Standardization of an enzyme-linked immunosorbent assay to detect anti-*Porphyromonas gingivalis*-peptidylarginine-deiminase antibodies

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• Conflicts of interest: none declared.

ABSTRACT

Objective: our aim was to standardize an indirect ELISA immunoenzymatic assay for the detection of serum anti-PPAD antibodies in adolescents with juvenile systemic lupus erythematosus (JSLE).

Material and Methods: serum of 50 patients, 25 with juvenile systemic lupus erythematosus (group A) (mean age 16.1 ± 1.6 years) and 25 healthy subjects (mean age 15.2 years ± 2.3 years) (group B) were analyzed. The method for anti-PPAD antibodies detection was chronologically performed by addition of: (1) diluted PPAD peptide (sensitization step), (2) bovine serum albumin (BSA) (blocking step), (3) serum from each participating patient and (4) diluted biotinylated monoclonal antibody anti-human IgG. The final steps consisted of the addition of (5) horseradish peroxidase-conjugated streptavidin protein, (6) hydrogen peroxide and (7) a chromogenic substance. Finally, (8) a stop solution was added to stop the reaction and the plaque was read in a spectrophotometric reader at 450nm. Results: serum anti-PPAD antibodies were detected in both groups without any difference between them. Conclusion: the detection of serum anti-PPAD antibodies was achieved in adolescents with juvenile systemic lupus erythematosus using the methodology proposed by the study.

Keywords: ELISA; *Porphyromonas gingivalis*; Anti-PPAD; Rheumatoid arthritis; Systemic lupus erythematosus.

Introduction

Periodontal infection with *Porphyromonas gingivalis* (*Pg*), a key pathogen in the development of periodontal disease, induces the production of anti-*Porphyromonas gingivalis* peptidylarginine deiminase (anti-PPAD) antibodies, which dramatically alter the structure and function of proteins, thus interfering in both cell signaling and immune response.1

Several studies point to a possible impact of periodontal infection by *Pg* on the initiation of autoimmunity in patients with rheumatoid arthritis (RA).2,3

However, just continuous studies that detected the presence of anti-PPAD antibodies in patients with RA did not use a standardized methodology, presenting mainly the following differences in: (a) dilution and amount of PPAD peptide previously inserted in the microtiter plate, (b) incubation temperature, (c) number of intermediate washes, (d) substances used in blocking steps, (e) the dilution of the sample, (f) the dilution liquid, (g) incubation time of the sample, and finally (h) dilution of the secondary antibody.6,9-11

Due to these variability of methodologies our aim was to standardize an indirect ELISA immunoenzymatic assay for the detection of serum anti-PPAD antibodies in adolescents with juvenile systemic lupus erythematosus (JSLE).

Material and Methods

Subjects and Sample Collection

The study consisted of twenty-five patients with JSLE (mean age 16.1 ± 1.6 years) (group A) and 25 healthy subjects (mean age 15.2 ± 2.3 years) (group B) undergoing treatment or medical follow-up at the Adolescent Health Studies Center (NESA) of Pedro Ernesto Hospital, Rio de Janeiro, Brazil. The study protocol was approved by the Research Ethics Committee of the Pedro Ernesto University Hospital (CAAE 380.686/2013, amendment 2.284.225/2017).

For the study, 20ml of blood was collected by peripheral venous puncture from all patients, and transferred to two blood tubes (BD Vacutainer®, REF 367820, BD Biosciences, San Jose, United States). The collection was conducted by a nursing who kept the samples refrigerated for 30 minutes, then centrifuged for 10 minutes at 1500rpm to separate the serum (Centrifugal CT-6000®, Cientec). Then, the serum was stored in 2ml microtubes and kept under refrigeration at -70°C until analysis.

Elisa

1.Antigen

For the detection of serum anti-PPAD antibodies the PPAD peptide (CLGTDALHC-Cit-THEVADKGC) (0.1µg/ml) (Aminotech Pesquisa e Desenvolvimento, São Paulo, Brazil) and a commercially available kit (Monoclonal Anti-human IgG (Fc-specific)-Biotin antibody: Sigma-Aldrich, Saint Louis, United States) were used.

2.Technical Procedure

As a first step, named sensitization step, a microtiter
plate was coated with 100μl of PPAD peptide (CLGTDAL- HC-Cit- THEVADKGC) (0.1μg/ml) (Aminotech Pesquisa e Desenvolvimento, São Paulo, Brazil) diluted in 0.1 M of coating buffer solution (BD OptEIA™, BD Biosciences, San Jose, United States) (pH 9.5) and maintained overnight in an oven (REF Q316M4 - Quimis Aparelhos Científicos LTDA, São Paulo, Brazil) at 37°C. The plate was protected with sealing film and aluminum foil to prevent light sensitization. Excess of peptide was removed from the wells by pouring the plate and washing three times (1 minute for each wash) with 200μl PBS containing Tween 20 (0.05%) (Sigma-Aldrich, Saint Louis, United States).

