

Full Length Research Paper

# Levels of IgM, IgG and IgA antibodies in gingival crevicular fluid of subjects with severe chronic periodontitis

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Humoral immune response is an important host mechanism against periodontopathogenic microorganisms, such as *Porphyromonas gingivalis*. *P. gingivalis* is the most frequent causative agent of chronic periodontitis in Mexico, but its detection presents difficulties. The aim of this study was to identify antibodies against semi-purified culture-secreted proteins of *P. gingivalis* in saliva and gingival crevicular fluid of patients with chronic periodontitis. Semi-purified culture-secreted proteins of *P. gingivalis* were immobilized in Enzyme-Linked Immunosorbent Assay (ELISA) plaque to detect IgA, IgG and IgM antibodies in saliva and gingival crevicular fluid from chronic periodontitis patients. IgG antibody against proteins from *P. gingivalis* was detected in 65% of the samples of patients, 27% of patients had IgA antibodies, while IgM was detected in 45% of patients. IgG, IgA and IgM antibodies were detected in saliva and gingival crevicular fluid mainly in patients with severe chronic periodontitis, confirming that *P. gingivalis* could be the causative agent of the infection.

**Key words:** Antibodies, gingival crevicular fluid, *Porphyromonas gingivalis*, and saliva.

## INTRODUCTION

Chronic periodontitis induces a chronic inflammatory response of the periodontium to bacterial plaque (Haffajee and Socransky 2000). Inflammation may lead to destruction of the tooth-supporting tissues and eventually possible tooth loss. Specific microorganisms or groups of microorganisms have been related to different forms of periodontitis. *Porphyromonas gingivalis* is a Gram-negative and anaerobic bacterium and it is considered to be a major periodontopathogen in chronic periodontitis (World Workshop in Periodontics, 1996; Haffajee and Socransky, 2000, 2005; Socransky et al., 1998). During chronic periodontitis both cellular and humoral immune responses are activated for infection control (Haffajee

and Socransky, 2000, 2005). The role of humoral immunity in chronic periodontitis has not been clarified, though a protective role has been demonstrated (Kinane et al., 2011; Kinane and Lappin, 2002). The rate of periodontal tissue breakdown has been associated with host susceptibility to periodontitis. Moreover, host predisposition to disease may relate to innate host defence and the subsequent activation of adaptive immune response (Deo and Bhongade, 2010).

The relationship between specific IgA, IgG and IgM antibodies against *P. gingivalis* and periodontal diseases is a controversial issue. High IgG antibody levels against *P. gingivalis* have been detected in adults with severe periodontitis, compared with healthy individuals (Mouton et al., 1981; Suzuki et al., 1984; Vincent et al., 1985; Califano et al., 1997; Ebersole, 1990). However, certain investigators have reported a significant decrease in specific IgG immunoglobulins in deep periodontal pockets (>4 mm) and therefore support the concept that humoral

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immune response is not protective (Kinane et al., 1993; Mooney and Kinane, 1997). Another study did not detect a significant increase in serum antibodies levels to *P. gingivalis* (Haffajee et al., 1995). It has been proved that antigenic components produced by *P. gingivalis* could induce host immune response. *P. gingivalis* antigens, such as arginine-specific gingipains (RgpA, RgpB) and lysine-specific gingipain (Kgp) have been demonstrated to induce a significant humoral response (Nakagawa et al., 2003). RgpA and Kgp are able to induce the production of high titers of IgG antibody in immunized rabbits (Nakagawa et al., 2001). Moreover, another study revealed that serum of generalized aggressive periodontitis patients had elevated levels of IgG anti-*P. gingivalis* and gingipain antibodies (Gibson et al., 2005). The aim of the present study was to determine the levels of IgM, IgG and IgA antibodies in gingival crevicular fluid or saliva of subjects with severe chronic periodontitis, in comparison with periodontally healthy subjects.

## MATERIALS AND METHODS

### Study population

The population of the study was divided into an experimental group, including 29 chronic periodontitis patients (15 females and 14 males), aged 30 to 68 years (mean age, 46.3±7.5 years) and a control group, comprising 10 periodontally healthy subjects (8 females and 2 males), aged 30 to 62 years (mean age, 46.5±10.5 years). Study participants were derived from the Department of Periodontics, Facultad de Odontología, Universidad Autónoma de Nuevo Leon, Monterrey, Mexico. The study protocol was approved by the Bioethics Committee at the Universidad Autónoma de Nuevo Leon and approved this project according to the International Review Board regulations. Informed consent was obtained from each patient. Selection criteria for patient inclusion in the experimental group were:

- 1) Patients with moderate to severe chronic periodontitis;
- 2) Systemically healthy;
- 3) Not having received antibiotic medication within the preceding three months;
- 4) Not having received periodontal therapy in the preceding six months;
- 5) Non-smokers; and
- 6) Non-pregnant women.

