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

Juliana Cristina Carlos,[1] Manuela Rubim Camara Sete,[1] Ronaldo Lira Júnior,[1] Bárbara Coelho,[1] Carlos Marcelo da Silva Figueredo[2]

[1]School of Dentistry, State University of Rio de Janeiro (UERJ), Rio de Janeiro, RJ, Brazil

[2]School of Dentistry and Oral Health, Griffith University, Queensland, Australia

• 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 (AR).[2-9]

However, just continuos 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.

PLATE

PEPTIDE BLOCKADE ANTI-PPAD ANTI-IgG STR-HRP REAGENTS H₂O₂ and TMB PHOSPHORIC ACID
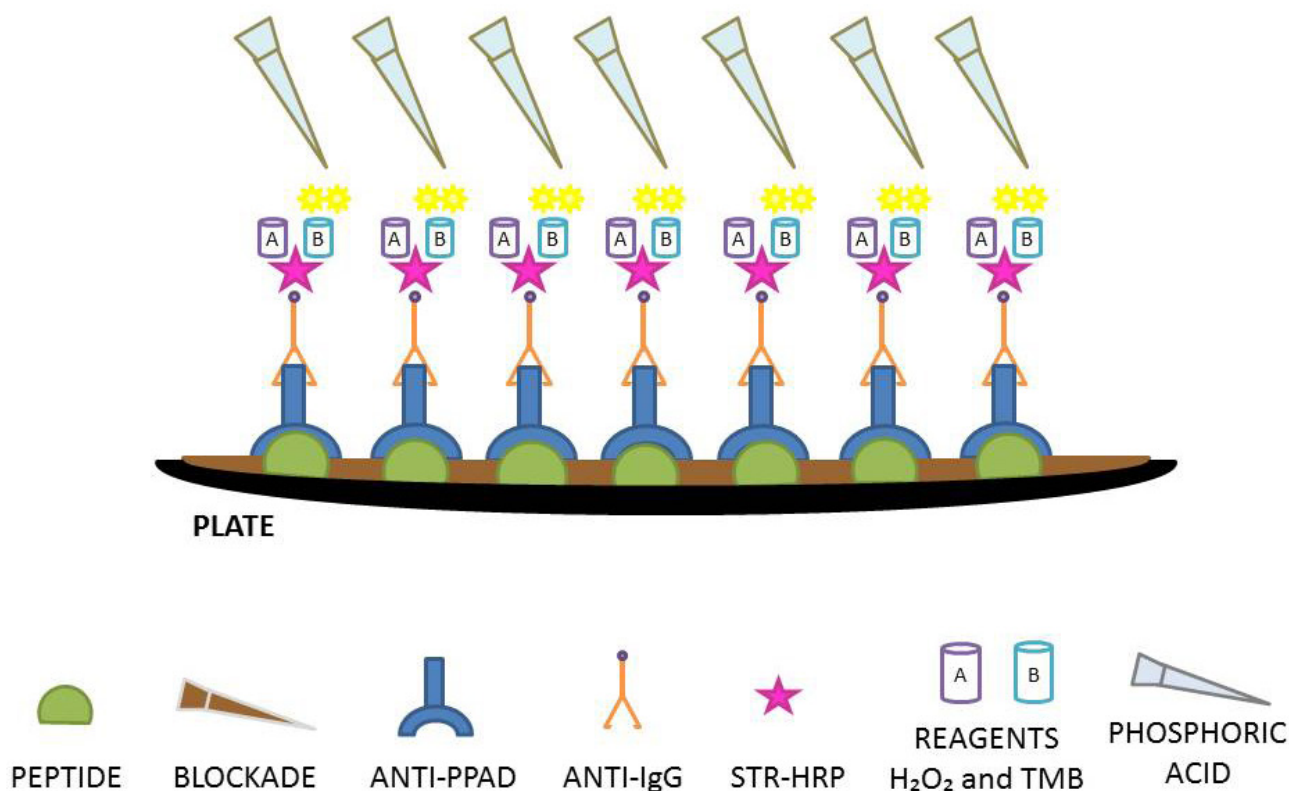
Figure 1. Indirect ELISA for serum detection of anti-PPAD antibodies
Subtitle:
Peptide: PPAD (CLGTDALHC-Cit-THEVADKGC) (0.1μg/ml) (Aminotech Pesquisa e Desenvolvimento, São Paulo, Brazil) diluted in 0.1M coating buffer solution (BD OptEIA™, BD Biosciences, San Jose, United States) (pH 9.5).
Blockade: 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).
Reagents A e B: solution composed of hydrogen peroxide (H_2O_2) and chromogenic substance 3,3', 5,5' tetramethylbenzidine (TMB) (BD OptEIATM (BD Biosciences, San Jose, United States)).
Phosphoric acid: stop solution (Stop Solution, BD OptEIATM (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 beteween groups were performed unsing the U Mann-Whitney test (*IBM SPSS Statistical Package for the Social Sciences* 21).

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).

Table 1. Serum levels of anti-PPAD antibodies from patients and controls.

ELISA	Group A (n=25)	Group B (n=25)
Mean	0.56	0.62
Median	0.53	0.56
Standard deviation	0.24	0.38
Minimum	0.19	0.17
Maximum	1.11	1.22

U de Mann-Whitney test.
Group A: patients with juvenile systemic lupus erythematosus; Group B: healthy patients.

