ANTIBODY RESPONSE TO A CHROMATOGRAPHIC FRACTION OF *Porphyromonas gingivalis* AND ITS CORRELATION WITH PERIODONTAL STATUS

RESPOSTA DO ANTICORPO PARA A FRAÇÃO CROMATOGRÁFICA DE Porphyromonas gingivalis E SUA CORRELAÇÃO COM O NÍVEL PERIODONTAL

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SUMMARY

Porphyromonas gingivalis *has been strongly associated to periodontal diseases severity, and elicits humoral and cellular host response.* ***Objective:*** *To correlate the IgA, IgG and IgG subclasses serum levels against a chromatographic fraction from* ***Porphyromonas gingivalis*** *ATCC33277 extract and the clinical periodontal parameter.* ***Material and Methods:*** *Periodontitis (29) and healthy control (26) subjects were evaluated according to probing depth, bleeding on probing, and clinical attachment level measurements.* ***Porphyromonas gingivalis*** *extract was fractionated by ion exchange chromatography and the humoral response against the fraction IV was assayed by enzyme linked immunosorbent assay.* ***Results and Conclusion:*** *Percentage of sites showing bleeding on probing (criterion 1) was significantly correlated only with IgG2 serum levels ($r = 0,385$; $p < 0,05$). Total IgG and IgG2 were significantly correlated with percentage of sites with clinical attachment level (CAL) ≥ 3 mm (criterion 2) ($r = 0,428$; $p < 0,05$ and $r = 0,510$; $p < 0,01$, respectively), CAL ≥ 5 mm (criterion 3) ($r = 0,499$ and $r = 0,518$, respectively; $p < 0,01$) and percentage of sites with CAL ≥ 3 mm associated to probing depth ≥ 4 mm and bleeding on probing at the same site (criterion 4) ($r = 0,607$; $p < 0,001$ and $r = 0,487$; $p < 0,01$, respectively). A statisticaly significant positive correlation was observed between IgGA and IgG1 levels and criterion 4 ($r = 0,339$ and $r = 0,345$, respectively; $p < 0,05$), and between IgG3 levels and criterion 3 ($p < 0,05$; $r = 0,370$). These results indicate that the more accurate is the diagnosis criterion employed in the periodontal disease determination, the higher are the serum levels of immunoglobulins.*

UNITERMS: *immunoglobulins; antibody; periodontal disease; probing depth; clinical attachment level; bleeding on probing.*

RESUMO

Porphyromonas gingivalis tem sido fotemente associado à gravidade das doenças periodontais e estimula respostas celulares e humorais no hospedeiro. **Objetivo:** Correlacionar os níveis de IgA, IgG e subclasses de IgG contra uma fração cromatográfica do extrato de *Porphyromonas gingivalis* ATCC33277 extract e descritores clínicos periodontais. **Material e**

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Métodos: Indivíduos com periodontite (29) e com saúde periodontal (26) foram avaliados de acordo com as medidas de profundidade de sondagem, sangramento à sondagem e nível de inserção clínica. O extrato de *Porphyromonas gingivalis* foi fracionado por cromatografia de troca iônica e a resposta humoral contra a fração IV foi avaliada por "enzyme linked immunosorbent assay". **Resultados e Conclusão:** O percentual de sítios com sangramento à sondagem (critério 1) estava correlacionado significativamente com os níveis séricos de IgG2 ($r = 0,385$; $p < 0,05$). Os níveis de IgG total e IgG2 correlacionaram-se significativamente com o percentual de sítios com nível de inserção clínica (NIC) ≥ 3 mm (critério 2) ($r = 0,428$; $p < 0,05$ e $r = 0,510$; $p < 0,01$, respectivamente), NIC ≥ 5 mm (critérion 3) ($r = 0,499$ e $r = 0,518$, respectivamente; $p < 0,01$) e percentual de sítios com NIC ≥ 3 mm associado a profundidade de sondagem ≥ 4 mm e sangramento à sondagem no mesmo sítio (criterion 4) ($r = 0,607$; $p < 0,001$ e $r = 0,487$; $p < 0,01$, respectivamente). Foi observada uma correlação positiva estatistivamente significante entre os níveis de IgGA e IgG1 e o critério 4 ($r = 0,339$ e $r = 0,345$, respectivamente; $p < 0,05$), e entre os níveis de IgG3 e o critério 3 ($p < 0,05$; $r = 0,370$). Estes resultados indicam que quanto mais acurado for o critério de diagnóstico empregado para a determinação da doença periodontal, maiores os níveis séricos de imunoglobulinas.