In the control group, the inclusion criteria were the same, except for criterion 1, where subjects should be periodontally healthy.

### Screening periodontal examination

The following clinical periodontal parameters were recorded for each subject: plaque index (Nakagawa et al., 2003), gingival index (Nakagawa et al., 2001), probing depth and clinical attachment level. Plaque was measured mesio-facial and disto-facial. Probing depth was registered with a North Caroline probe. The probing force applied was standardized and all clinical parameters were performed by a single investigator. The aforementioned indexes, as well as mean plaque and gingival index were used as diagnosis criteria for periodontal disease rather than clinical criteria.

### Sample collection

Gingival crevicular fluid (GCF) samples were collected in the experimental and control groups. The collection of gingival crevicular fluid was performed, using standard methods previously described in the literature (Löe and Holm-Pedersen, 1965; Daneshmand and Wade, 1976; Hancock et al., 1979). Before GCF samples were collected, the tooth selected for sampling was cleaned of supragingival plaque, dried with gauze to remove saliva and isolated with cotton rolls placed in the mucobuccal fold. Subsequently, one filter strip (Periopaper strips) was gently placed at the entrance of the gingival crevice of the tooth avoiding blood inclusion. The filter strips were subsequently removed, placed in microtubes and stored at -20°C until used. Six samples were randomly collected from each subject. The unstimulated saliva from the floor of the mouth was collected directly into microtubes with sterile Pasteur pipettes (Avitrolab, China). A volume of 1 ml of saliva was collected from each subject and stored at -20°C until used.

### *P. gingivalis* culture

*P. gingivalis* (Pg) ATCC 33277 was grown on blood agar plates for 5 to 6 days in an anaerobic chamber with Gas Pack System (BD, Franklin Lakes, NJ USA) in an atmosphere containing 80% N<sub>2</sub>, 10% H<sub>2</sub> and 10% CO<sub>2</sub>. subsequently, *P. gingivalis* ATCC 33277 (Manassas, VA, USA) was inoculated on thioglycolate medium and after 48 h incubation, the culture secreted protein of the bacteria was obtained by centrifugation at 10,000 x g for 15 min at 4°C and the cellular pellet was sonicated to obtain the protein of cellular extract.

### Semi-purified culture-secreted proteins from *P. gingivalis*

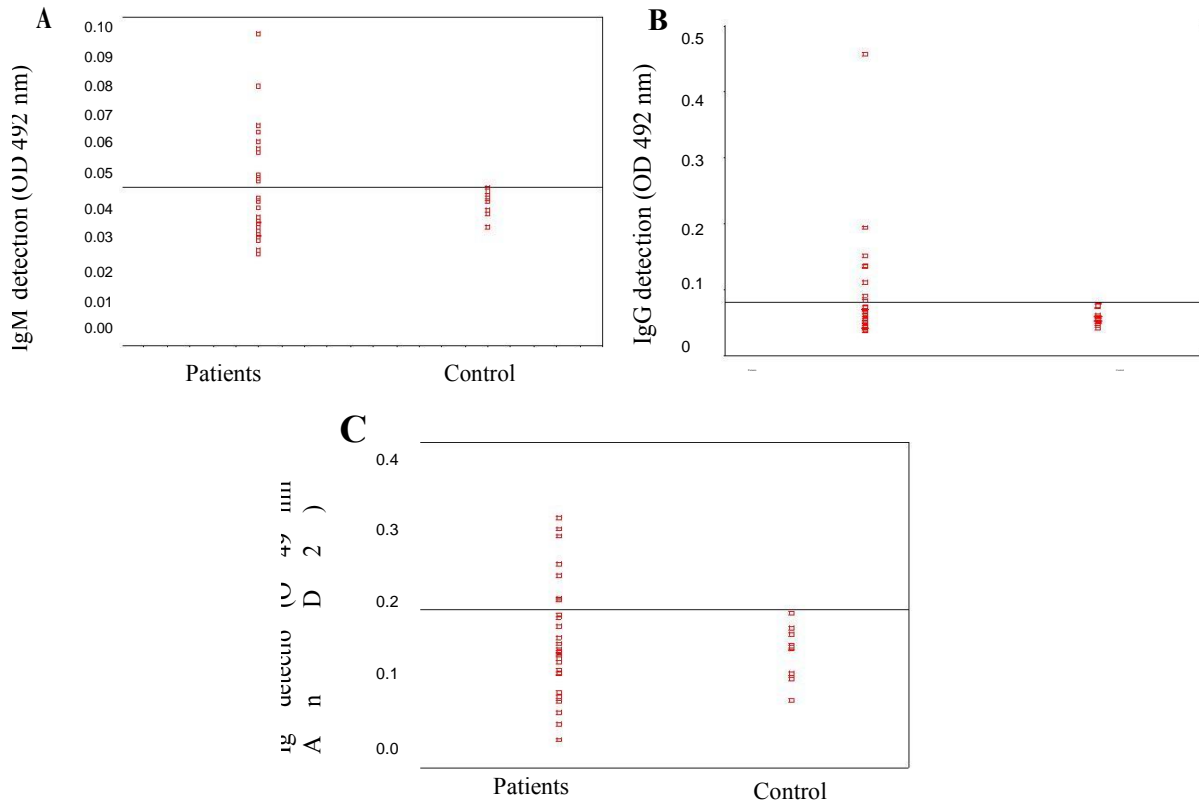
Proteins were obtained by precipitation using 50% ammonium sulfate (v/v) at 4°C. The precipitate was resuspended in distilled water and dialyzed against distilled water until absence of salts was observed. Protein concentration was determined by the Bradford method (Gibson et al., 2005), and proteins were lyophilized and then sterilized by filtration through a 0.2 µm-pore size filter (Millipore, Billerica, MA, USA) and stored at -20°C until its use.

