

Multiserotype Enzyme-Linked Immunosorbent Assay using Microtiter Cliniplate

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The aim of the study was to measure serum immunoglobulin G class antibodies against *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* by an enzyme-linked immunosorbent assay (ELISA) in which mixtures of several serotypes of the pathogens were used as antigens to avoid biasing of the results in favor of a particular strain. Based on our results, we consider the serotype mixture ELISA to be a suitable tool to assess the periodontal status from serum samples in epidemiological studies.

Introduction

Periodontal diseases are characterized by inflammatory changes in the periodontium caused by bacterial infections. Inflammation may lead to destruction of the tooth-supporting tissues and eventually to tooth loss. The finding that periodontitis is a plausible risk factor for cardiovascular diseases, rheumatoid arthritis, and premature birth adds a new perspective to the importance of oral health. The increased bacterial burden in the inflamed periodontal pockets may often lead to the presence of oral bacteria and their components in the systemic circulation.

The aim of the study was to measure serum immunoglobulin G (IgG) class antibody responses against *A. actinomycetemcomitans* and *P. gingivalis* by an ELISA in which mixtures of several serotypes of the pathogens were used as antigens. The immunoassay was designed to be used as a serological marker of periodontitis in large epidemiological studies in which no clinical or radiographic information on the periodontal status of the subjects is available.

Material and Methods

Study subjects

Serum samples from 90 subjects were included in the study. Out of these, 35 samples were from patients with diagnosed periodontitis (referred to simply as “patients”). Ten samples were from controls with clinically healthy



periodontal tissues (referred to as “healthy controls”) with no periodontal attachment loss. The third group comprised 45 samples from randomly selected apparently healthy volunteers (referred to herein as “random controls”) who worked at a research institute in Helsinki, Finland.

ELISA assay

Serum IgG antibodies against *A. actinomycetemcomitans* and *P. gingivalis* were determined by an ELISA using mixtures of six strains of *A. actinomycetemcomitans* and three strains of *P. gingivalis* as antigens. The strains were ATCC 29523, ATCC 43718, ATCC 33384, IDH 781, IDH 1705, and C59A for *A. actinomycetemcomitans*, representing serotypes a, b, c, d, and e and one nonserotypeable (x) strain, and ATCC 33277, W50, and OMGS 434 for *P. gingivalis*, representing serotypes a, b, and c, respectively.

A. actinomycetemcomitans strains were grown on supplemented Brucella agar plates (containing 5% horse blood, hemin [5 µg/ml], vitamin K1 [100 mg/ml], and Brucella agar) and incubated in an atmosphere of 5% CO₂ at 37°C for 3 days. The cultures were transferred into Todd-Hewitt broth (3% TH, 1% yeast extract), where they were further grown for 2 days (1 day in 5 ml and 1 day in 200 ml) under the conditions mentioned above. After removing the broth by centrifugation at 5,500 × g at room temperature for 15 min, the bacteria were washed with phosphate-buffered saline (PBS) (10 mM phosphate [pH 7.4], 150 mM NaCl). *P. gingivalis* strains were cultured on supplemented Brucella agar plates anaerobically for 5 to 6 days. The purity of the cultures was checked by colony morphology and Gram staining.

All strains to be used as antigens in the ELISA were fixed in 0.5% formalin–PBS overnight at 4°C and washed three times with PBS⁽¹⁾. The density of the bacterial suspensions in the antigen buffer (PBS, 0.5% bovine serum albumin, 0.05% Tween 20) was adjusted to give an absorbance of 0.15 at 580 nm. For serotype mixture ELISA, equal

volumes of the six *A. actinomycetemcomitans* or three *P. gingivalis* strains were mixed and used to coat microtiter plates (Microtiter Cliniplate; Thermo Fisher Scientific).

The unspecific binding was blocked by 5% bovine serum albumin in PBS at room temperature for 30 min. Four dilutions of the serum samples in duplicate were added on the plate and incubated for 2 h at room temperature. The dilutions used were 1/500, 1/1,500, 1/4,500, and 1/13,500 for *A. actinomycetemcomitans* and 1/100, 1/400, 1/1,600, and 1/6,400 for *P. gingivalis*.

The bound antibodies were visualized using horseradish peroxidase-coupled goat anti-human IgG (Sigma) diluted 1/20,000 and measured spectrophotometrically at 492 nm. Unspecific binding was monitored by blank wells which contained no sample, and four dilutions of a “low” (pool of core blood) and a “high” (a high-level responding) control serum in duplicate were measured on each plate. The IgG levels are expressed as the areas (square millimeters) under the dose-response curves (AUCs) of the test and reference sera as suggested earlier by Sedgwick et al. (2).

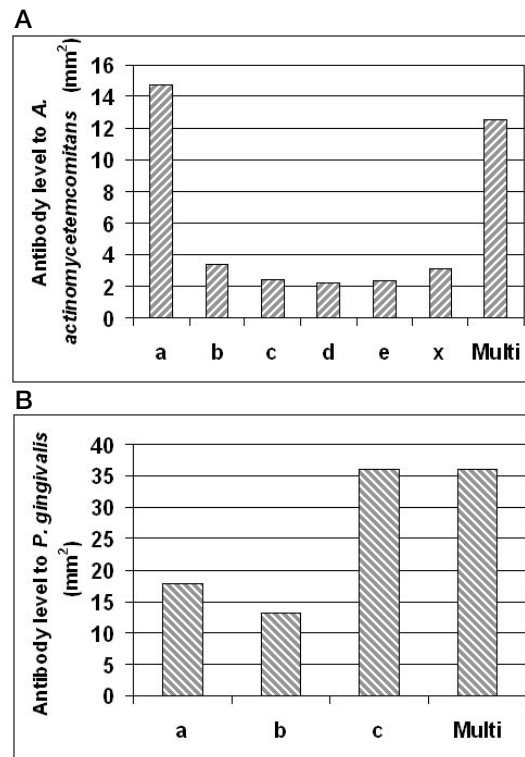


Figure 1. Serum serotype-specific antibody levels to periodontal pathogens. Serum IgG-class antibody levels to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* were measured from a patient with periodontitis and culture-positive for both pathogens. The antigens used in the assays were A) *A. actinomycetemcomitans* strains representing serotype, a, b, c, d, e, and a non-serotypeable strain (x) separately and in combination (Multi), and B) *P. gingivalis* strains representing serotypes a, b, and c separately and in combination (Multi).

