Journal of Pharmaceutical Research International

33(64A): 108-115, 2021; Article no.JPRI.78797
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Comparative Assessment of *P. gingivalis* Level in Periodontit is Patients with and without Diabetes Mellitus- A PCR based Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i64A35308

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/78797

Original Research Article

Received 27 October 2021
Accepted 29 December 2021
Published 30 December 2021

ABSTRACT

Introduction: Periodontal diseases if left untreated can lead to tooth loss with the main cause being bacterial plaque. Among the subgingival plaque bacterial species, Porphyromonasgingivalis has been implicated as a major etiological agent causing tooth loss. Diabetic patients are at high risk for periodontal disease. Our aim was to compare the involvement of *P. gingivalis* in diabetes mellitus (DM) patients associated with periodontitis and to compare them with periodontitis patients having no other systemic pathologies.
Materials and Methods: Subgingival plaque samples from a total of 8 patients were collected. DNA was isolated from the collected samples and was quantified using RT-PCR for standardizing the polymerase chain reaction. Paired t test was performed using the statistical software Graphpad prism (Version 7.0).
Results: There was a statistically significant level of *P. gingivalis* seen in periodontitis patients having DM (p=0.0053), whereas the least score was seen in periodontitis patients without DM.
Conclusion: Poor glycemic control, as indicated by HbA1c≥7%, is associated with increased levels and frequencies of periodontal pathogens in the subgingival biofilm of subjects with DM.

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Keywords: Diabetes mellitus; innovative technology; periodontitis; porphyromonasgingivalis; real-time polymerase chain reaction.

1. INTRODUCTION

Periodontal disease is one of the most common diseases of the oral cavity and is the major cause of tooth loss in adults [1]. Recently, there have been studies correlating the relationship of periodontal disease to important systemic diseases, such as cardiovascular disease and complications in pregnancy [2]. There are two main categories of periodontal disease in which loss of supporting structures around the tooth occurs: periodontitis and aggressive periodontitis [3]. Periodontitis is the result of a polymicrobial infection with variable microbial patterns. periodontitis is an inflammatory disease of the supporting tissues of the teeth associated with bacteria. It results in either localized or generalized destruction of the supporting tissues of the teeth; the periodontal ligament, bone, and soft tissues. In contrast, aggressive periodontitis involves rapid attachment loss and bone destruction, and the destruction seen is usually not commensurate with the amount of microbial deposits [4].

Bacteria are the primary etiologic factor of periodontal diseases, however, recent evidence also lists yeast and herpesviruses as putative pathogens responsible for periodontitis [5,6]. Porphyromonasgingivalis (*P. gingivalis*) being Gram-negative non spore forming, non-motile, obligate anaerobe, rod-shaped and highly virulent organism has been implicated as a major pathogen in destructive periodontal disease since it has the ability to adhere and invade oral epithelium. These pathogens gain entry into circulation through the ulcerated epithelium and exposed capillaries during periodontal inflammation and may induce systemic symptoms. It is also implicated to be involved in the development of systemic diseases due to systemic inflammation with increased circulating cytokines and mediators, direct infection and cross-reactivity/molecular mimicry between bacterial antigens and self-antigens [7–10].

Diabetes Mellitus has been undoubtedly confirmed as a major risk factor for periodontitis [11]. In the early 1990s periodontitis was sometimes referred to as the ‘sixth complication of diabetes’ [12], and in 2003 the ADA acknowledged that periodontal disease is often found in people with diabetes [13]. There has recently been much emphasis on the ‘two-way’ relationship between diabetes and periodontitis. That is, not only is diabetes a risk factor for periodontitis, but periodontitis could have a negative effect on glycaemic control [14]. In addition, various studies have reported that the prevalence and severity of non-oral diabetes-related complications, including retinopathy, diabetic neuropathy, proteinuria and cardiovascular complications, are correlated with the severity of periodontitis [15,16]. However there is no clear cut idea about the bacterial species most commonly involved in causing periodontitis in diabetes and also if there is any increased involvement of the pathogens. Although being able to determine which subjects are at greater risk of future periodontal breakdown is undoubtedly beneficial to the patient and clinician, determination of increased risk of disease most commonly by a specific bacteria within a patient would be the ideal in periodontal diagnosis. There is also a lack of prospective longitudinal data regarding the association of levels of pathogenic species in subgingival plaque with the progress. In this way this study fulfills the lacunae created by the previous studies and sheds light for the dentists and general population. With this background in mind, the current study aims to quantify and compare the *P. gingivalis* levels in periodontitis patients with/ without diabetes.

Our team has extensive knowledge and research experience that has translate into high quality publications [17–29,30–34,35,36].

2. MATERIALS AND METHODS

Group I periodontitis patients without diabetes mellitus and Group II periodontitis patients with diabetes mellitus were selected by assessing the periodontal status, HbA1c and blood glucose levels. The enrollment criteria for the study are as follows

Inclusion criteria:

1. For periodontitis included individuals with not >2 teeth missing in each quadrant; ≥ 30% of periodontal sites with PD ≥ 4 mm; ≥20% of periodontal sites with interproximal clinical AL >2 mm; ≥30% of sites show- ing BOP; and radiographic evidence of bone loss visible in posterior vertical bitewing films.