### Cell extracts from *P. gingivalis*

The bacterial cell pellet was washed in phosphate-buffered saline (PBS) with a concentration of 0.1 M and pH=7, and resuspended in sterile saline solution to an optical density of 1.0 at a wavelength of 690 nm (model 750 spectrophotometer; Beckman, Brea CA, USA). The bacterial suspensions were sonicated using a Branson Sonifier 450 (Branson, Danbury, Connecticut, USA). The insoluble debris was removed by centrifugation at 10,000 x g for 30 min at 4°C. The protein concentration was determined by the Bradford method (Gibson et al., 2005), lyophilized, sterilized by filtration through a 0.2 µm-pore size filter (Millipore, Billerica, MA, USA) and stored at -20°C until its use.

### IgA, IgM and IgG antibodies detection in saliva and gingival crevicular fluid by enzyme-linked immunosorbent assay (ELISA)

Culture-secreted proteins (0.01 µg/well in 200 µl acetate buffer with pH=5) and cell extract (0.05 µg/well in 200 µl PBS with pH=7.4) from Pg ATCC 33277 were immobilized on 96-well polystyrene plates overnight at 4°C. Five washes using PBS with a



**Figure 1.** Antibodies in gingival crevicular fluid of chronic periodontitis patients, compared with periodontally healthy subjects. Presence of antibodies against semi-purified culture filter antigens from *Porphyromanus gingivalis* A) Ten patients with chronic periodontitis shown IgM levels up of control; b) 8 patients had IgG antibodies and c) 8 patients had IgA antibodies against semi-purified culture filter antigens.

concentration of 0.1 M and pH=7.2 were performed and 5% skim milk (BD Difco™, Sparks MD, USA) dissolved in PBS was used to block unspecific binding by incubation at 37°C for 1.5 h. Saliva (for IgA detection) and gingival crevicular fluid (for IgM and IgG detection) obtained from patients and healthy controls were added on the plate and incubated for 1 h at room temperature, using shaking to 300 rpm. Three washes were performed using washing solution, PBS with a concentration of 0.1 M and pH=7.2, and a 0.05% Tween 20, (Sigma Aldrich, St. Louis, Mo, USA).

Bound antibodies were visualized using horseradish peroxidase-coupled goat anti-human IgG ( $\gamma$ -chain specific, 1:3000, Chemicon, Temecula, CA, USA), anti-human IgM ( $\mu$ -chain specific, 1:5000, Chemicon, Temecula, CA, USA) or anti-human IgA ( $\alpha$ -chain specific, 1:10000, Sigma, St. Louis, Mo, USA) were used as secondary antibodies and incubated at 37°C for 1 h. Three washes were performed using the washing solution and a chromogen substrate solution (Sigma FAST™ OPD, Sigma Aldrich, St. Louis, MO, USA) was added. After a 30 min reaction at room temperature in the absence of light, the reaction was terminated using 1 N sulfuric acid. The microplate was read at A<sub>492</sub> using a semi-automatic ELISA plate reader.

