Screening the Antigenic Properties of *Porphyromonas gingivalis* and Level of Raised Antibodies after Antigenic Injection into Balb/c Mice

**Abstract**

**Background:** Different diagnostic screening tests have been developed to detect periodontal disease in the early stages. Despite these advances still, there is a need for a more practical and beneficial diagnostic test. **Aim:** The aim of this study was to investigate the possibility of developing such a kit based on the body immune response against *Porphyromonas gingivalis*. **Method and Materials:** This experimental study was conducted by culturing *P. gingivalis* and extracting its antigens. These antigens were injected into peritoneal cavity of four Balb/c mice. Finally, the pattern, type, and quantity of antibody response against *P. gingivalis* antigen were detected. Results of the study showed that $3.0 \times 10^8$ cells of *P. gingivalis* are an appropriate count for stimulating the immunization in Balb/c mice and the subsequent amount of antibody (IgG) production was 81.5 µg/ml. **Result:** The antigenic injections which were done in the current study could mimic the condition of periodontal disease and the raise of *P. gingivalis* in the body. **Conclusion:** The obtained data can be used in future attempts to develop practical and usable test kits against *P. gingivalis*.

**Keywords:** Antibody, antigenic properties, diagnostic screening tests, *Porphyromonas gingivalis*

**Introduction**

Periodontitis is considered as a multifactorial polymicrobial infection, characterized by a destructive inflammatory process resulting in the loss of tooth-supporting tissues. If it is left untreated, the disease may result in tooth loss and systemic complications.[1]

It has been estimated that less than half of the bacterial species present in the oral cavity can be cultivated using anaerobic microbiological methods and that there are likely 500–700 common oral species.[2]

Although the subgingival microenvironment in the periodontal pocket is characterized by a wide diversity of organisms, only a few species have been associated with the disease.[3]

The black pigmented Gram-negative anaerobic bacterium *Porphyromonas gingivalis* has been frequently isolated in several oral infections, including pulpal infection, oral abscesses, and periodontitis.[4,5]

*P. gingivalis* can adhere to cellular and acellular surfaces and form a biofilm, which contributes to its colonization in the oral cavity.[6‑8]

This microorganism also produces soluble and cell bound proteases, which can degrade various tissue and plasma proteins and propagate to the invasion of the periodontal tissue.[9‑11]

It also produces some virulence factors such as arginine and lysine-specific cysteine proteinases, lipopolysaccharides, fimbriae, hemagglutinins, which are responsible for bacterial colonization and increase in humoral and cellular host response.[12‑16]

Considering its culture medium, *P. gingivalis* specifically grows in a selective medium, which is brain heart infusion (BHI) and is further enhanced by a percentage of beef extract in the culture media. Beta hemolysis is a confirmatory characteristic of *P. gingivalis*. Hemolysis is classified into alpha (partial or incomplete) where colonies appear to be opaque and semitranslucent, beta (complete) where the colonies appear transparent and gamma (no hemolysis).

Microbiologic tests to identify putative pathogens for periodontal disease have the potential to support the diagnosis of the various forms of the periodontal diseases, to serve as indicators of disease initiation.
and progression, and to determine which periodontal sites are at higher risk for active destruction.[11]

These tests can also be used to monitor periodontal therapy directed at the suppression or eradication of periodontal pathogenic microorganisms.[17]

Many methods have been introduced and examined for this purpose such as bacterial culturing, direct microscopy, immunodiagnostic tests, enzymatic method of bacterial identification, and deoxyribonucleic acid probe technology. These tests are not completely reliable. Furthermore, they are expensive and technique sensitive or need to be operated by a specialist, so their use in the clinical office or home is limited.

Screening of the antigenic property of *P. gingivalis* in producing antibodies gives a tremendous assistance to clinicians in understanding the condition of the periodontium and/or level of periodontitis.[9]

Limited resources and availability of diagnostic tests for dental diseases in developing countries have led to the use of inadequate treatment modalities. Hence, there is a need for developing a simple and more practical method as an aid in diagnosis and treatment of periodontal diseases.