2. For Diabetes mellitus patients included RBS >200 mg /dl
Fasting > 110 mg/ dl
HbA1C >7

Exclusion criteria: Individuals with pregnancy, previous or current smokers, menopause, cardiovascular disorders, thyroid disorders, use of antioxidant supplements, long-term steroid medications, patient who had taken anti - inflammatory or antibiotics within previous 3 months or underwent periodontal treatment in the past 6 months were excluded from the investigation.

Sample collection: Supragingival and subgingival plaque samples were collected from 20 patients (10 patients in each group) with the help of curette and transported to phosphate buffered saline and stored at -80 degree celsius for further analysis.

Isolation and Quantification of DNA: Genomic DNA was extracted from subgingival plaque of both the patients group using a QIA amp DNA Mini kit (QIAGEN Inc., USA, 9300 Germantown Road, Germantown, MD 20874). The DNA concentration and purity was maintained using Nano-Drop Spectrophotometer (NanoDrop™ 2000/2000c Spectrophotometer, 168 Third Avenue. Waltham, MA USA 02451) with a multi-wavelength programme (260/280 nm).

Quantification of *P. gingivalis* by Real Time-PCR: Quantitative RT-PCR was performed with the CFX 96 Real Time system (Bio-Rad, USA) with SYBR Premix Ex Taq (Takara, Japan) in triplicates using the primers listed. The double standard DNA-binding dye SYBR Green I (KAPA SYBR FAST q-PCR Kit) using species-specific primers used for *P. gingivalis* (Forward: 5'-AGG CAG CTT GCC ATA CTG CG-3' Reverse:5'-ACT GTT AGC AAC TAC CGA TGT-3'). and the reaction efficiency was optimized as follows: Enzyme activation (PCR initial activation): 95°C for 30 min; denaturation 95°C for 55 sec; Annealing: 60°C & 57°C for 45 sec and extension: 72°C for 30 sec with 40 cycles. All the reactions were performed in triplicate along with no template control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 59°C - 95°C for each sample to determine the presence of multiple amplicons, non-specific products, and contaminants. Random samples (PCR products) from control and treated groups were resolved on a 2% agarose gel electrophoresis along with 100 bp molecular marker DNA and visualized with the use of ethidium bromide as a quality control. Agarose gel was compared with melting curves for the presence of the appropriate sized amplicon as well as the presence of a single PCR product. The relative amount of genes was calculated by using the comparative CT method.

Statistical analysis: The data was tabulated in Microsoft Excel sheet. Data analysis was performed using SPSS software version 23. Paired t test was performed for comparison of *P. gingivalis* expression among the diabetic and non diabetic groups.

3. RESULTS AND DISCUSSION

As periodontal diseases are polymicrobial infections with various etiologic factors, accurate quantitation of the number of cells of individual bacterial species in dental plaque samples is needed for understanding the bacterial etiology of periodontitis. Hence, in our study, we intend to quantify the *P. gingivalis* count using real-time polymerase chain reaction (RT-PCR), since it is more efficient and sensitive compared to other methods and provides precise counts through direct monitoring of the increasing amount of PCR product throughout the enzymatic assay [37]. Also, in our study subgingival plaque samples were chosen over other samples such as saliva and gingival crevicular fluid for better yield and to understand bacterial etiology of periodontitis.

The results of the study revealed that the *P. gingivalis* are of statistically significant level (p=0.0053) in the periodontitis patients with diabetes mellitus (Table 1); Fig. 1 reveals the three fold increase of *P. gingivalis* level in periodontitis patients with diabetes mellitus than patients without diabetes.

Table 1. *P. gingivalis* (Fold change) among periodontitis with diabetes mellitus and non diabetic groups

Group	Periodontitis	Periodontitis +DM	P value
P. gingivalis mRNA (Fold change over control)	1±0.000	1.350±0.02887	p<0.0067

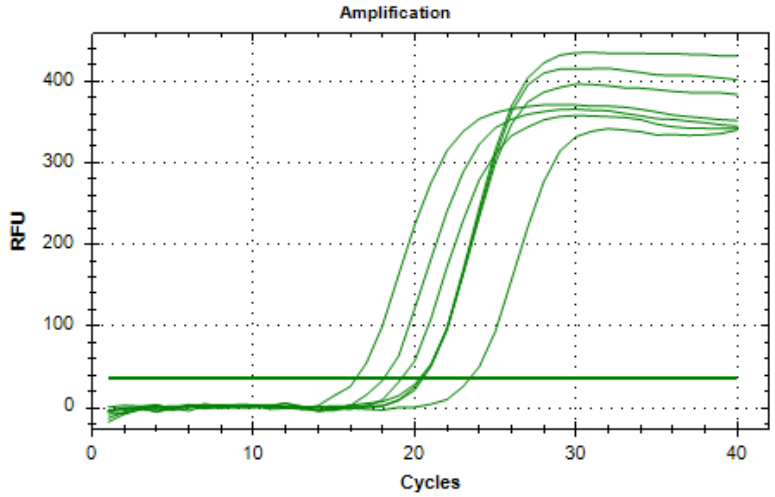

Fig. 1. Amplification plots showing the mRNA levels of *P. gingivalis* in periodontitis without diabetes mellitus using gene specific primers. Each bar represents mean ± SD (n=20). Significance at P <0.05, **- Significantly different from the control group (Periodontitis)