Discussion

The identification of anti-PPAD antibodies has also been demonstrated by other studies, but without a padronization. The greatest variability between the proposed methodology and the protocols used previously concerns the step of sensitizing the microtiter plate. The PPAD peptide was maintained in the plate overnight in a temperature of 4°C in two studies, but using different dilutions (5μg /ml and 10μg/ml respectively)[9,10], and at a temperature of 37°C in two other studies, however at the concentration of 0.1 μg /ml or 100 ng.[6,11]

The blocking substance used to prevent non-specific antibody binding was also a diverging point between previous studies, ranging from bovine serum albumin in two studies,[9,10] to the same used in the protocol of the present study (0.05% PBS solution and Tween 3%).[11] The generic term "blocking buffer" was also cited with reference to the blocking substance, but without specification of the substance used for this purpose.[6]

Another difference found between the studies was the dilution of the sample and its respective dilution solution, ranging from RIA buffer (10 mM Tris + 1% BSA + 350 mM NaCl + 1% Triton-X + 0.5% Nadeoxycholate + 0.1% SDS)[9,10] to PBS with 1% Tween[11]. Not always the solvent used for dilution of the samples was disclosed in the methodology.[6] The variability in the sample's dilutions used can be justified by the standard curve with absorbance and concentration values for serial dilution performed in each of the studies.

The dilution of the anti-IgG monoclonal antibody and its respective solvent also differed between methodologies (1:1000 and 1:3000 RIA buffer[9,10] and 1:1000 in PBS + Tween 1%[6]), as well as the number of intermediate washes, which ranged from 4 times[9] to 3 times for 10 minutes each wash.[6]

We acknowledge that the weak point of the study is the lack of sensitivity, specificity and reliability tests that could guarantee the reproducibility of the technique suggested for the detection of anti-PPAD antibodies.

Conclusion

The detection of serum anti-PPAD antibodies was achieved in adolescents with juvenile systemic lupus erythematosus using the methodology proposed by the study.

Acknowledgments

References

1. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. Nat Rev Immunol. 2015;15(1):30-44.

2. Maresz KJ, Hellvard A, Sroka A, Adamowicz K, Bielecka E, Koziel J, et al. Porphyromonas gingivalis facilitates the development and progression of destructive arthritis through its unique bacterial peptidylarginine deiminase (PAD). PLoS Pathog. 2013;9(9):e1003627.

3. Wegner N, Wait R, Sroka A, Eick S, Nguyen KA, Lundberg K, et al. Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis. Arthritis Rheumatol. 2010;62(9):2662-72.

4. Mikuls TR, Payne JB, Yu F, Thiele GM, Reynolds RJ, Cannon GW, et al. Periodontitis and Porphyromonas gingivalis in Patients with Rheumatoid Arthritis. Arthritis Rheumatol. (Hoboken, NJ). 2014;66(5):1090-100.

5. Koziel J, Mydel P, Potempa J. The Link Between Periodontal Disease and Rheumatoid Arthritis: An Updated Review. Curr Rheumatol Rep. 2014;16(3):408.

6. Shimada A, Kobayashi T, Ito S, Okada M, Murasawa A, Nakazono K, et al. Expression of anti-Porphyromonas gingivalis peptidylarginine deiminase immunoglobulin G and peptidylarginine deiminase-4 in patients with rheumatoid arthritis and periodontitis. J Periodontal Res. 2016;51(1):103-11.

7. Montgomery AB, Kopec J, Shrestha L, Thezenas ML, Burgess-Brown NA, Fischer R, et al. Crystal structure of Porphyromonas gingivalis peptidylarginine deiminase: implications for autoimmunity in rheumatoid arthritis. Ann Rheum Dis. 2016;75(6):1255-61.

8. Lundberg K, Wegner N, Yucel-Lindberg T, Venables PJ. Periodontitis in RA-the citrullinated enolase connection. Nat Rev Rheumatol. 2010;6(12):727-30.

9. Quirke AM, Lugli EB, Wegner N, Hamilton BC, Charles P, Chowdhury M, et al. Heightened immune response to autocitrullinated Porphyromonas gingivalis peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis. Ann Rheum Dis. 2014;73(1):263-9.

10. Fisher BA, Cartwright AJ, Quirke AM, de Pablo P, Romaguera D, Panico S, et al. Smoking, Porphyromonas gingivalis and the immune response to citrullinated autoantigens before the clinical onset of rheumatoid arthritis in a Southern European nested case-control study. BMC Musculoskelet Disord. 2015;16:331.

11. Konig MF, Paracha AS, Moni M, Bingham CO, Andrade F. Defining the role of Porphyromonas gingivalis peptidylarginine deiminase (PPAD) in rheumatoid arthritis through the study of PPAD biology. Ann Rheum Dis. 2015;74(11):2054-61.

Mini Curriculum and Author's Contribution

1. Juliana Cristina Carlos – DDS; MSc. Contribution: effective scientific and intellectual participation for the study; writing of the manuscript. ORCID: 0000-0002-0051-5392

2. Manuela Rubim Camara Sete – DDS; PhD. Contribution: effective scientific and intellectual participation for the study; critical review. ORCID: 0000-0001-5538-947X

3. Ronaldo Lira Júnior – DDS; PhD. Contribution: effective scientific and intellectual participation for the study; critical review. ORCID: 0000-0003-0297-5138

4. Bárbara Coelho – DDS and MSc. ORCID: 0000-0001-9501-3944

5. Carlos Marcelo da Silva Figueredo – DDS; PhD. Contribution: effective scientific and intellectual participation for the study; critical review and final approval. ORCID: 0000-0003-0222-9231

Corresponding Author
Juliana Cristina Carlos
E-mail: jcc_ufrj@yahoo.com.br