UNITERMOS: imunoglobulinas; anticorpo; doença periodontal; profundidade de sondagem; nível de inserção clínica; sangramento à sondagem.

INTRODUCTION

Immuno-inflamatory response to Gram negative bacterial pathogens that inhabit the subgingival biofilm, in a susceptible host, can result in soft- and hard- tissues destruction. *Porphyromonas gingivalis* (*Pg*) is one of the most etiologically important agents associated with periodontal diseases[1] and play an important role in the severity of periodontal destruction, mainly in coinfection with other bacteria.[2,3] *Pg* produces a number of virulence factors such as arginine- and lysine-specific cysteine proteinases (RGP and kgP, respectivelly),[4] lipopolysaccharides (LPS),[5] and fimbriae[6] that rise humoral and cellular host response.

As regards the humoral response, various studies have demonstrated that patients with periodontitis present high antibody titers.[7-11] Kojima, Yano and Ishikawa[12] suggest a direct relationship between the serum anti-*P. gingivalis* IgG levels and subgingival *P. gingivalis* colonization; however, the functional capabilities of IgG antibodies may vary among the various types of periodontitis patients. Interestingly, the serum antibody reactivity to *Porphyromonas gingivalis* lipopolysaccharides (LPS) by western blotting analysis seems to correlate particularly well to the clinical parameters.[13] In addition, evaluation of serum IgG1 and IgG2 antibody responses of periodontitis patients to protein and carbohydrate-rich antigens of *P. gingivalis* showed that both IgG1 and IgG2 antibodies recognized a dominant antigen of 47 kDa, probably Arg-gingipain.[14] In this perspective, the aim of this stuy is to investigate the role of isotypes of antibodies reactive to Pg in periodontal status.

MATERIAL AND METHODS

Fifty-five subjects (mean age 38.26 years) from the Dental Ambulatory of Feira de Santana State University, diagnosed as having moderate or severe chronic periodontitis (CP, n = 29), or being periodontally healthy (HC, n = 26), were selected for inclusion in the study. Periodontal examination included bleeding on probing (BOP), clinical attachment level (CAL) and probing depth (PD) at six sites for each tooth, measured by a periodontologist using a Williams calibrated periodontal probe (Trinity, SP). Study volunteers signed the informed consent form. The research was approved by the Feira de Santana State University Ethics Committee (Protocol nº 007/2003).

Subjects with any of the following conditions were excluded from the study: systemic antibiotic use in the previous 6 months, systemic anti-inflammatory use in the previous 2 months, systemic disease, and pregnancy. The evaluated subjects were non-smokers and not habitual alcohol drinkers. The patients examined in this study had never had any previous periodontal treatment. There was no statistical difference between women and men.

Disease classification

Individuals who had 4 or more teeth showing one or more sites with probing depth 4 mm or higher, and with clinical attachment loss 3 mm or higher at the same site, were diagnosed as having periodontitis, according to López et al.[15] The chronic character of disease was based on the American Academy of Periodontology[16] criteria. Periodontal status of each subject was evaluated according to the following criteria: Criterion 1 – percentage of sites with BOP; Criterion 2 – percentage of sites with CAL ≥ 3 mm; Criterion 3 – percentage of sites with CAL ≥ 5 mm, and Criterion 4 – percentage of sites with CAL ≥ 3 mm, PD ≥ 4 mm and BOP at the same site. Individuals with no clinical attachment loss and bleeding on probing in less than 25% of the sites were considered healthy.

Antigen preparation

P. gingivalis ATCC33277 strain was grown in Brucella broth supplemented with 0.5% yeast extract, 0.1% hemin, 0.1% menadione and 0.05% L-cistein (Supplemented Brucella Broth – SBB) until late log phase, under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37ºC (Anaerobac, PROBAC, São Paulo, SP, BRA) and sonicated on ice at 60 Hz (ten cycles for one minute).

Six fractions were obtained from *P. gingivalis* sonicate by ion-exchange chromatography on MonoQ column (Pharmacia-Biotech, Sweden). A column was attached to a Pharmacia gradient FPLC (Fast Performance Liquid Chromatography) system and the effluent was monitored at A_{280} using a UV-M detector. Salt was removed by dialysis through distilled water at 4ºC, under agitation. Protein concentrations were determined by using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin (BSA) as standard.