This study focused on determining the quality and type of immunoglobulin G as the antibody raised against *P. gingivalis* based on the formation of ascites. Ascites fluid is a watery fluid that accumulates in the peritoneal cavity and is the result of antibodies produced in the peritoneal cavity in association with certain diseases, such as liver disease or congestive heart failure. Formation of such fluid after injection of antigens would indicate the production of antibodies in suspension form. After secretion of antibodies, they can be classified according to their type. This in turn would determine the method of collecting and processing the antibodies.

The aim of this study was to develop a different diagnostic screening test kit against *P. gingivalis* with the property to show antigenic property and level of raised antibodies. This kit is anticipated to be a practical and usable test kit for dental students, clinical instructors, dental researchers, dental manufacturers, and dental specialists.

### Methods

The study was an experimental one, and purposive sampling technique was used in the study. After obtaining the approval permission from the Institutional Animal Care and Use Committee of Centro Escolar University, the 6 Balb/c mice of 6–8 weeks old, were used as hosts for the production of the antibody.

Agar plates which contained a mixture of BHI agar, blood agar base and beef extraction were used for the culture of *P. gingivalis*. The microorganism was obtained from the vials of pure culture of lyophilized *P. gingivalis* (Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, 125 Ghawak_ro, Yuseong_gu, Daejeon, Korea, 305-806).

The vials were kept at the room temperature before their reviving procedure. *P. gingivalis* was then subcultured and preserved in the vials. Hemolysis test was done to check the growth of *P. gingivalis* on blood agar plates. Furthermore, cell count was done to compute the number of bacterial cells in the subculture test tube. The data obtained, were used to indicate cell density and to determine the number of required bacteria for immunization.

After culturing *P. gingivalis* in agar plates and incubating, antigens were extracted and injected into the peritoneal cavity of Balb/c mice.

The aim of antigenic injections was to measure the antibody response against them. The antigenic injections mimic the condition of periodontal disease and the raise of *P. gingivalis* in the body.

The mice were injected peritoneally with 0.20 ml of vehicles in Freund’s complete adjuvant three times. First and second immunizations were administration at 30-day intervals. Afterward, the third injection was done in 14 days. Four days later, samples from blood and spleen were obtained from the immunized mice for the measurement of the antibodies.

Four Balb/c male mice (7 weeks old) were chosen for multiple immunizations. 0.2 ml of the fluid containing $3 \times 10^7$ bacteria was extracted from the vials and used for the primary immunization. Then the cells were suspended in normal saline solution and heat inactivated. After that the test tubes were placed on Sonicator (Vortex Shaker), for 5–10 min at 8000 rpm and centrifuged for 15 min. Then the centrifuged suspension was injected into 2 of mice with a #25 gauge needle. The status of the health of the mice was monitored for the succeeding days. Other 2 mice were injected with 0.2 ml of the supernatant suspension.

For the second immunization, 0.2 ml of the heat inactivated and sonicated fluid containing *P. gingivalis* was injected into the same 2 mice that had been primary immunized 4 weeks ago, and 0.2 ml of the supernatant was injected into the mice that had primary immunization 4 weeks ago with the same suspension. Again, the mice were monitored for the succeeding days.

After 2 weeks, the same procedure was repeated for the third immunization as done in the second stage and the mice were monitored after the injection procedure.

After immunization, blood samples were obtained from the mice for the measurement of the antibodies. Serum antibody titer was determined by the enzyme-linked immunosorbent assay (ELISA) technique.
Later, the mice were euthanized and splenectomized for the performance of cell fusion procedure and measurement of possible antibody production.

**Results**

A minimum cell population of $1 \times 10^8$ cells is needed to produce an immune response in the form of an antibody in mouse; therefore, in the current study, *P. gingivalis* was grown in several tubes, subcultured and then harvested. Subsequently, the obtained cell density was determined by the aid of a spectrophotometer.

The number of *P. gingivalis* cells collected per vials ranged from $2.7 \times 10^7$ to $3.3 \times 10^7$ in subcultures with BHI as culturing media, with a total cell of $3 \times 108$ cells. Table 1 shows the amount of prepared *P. gingivalis* cells contained per vial and Table 2 illustrates average cell density, the average amount of cells, and optical density values per vial during culturing period.