#### Statistical analysis

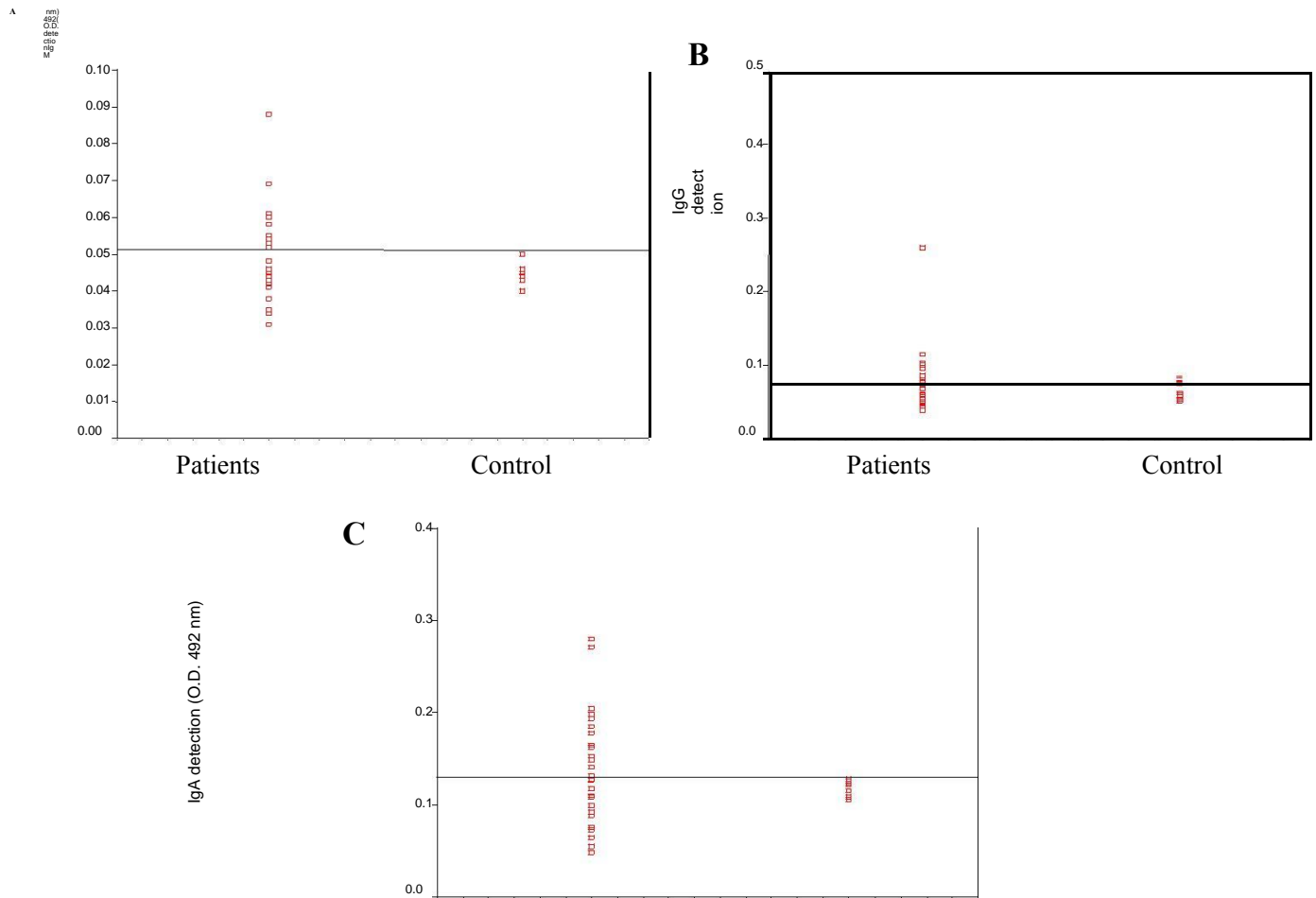
The data was analyzed using the t test. The statistical analysis was performed using commercially available software (Statistical Package for the Social Sciences, version 10.0, SPSS Inc., Chicago, IL, USA). Differences between the experimental and control group

were considered to be statistically significant, when the P value was <0.05.