After antigenicity assays, fraction IV was chosen for late studies because it showed a better protein recognition difference between the healthy and diseased subjects than the others fractions, as shown in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting.

SDS-Page and Western Blotting

Chromatographic fraction IV proteins from *Pg* were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli[17] and revealed by silver nitrate.[18] Western blotting on nitrocellulose was performed according to Towbin et al.[19] Nitrocellulose strips with 2 mm-wide (Millipore, USA) were incubated with 1:100 diluted subject sera in phosphate buffered saline-0,05%Tween 20 (PBS-T) containing 0.5% bovine serum albumine (BSA). After washing in PBS-T, strips were incubated with rabbit anti-human IgG conjugated with peroxidase (Sigma A-8792), diluted 1:500 for 1h, at 37ºC. The presence of the bands was revealed by 4-chloro-1-naphtol diluted 1:5 in Phosphate Buffered Saline (PBS), with 0.33 mL/mL H_2O_2. Adding water stopped the resulting reaction.

Serum antibody analysis

Sera from all individuals were obtained by venipuncture. Serum antibodies reactive to fraction IV were measured by enzyme-linked immunosorbent assay (ELISA). Fraction IV from *Pg* ATCC 33277 (5 mg/mL) was used to coat 96 well polystyrene microtiter plates (Costar, Corning, NY, USA) overnight at 4ºC. After blocking with 1%BSA at 37ºC for 2 hours, serum was diluted in PBS containing 0.05% BSA and then incubated at 37ºC for 1 hour. Plates were then incubated at 37º for 1 hour with peroxidase-conjugate goat anti-human IgA (Bethyl A-80-102P, Montgomery, Texas, USA), peroxidase-conjugate rabbit anti-human IgG (A-8792, SIGMA, St Louis, Missouri, USA), or mouse anti-human IgG1-4 monoclonal antibodies followed by biotin-conjugated goat anti-mouse IgG and thereafter with streptavidin-peroxidase. Reactions were revealed with o-phenylene diamino (OPD) for IgG and IgA or tetramethylbenzidine (TMB) for IgG1-4 and stopped with H_2SO_4 4N and H_2SO_4 2N, respectively. The optical density (OD) was read at 450/630 nm with a microplate reader (Diasorin, USA).

Statistical analysis

Descritive analysis was realized to characterize the sample. T student test was used to evaluate the differences in age between the two groups. Central tendency of the $OD_{450/630\ nm}$ achieved in the two groups – chronic periodontitis patients (CP) and healthy control (HC) were compared by the Mann-Whitney U test. A nonparametric (distribution-free) rank statistic, the Spearman's rank correlation coefficient (r_s) test was used to correlate antibody levels of 29 periodontitis patients against each clinical periodontal status, according to the four criteria established.

RESULTS AND DISCUSSION

The description of the clinical findings, such as gender, age, number of teeth, bleeding on probing (BOP), probing depth (PD) and clinical attachment levels (CAL) are shown in the Table 1. There is a statistically significant difference in age between the studied groups (T Student; $p < 0.001$).

The chromatographic fraction IV presented protein bands ranging from 15 kDa to 43 kDa in the SDS-PAGE profile (Figure 1A). Six fractions were obtained from *Pg* ATCC 33277 sonicate, eluted from the ion-exchange column with a continuous gradient of NaCl (fraction I: 0%; fraction II: 10%; fraction III: 20%; fraction IV: 30%; fraction V: 40% and fraction VI: 60%). Fraction IV was chosen because its antigenicity ability, as showed in the Figure 1B: IgG from chronic periodontitis pooled sera recognized by Western blotting bands which ranging from 58, 54, 50, 45, 37 and 35 kDa while IgG from healthy control pooled sera did not recognize any. These finds shows that the fraction IV has a good potential of immunogenicity assessment. According to Booth et al.,[14] IgG1 and IgG2 antibodies recognized a dominant antigen of 47 kDa from *P. gingivalis*, probably Arg-gingipain.