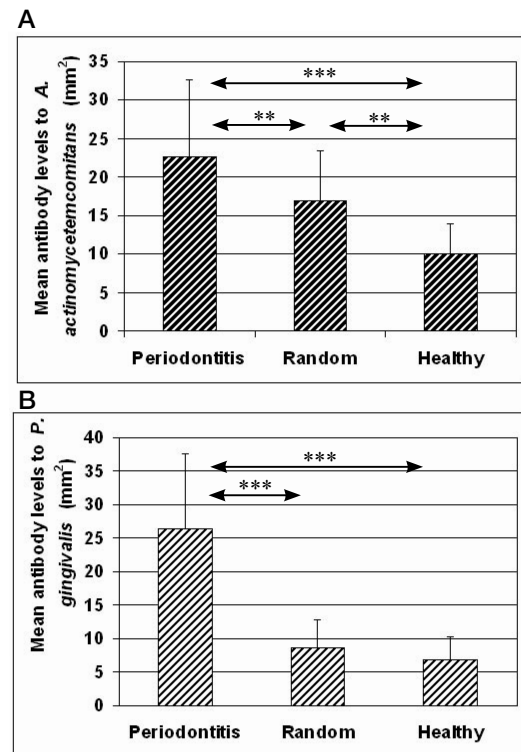


Figure 2. Mean antibody levels to periodontal pathogens as determined by multiserotype-ELISA. Serum samples were collected from 35 patients with clinically diagnosed periodontitis, 10 periodontally healthy subjects, and 45 randomly chosen volunteers. The IgG-class antibody levels to *A. actinomycetemcomitans* (A) and *P. gingivalis* (B) were determined by multiserotype-ELISA. Mean results and SD are shown, ** $p < 0.01$, *** $p < 0.001$ between the groups indicated.

Results

In the multiserotype ELISA the inter-assay coefficients of variation (CV%) were 4.1 and 8.1, and the intra-assay reproducibility (SD) between duplicate assays was 0.20 and 0.15 for *A. actinomycetemcomitans* and *P. gingivalis*, respectively. The detection limits for *A. actinomycetemcomitans* and *P. gingivalis* were 0.53 and 0.65 mm², respectively.

To analyze how individual serum IgG levels differ in serotype-specific and serotype mixture ELISAs, serum samples were evaluated by both types of assays. As an example from these analyses, Fig. 1 shows the serum serotype-specific antibody levels to periodontal pathogens measured from a patient with periodontitis and culture-positive for both pathogens.

In the serotype mixture ELISA the IgG level (mean \pm SD) of all patients with periodontitis (n = 35) against *A. actinomycetemcomitans* was 22.60 \pm 9.94 mm² (Fig. 2). The mean AUCs of healthy controls and random controls were 9.99 \pm 3.92 mm² and 16.85 \pm 6.67 mm², respectively. When the titers of all patients were analyzed as one group, the results were significantly higher than those of healthy controls (P < 0.001) or random controls (P < 0.01). In the *P. gingivalis* ELISA the IgG level of all patients (mean \pm SD) was 26.72 \pm 11.13 mm², and those of random controls and healthy controls were 8.51 \pm 4.23 mm² and 6.90 mm² \pm 3.38, respectively. The patients had higher antibody levels than random controls (P < 0.001) and healthy controls (P < 0.001), which in turn did not differ significantly from each other (P = 0.21).

Discussion

Like this study demonstrates, the serotype mixture ELISA distinguishes as positive also the subjects whose antibody response against only one serotype is clearly dominant. Therefore, serotype mixture ELISA helps to minimize the risk of false negative results which are easily obtained, if only one strain representing one serotype is used as an antigen. Despite of the low number of study subjects included, the mean serum antibody levels to both pathogens differed significantly between the periodontitis patients and periodontally healthy subjects.

To calculate the results obtained by the ELISA, we recruited an earlier-devised method, which is currently only seldom used (2). Although serial dilutions are

laborious, the AUC gives a result, which is linearly proportional to the antibody concentration (2). In this way the results are as reliable as possible without a proper affinity-purified standard antibody. By exploring dilution curves, one also gets an idea about the affinity of the antibodies measured, which vary individually.

By calculating the AUCs, even small differences in the antibody levels are found, because the scale ranges between 0.65 and about 55 mm².

In immunological analyses the most essential point is the choice of antigen. For both *A. actinomycetemcomitans* and *P. gingivalis*, several serotypes have now been designated, and the individual antibody response depends on the amount and virulence of the infecting strains. There is still some disagreement on the localization and immunodominant nature of the antigen for both these species, which justifies the use of whole cells as antigens in the assay.

Based on our results, we consider the serotype mixture ELISA to be a suitable tool to assess the periodontal status from serum samples in epidemiological studies. The assay is particularly valuable in the study of the association between periodontal infections and systemic health.

References

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