This study confirmed that *P. gingivalis* could grow on Beef Extract, BHI Broth with Beef Extract and blood agar plate at $37^\circ C$ for 48 h. Beta hemolysis (complete hemolysis) was demonstrated on blood agar plate, which confirmed the cultural characteristics of *P. gingivalis* [Table 3].

3.0 $\times$ 10$^7$ cells were injected to the mice each time for the immunization sequences. Third immunization followed 2 weeks after the second one which took place 4 weeks after the initial immunization. None of the subjects showed ascites formation [Table 4].

Negative ascites formation further explained the characteristics of *P. gingivalis* as a membrane-bound microorganism which elicits IgG and IgE production. These antibodies are formed primarily in bone marrow, blood, and other organs and then mature in the spleen.

Y-shape pattern Ouchterlony immunodiffusion assay was used to identify the type of antibodies. Y-shape pattern (partial identity) indicates when the antibodies in the antiserum react with one of the antigens more than the others.

The spur is supposed to result from the determinants present in one antigen but lacking in the other antigen. The Y-shape pattern Ouchterlony immunodiffusion assay showed some circles, which were the walls and provided to act as containing space within the semisolid agar gel (medium) for the reagents used in the test (antigen or antibody in this research).

Partial identity reaction between *P. gingivalis* and the antibodies collected from the mouse 1 and 2 confirmed the unique reactivity between the antigen and antibody. It also showed the nonreactive precipitation lines between the *P. gingivalis* and the supernatant production from mouse 3 and 4. This was indicative of that there was no significant antigenic property present in the supernatant medium to elicit antibody production. After confirmation of the partial identity Ouchterlony immunodiffusion assay, the amount of protein (antibody) was quantified by ELISA technique. That was performed for determining the actual quantitative amount of antibody. This test quantified the amount of antibodies that could be harvested from Balb/c mice. This in turn can be used in antibody purification for further studies.

Finally, concentration of antibody production from the mice was determined [Table 5]. Based on ELISA readings, the acquired data using the Standard Optical Density and the average of the different samples, 81.5 µg/ml of protein was found in the sample collected from the sacrificed mice. Table 5 shows that 81.5 µg/ml antibody was sufficient enough to react with the cultured antigens which were injected into the mice.

The concentration of *P. gingivalis* that was harvested in the current study resulted in $3.0 \times 10^4$ cells. This cell count satisfied the number of cells needed for immunization. Positive growth in Beef extract, BHI enriched with beef extract and demonstration of β-hemolysis in blood agar plate confirmed the manifestations unique for *P. gingivalis*.
In this research, there was no ascites fluid formation in Balb/c mice after multiple immunizations. The Ouchterlony immunodiffusion assay showed partial identity for the antibody production. The concentration of antibody produced from Balb/c mice using ELIZA was 81.5 µg/ml.

**Discussion**

The oral cavity appears as an open ecosystem with a dynamic balance between the entrance of microorganisms, colonization modalities, and host defenses.\[18\]

The primary purpose of periodontal examination and evaluation is to identify and quantify the clinical signs and symptoms of present inflammation. This accurate judgment would lead to more definite treatment. Actually, clinical evaluation alone cannot be reliable in identifying the sites affected by the disease and does not provide the needed information on the etiology of the condition. It also does not report the patients’ susceptibility to the disease.\[1\]

Microbiological tests have the potential to support the diagnosis of different types of periodontal diseases and so serve as a guide for the diagnosis of disease etiology, activity, and progression. They also can have a role in determining the sites with a higher risk of active destruction. These tests can also be useful in monitoring the progress of the therapy and eradication of periodontopathic microorganisms.\[1\]

Comparing the laboratory diagnostic techniques, cultural techniques are more efficient to distinguish species, but they are too expensive.

