis which are periodontal pathogens difficult to be cultivated, could be easily detected by this standardized qPCR assay (10).

Limitations of the study: considering the sample population of the current study, further research is necessary to validate our findings. Nowadays, in the era of new technology, there are other modern molecular assays which are used for the detection of periodontal pathogens, understanding new pathogenic mechanisms, finding therapeutic target genes (11) or testing biomarkers in periodontal disease associated conditions, such as diabetes (12). For example, a research team from Greece combined Real Time PCR with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) and they detected Aspergillus and C. albicans in 60 cases of apical periodontitis (13). Rams TE et al., from the USA, used MALDI TOF for subgingival isolates from 38 adults with chronic periodontitis and all P. gingivalis subgingival isolates were confirmed as P. gingivalis with MALDI-TOF. The authors considered MALDI-TOF as fast phenotypic identification of cultivable P. gingivalis in human subgingival biofilm specimens (14).

Jeong J et al., from Korea used next-generation sequencing (NGS)-based 16S metagenome profiling data to confirm the presence of Porphyromonas gingivalis in periodontal patients (15).

CONCLUSIONS

Molecular methods as Real Time PCR are compulsory in optimal management of periodontal diseases since many of the etiological agents are non-cultivable or require accurate quantitative assessment to understand their clinical significance. With sensitive, specific, and clinically validated detection assays and targeted therapy, evo-
lution to more severe consequences could be stopped.

CONFLICT OF INTEREST AND FUNDING
The authors declare that there is no conflict of interest and they received no funding.

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REFERENCES