## RESULTS

### Plaque index and gingival index

The mean plaque and gingival index are shown as a reference to differentiate between healthy and periodontally affected subjects. Mean Plaque Index (Nakagawa et al., 2003) for the experimental group was  $2.45 \pm 0.50$  and for the control group was  $1.68 \pm 1.20$ . The values for the Gingival Index (Nakagawa et al., 2001) were  $1.09 \pm 0.50$  for the experimental group and  $0.60 \pm 0.50$  for the control group. In this study, we demonstrated that 36% of patients who were positive to any immunoglobulin had higher plaque index and 72% had higher gingival index than patients with negative results. IgA, IgM and IgG antibodies detection anti-semi-purified culture-secreted proteins from *P. gingivalis* by ELISA. In 34.5% of chronic periodontitis patients, IgM antibody was detected in the gingival crevicular fluid. In 27.6% of chronic periodontitis patients, IgG antibody was detected in the gingival crevicular fluid. Finally, in 27.6% of chronic



**Figure 2.** Presence of antibodies against cell extract from *Porphyromonas gingivalis* ATCC 33277. A) 9 patients with chronic periodontitis shown IgM levels up controls b) 6 patients had IgG antibodies and c) 13 patients had IgA antibodies against semi-purified culture filter antigens.

periodontitis patients (that is the same proportion as that revealed for IgG antibody), IgA antibody was detected in the saliva.(Figure 1).

**IgA, IgM and IgG antibodies anti-extract cell from *P. gingivalis* in saliva and gingival crevicular fluid by ELISA**

In 31.0% of chronic periodontitis patients, IgM antibody was detected in the gingival crevicular fluid. In 20.7% of chronic periodontitis patients, IgG antibody was detected in the gingival crevicular fluid. Finally, in 44.8% of chronic periodontitis patients, IgA antibody was detected in the saliva (Figure 2).

**DISCUSSION**

Periodontal diseases comprise a heterogeneous group of infections that are caused by microorganisms that

colonize the tooth surface in the gingival crevicular area (Loesche and Grossman, 2001). Inflammation of the gingiva extending into the adjacent attachment apparatus is known as chronic periodontitis and is characterized by loss of the periodontal ligament and the adjacent supporting alveolar bone (Loesche and Grossman, 2001; Caton and Greensteinm, 2000, 1993). Microorganisms, host response, and environmental factors are involved in or associated with the pathogenesis of periodontitis (Haffajee et al., 1995). The presence of certain specific periodontal pathogens, such as *P. gingivalis*, has been associated with periodontal tissue breakdown and progression of periodontitis (Haffajee and Socransky, 2000; World Workshop in Periodontics, 1996; Haffajee and Socransky, 2000, 2005; Socransky et al., 1998; Laemmli, 1970). Antibody detection with enzyme-linked immunosorbent assay seems to reflect both past exposure and present level of bacterial challenge (Bascones and Figuero, 2005; Haffajee et al., 1995). Animal models have been used in the humoral immune response evaluation in periodontitis (Towbin et al., 1979;

Socransky and Haffajee, 1992).

In a study, high levels of IgG and IgM antibodies were found in mice after *P. gingivalis* immunization with whole or the outer membrane fraction using ELISA (Gemmell et al., 2004). In this study, IgA, IgG and IgM antibodies were detected against semi-purified culture-secreted proteins and extract cell from *P. gingivalis*. In another study, immunodominant antigens from *P. gingivalis* ATCC 33277 in whole-cell sonicates, proteinase K-digested sonicate, lipopolysaccharide, capsular polysaccharide, and whole-cell protein fractions were studied and it was revealed that whole-cell protein fraction was immunodominant and induced a more effective antibodies response (Leone et al., 2006). In other studies, several immunodominant antigens were demonstrated to be present in extract cell from *P. gingivalis*, which have a molecular weight of 55 and 40 kDa (Kesavalu et al., 1992; Chen et al., 1995). It is possible that those antigens could induce the production of antibodies at a local or systemic level in humans. The levels of IgG and IgM antibodies in gingival crevicular fluid were not significantly different between periodontitis patients and healthy controls. This finding could be explained by:

1. The low number of patients enrolled in the study, which was a limitation of this study; and
2. Most antibodies are forming immune complexes and thus stimulating the inflammation process.

IgG antibodies found in healthy controls could indicate an immunological memory protecting the host from *P. gingivalis* infection. IgM detection in experimental group is indicative of an active infection by *P. gingivalis*. Proinflammatory cytokines have been associated with destruction in periodontal tissues in patients, in which antibodies to *P. gingivalis* were not found (Chen et al., 1995). In conclusion, the present study supports the concept of a dual role of the humoral immune response: First, a protective effect of IgG antibodies and, second, the probability that immune complexes might have a destructive effect upon periodontal tissues in patients with low levels of antibodies in gingival crevicular fluid, while IgM levels are high. This study demonstrated a potential application of ELISA test to detect bacterial-specific antibodies in the gingival crevicular fluid and its use at least in *P. gingivalis* detection.

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