The plate was blocked with 150μl of bovine serum albumin solution (BSA) (1%) (PBS + Tween 20 0.05% + BSA 1%), so that the spaces between the peptides were filled avoiding nonspecific binding of the antibody. The plate was protected with sealing film and aluminum foil and kept at room temperature for 2 hours. The excess of blocking solution was removed by pouring the plate and tapping firmly onto an absorbent paper.

In the following step, the plate was incubated with 100μl of serum at 1:500 dilution in PBS (serial dilution), and after 30 minutes at room temperature, each well was washed three times (1 minute for each wash) with 200μl of PBS and 0.05% Tween 20.

The next step corresponded to addition of 100μl of diluted specific Fc Biotinylated Human Anti-IgG monoclonal antibody (1:1000) (Sigma-Aldrich, Saint Louis, United States), plate protection and incubation for 1 hour at room temperature. After removal of the excess, the wells were washed as described before.

In the penultimate step, 100μl of the streptavidin protein conjugated with diluted horseradish peroxidase enzyme (HRP) (BD Biosciences, San Jose, United States) (1:1000 were added to the plate). The streptavidin-biotin-HRP complex was formed after binding of streptavidin to biotin present in the biotinylated monoclonal antibody. The plate was incubated for 1 hour in an oven at 37°C (REF Q316M4 - Quimis Aparelhos Científicos LTDA, São Paulo, Brazil) and then repeated washed as previously described.

Finally, 100μl of hydrogen peroxide solution (Reagent A, BD OptEIA™ (BD Biosciences, San Jose, United States)), and 3,3′,5,5′ tetramethylbenzidine chromogenous substance (TMB) (Reagent B, BD OptEIA™ BD Biosciences, San Jose, United States) were added to each well. After 2 minutes, the interaction of HRP with hydrogen peroxide acted on the chromogenic substrate (TMB) and a turquoise-blue compound formed. In order to stop the reaction, 100μl of phosphoric acid solution was added (Stop Solution, BD OptEIA™ BD Biosciences, San Jose, United States), which changed the color yellow. The intensity of this staining varies according to the amount of antibody present.

The summary of all steps of the anti-PPAD antibody detection method proposed can be visualized in Figure 1.
Figure 1. Indirect ELISA for serum detection of anti-PPAD antibodies

Subtitle:
Peptide: PPAD (CLGTDALHC-Cit-THEVADKGC) (0.1µg/ml) (Aminotech Pesquisa e Desenvolvimento, São Paulo, Brazil) diluted in 0.1M coating buffer solution (BD OptEIA®), BD Biosciences, San Jose, United States) (pH 9.5).
Blockade: bovine serum albumin solution (BSA) (1%) (PBS + Tween 20 0.05% + BSA 1%).
Anti-PPAD: Anti-Porphyromonas gingivalis peptidylarginine deiminase antibody present in 100µl of serum diluted in PBS (1:500).
Anti-IgG: monoclonal antibody Anti-Immunoglobulin G Human Biotinylated Fc specific diluted (1:1000) (Sigma-Aldrich, Saint Louis, United States).
ESTR-HRP: diluted protein streptavidin conjugated with horseradish peroxidase enzyme (HRP) (BD Biosciences, San Jose, United States) (1:1000).
Phosphoric acid: stop solution (Stop Solution, BD OptEIA®) (BD Biosciences, San Jose, United States).

3. Cutoff
Prior to peptide sensitization, two wells received phosphate buffered saline only (PBS pure - Sigma-Aldrich, Saint Louis, United States - pH 7.5) instead of serum (true negative control). To determine the serum levels of anti-PPAD antibodies in the study patients, the mean absorbance scores of duplicate wells were subtracted from the mean values found in the wells considered true negative control.

4. Spectrophotometric Reading
The spectrophotometric reading was performed immediately after the reaction was stopped, at a wavelength of 450nm (Microplate reader, TP-reader® - Thermo Plate, China). Serum levels of anti-PPAD antibodies were expressed in ELISA units (EU) and the analyzes of the samples were performed in duplicate.

Statistical Analyses
Numerical variables were examined for normality by the Kolmogorov-Smirnov test and the comparisons between groups were performed using the U Mann-Whitney test.

Results
The proposed methodology was able to detect anti-PPAD antibodies in patients with JSLE (group A) and healthy patients (group B). The comparison of these levels showed no significant difference between groups (p = 0.9) (Table 1). The mean age was 16.1 (± 1.6) years in group A and 15.2 (± 2.3) years in group B with no statistically significant difference (p = 0.195). The similarity found in serum anti-PPAD antibody levels in can be explained by the presence of P. gingivalis in both groups (data not shown).
Conclusion
The detection of serum anti-PPAD antibodies was achieved in adolescents with juvenile systemic lupus erythematosus using the methodology proposed by the study.

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References

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