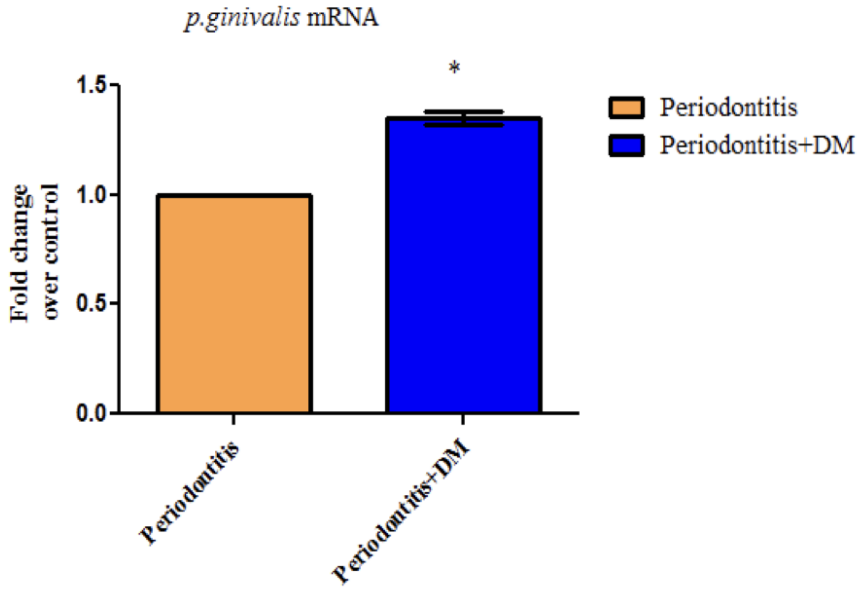

Fig. 2. Assessment of mRNA expression of *P. gingivalis*. The mRNA expression was assessed by Real Time-PCR using gene specific primers. Each bar represents mean ± SD (n=20). Significance at P <0.05, **- Significantly different from the control group (Periodontitis)

The reason for this increased level of *P. gingivalis* is the fact that diabetes increases the glucose concentration in the gingival crevicular fluid and decreases the salivary levels of epidermal growth factor which plays an important role in wound healing. These modifications in GCF affect plaque composition which is supported by an increased amount of plaque and increased numbers of Gram-negative anaerobes. So in addition to diabetes, if the patient suffers from periodontitis, it will impair cellular functions, impair host defense, vascular alterations, prolonged inflammation, impair bone formation or repair ultimately resulting in tooth mobility and premature loss of teeth [38].

Since there is a two-way relationship between DM and periodontitis, reduction of bacterial

burden by periodontal therapy may show a greater impact in the prevention of periodontal disease progression, which in turn can lead to the reduction in the glycemic control in diabetic patients. There have been many recent studies with good evidence to support this hypothesis. Grossi and others have suggested that effective control of periodontal infection in diabetic patients reduces the level of AGEs in the serum [39]. The level of glycemic control seems to be the key factor. Tervonen and Karjalainen followed diabetic patients and nondiabetic controls for 3 years; They found that the level of periodontal health in diabetic patients with good or moderate control of their condition was similar to that in the nondiabetic controls. Those with poor control had more attachment loss and were more likely to exhibit recurrent disease [40]. This phenomenon has been pointed out by several other researchers also. From this, we can conclude that prevention and control of periodontal disease must be considered an integral part of diabetes control. Hence it is suggested that periodontal therapy should be included as a part of prevention program in systemic diseases such as diabetes [41–43].

The only limitation of this study is smaller sample size. Hence, further studies need to be done with a large sample size to get even more statistically significant results. Larger studies of this kind can throw light on a variety of etiological microorganisms and also will confirm the findings of our study.

4. CONCLUSION

In our study, periodontitis individuals with diabetes mellitus harbor increased *P. gingivalis* bacteria (p= 0.0053) than patients without diabetes. This states the strong association of periodontitis and diabetes mellitus. Hence, reduction of bacterial burden by periodontal therapy may show a greater impact in the prevention of periodontal disease progression, especially in diabetic individuals. Therefore it is suggested that periodontal therapy should be included as an integral part of prevention programs in systemic diseases such as diabetes.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

ACKNOWLEDGEMENT

The authors would thank all the participants for their valuable support and authors thank the dental institution for the support to conduct.

SOURCE OF FUNDING

The Present project is funded by

- Saveetha Institute of Medical and Technical Sciences
- Saveetha Dental College and Hospitals
- Saveetha University.
- Office Needs inc.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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