Polymerase chain reaction (PCR) is a convenient technique that can detect low numbers of cells but lack the ability to provide quantitative data. Real-time PCR overcomes this limitation but is expensive and time-consuming.\[19\]

DNA probe test and/or ELISA antibodies can be reliable in detecting *P. gingivalis*, *Actinobacillus actinomycetemcomitans*, *Treponema denticula*, and *Bacteroides forsythus*.\[20\]

Both PCR and N-benzoyl-dl-arginine-b-naphthylamide (BANA) techniques are useful for detecting pathogenic bacteria that promote alveolar tissue destruction, but the former is more sensitive than the latter.\[19,21\]

BANA test is an effective technique for detecting the red complex pathogens, so it can be used for the diagnosis of chronic periodontitis. But since the specificity of the BANA test increases after scaling and root planning, there are some limitations for its posttherapy applications.\[22\]

Antibody-based techniques such as immunofluorescence and ELISA are very specific and can provide quantitative data.\[19\]

The latter technique was used in the current study. In this *in vivo* study, we aimed to determine the quantity of antibodies raised against *P. gingivalis* in Balb/c mice.

The presence of IgG as one of the earliest immunological responses to the infection has been confirmed in gingival crevicular fluid of the patients with periodontitis. This has been demonstrated, especially at the site of the infection.

There are promising results regarding the use of ELISA for the assessment of periodontal disease by measuring the antibody response to the different serotypes of periodontal pathogens in the serum.\[22,23\]

Some researchers have observed an association between *P. gingivalis* isolation and detection of immunoglobulins, such as specific IgG, especially IgG\(_1\) subclass. In the serum of adults with periodontitis IgG was the predominant Ab.\[24-29\]

Several other studies have reported higher antibody titers (IgG, IgM, and IgA) to *P. gingivalis* whole cells and outer membrane preparations in the sera from the healthy controls.\[30-33\]

The severity of periodontitis has been associated with an increase in IgG response to *P. gingivalis*.\[34,35\]

Cases with high antibody levels and variations in strain type have been reported in association with systemic conditions. Regarding serum IgG response in several cardiovascular attack and periodontal categories, it can be postulated that there is a relationship between periodontal disease and cardiovascular diseases. This provides further support for the role of immune response to *P. gingivalis* in the systemic health of individuals.\[36,37\]

Several investigators have attempted both active and passive immunization of nonhuman species and human to protect against periodontal disease using antibodies against *P. gingivalis*.\[38-43\]

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**Table 4: Number of injected cells, immunization sequences, and ascites formation results**

| Mouse number | Amount of injected cells | First immunization | Second immunization | Third immunization | Ascites formation |
|--------------|--------------------------|--------------------|---------------------|--------------------|------------------|
| 1            | 3.0×10^5                | February 27, 2012  | March 26, 2012      | April 9, 2012      | Negative         |
| 2            | 3.0×10^5                | February 29, 2012  | March 28, 2012      | April 11, 2012     | Negative         |
| 3            | 3.0×10^5                | March 2, 2012      | March 30, 2012      | April 13, 2012     | Negative         |
| 4            | 3.0×10^5                | March 2, 2012      | March 30, 2012      | April 13, 2012     | Negative         |

**Table 5: Concentration of antibody production**

| Sample | Raw OD | OD | Average | Dilution | Value |
|--------|--------|----|---------|----------|-------|
| 1      | 0.238  | 0.145 | 12.287  | 5120     | 62.9  |
| 2      | 0.052  | 0.082 | 4.886   | 20,480   | 100.1 |

Concentration of protein present: 81.5
In the current study, several tubes were grown, subcultured, and harvested to produce minimum cell population of *P. gingivalis* needed to provoke an immune response in the form of antibody formation in mice.

Since the host response to *P. gingivalis* through IgG evaluation from the early onset to later stages of periodontitis is evidential, it seems to be an ideal immunologic medium in screening these pathogenic microorganisms. This study aimed to screen the antigenic property of *P. gingivalis* in producing antibodies, and the results can be used as a reference for diagnosis and examination of periodontal pathogens in individuals before, during, and after periodontal treatment.

This study showed that 3.0 × 10⁸ cells of *P. gingivalis* is an appropriate count for stimulating the immunization in Balb/c mice and the subsequent amount of antibody (IgG) production was 81.5 µg/ml. The antigenic injections may mimic the condition of periodontal disease and the raise of *P. gingivalis* in the human.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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