Serum antibody levels assay (ELISA $OD_{450\text{-}630\ nm}$) were compared using Mann-Whitney U test and showed that IgA ($p < 0{,}001$), IgG ($p < 0{,}001$), IgG1 ($p < 0{,}01$), IgG2 ($p < 0{,}001$), IgG3 ($p < 0{,}001$) and IgG4 ($p < 0{,}001$) against fraction IV from *Pg* were higher in CP when compared to HC groups (Figure 2A-F). When assessing the humoral response against the recombining PrtC of *P. gingivalis*, Beikler et al.[20] identified the production of IgA and IgG, with a distribution of the sub-classes of IgG of IgG2 > IgG3 > IgG1 > IgG4. The reactivity of the sub-classes of IgG against PrtC of *P. gingivalis* was not, however, related to progression of periodontal attachment loss. According to Booth et al.[14] much of the response to carbohydrate antigen is of the IgG2 subclass. Neither the level of IgG1 nor the IgG2 antibody specific to *P. gingivalis* was related to the total serum concentration of that antibody.

The antibody response against *Porphyromonas gingivalis* depended on the periodontal status, which in this investigation was categorized in accordance with four previously described clinical criteria. The correlation between the different criteria, classified with regard to the clinical parameters used, and the serum antibody levels against *Porphyromonas gingivalis* indicated

TABLE 1 – Characterization of clinical findings data: gender, age, number of present teeth and periodontal parameters.

	HC (n = 26)	CP (n = 29)
Male/Female	14/12	20/9
Age years (mean ± SD) $^{\varphi}$*	29.72 ± 9.85	40.61 ± 12.90
Number of teeth (mean ± SD)	27.93 ± 2.11	20.41 ± 6.43
BOP	10.07 ± 6.23	21.41± 6.17
% PD ≥ 4 mm	0.0 ± 0.0	24.42 ± 17.19
% CAL ≥ 3 mm	0.0 ± 0.0	71.35 ± 16.88
% CAL ≥ 5 mm	0,0 ± 0,0	31,76 ± 22,65
% CAL ≥ 3 mm; PD ≥ 4 mm; BOP	0,0 ± 0,0	17,14 ± 12,01

* $p \leq 0.01$; φ T Student test; SD: standard deviation; BOP: Bleeding on Probing; PD: Probing Depth; CAL: Clinical Attachment Level. HC: Healthy Control; CP: Chronic Periodontitis.

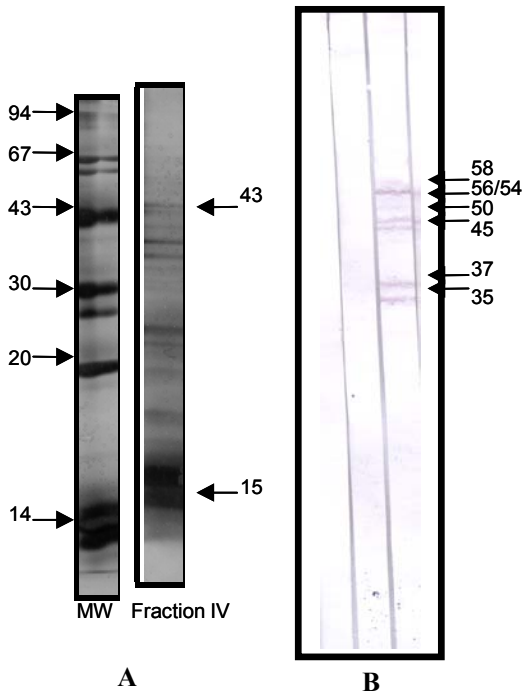

Figure 1 – Profile of the chromatographic fraction IV from *P. gingivalis* ATCC33277 sonic extract in 12%SDS-PAGE stained by silver nitrate. MW: Molecular weight. (A); Western Blotting showing the recognition profile of the chromatographic fraction IV from *P. gingivalis* ATCC33277. W: white; HC: healthy control; CP: chronic periodontitis pooled sera; AP: aggressive periodontitis pooled sera.

that there was statistically significant positive correlation between the criterion 1 and IgG2 levels ($r = 0{,}385$; $p < 0{,}05$ – Figure 3A) and between the criterion 2 and IgG ($r = 0{,}428$; $p < 0{,}05$ – Figure 3B) and IgG2 ($r = 0{,}510$; $p < 0{,}01$ – Figure 3C) levels. The criterion 1 represents the percentage

of sites with bleeding on probing, which indicates an inflammatory process at that site. Although it would seem difficult to establish the relation between this clinical parameter and periodontal disease progression, Rahardjo et al.[21] related a highly significant correlation between increased frequency of BOP and periodontal disease progression. On the other hand, the criterion 2 represents the percentage of sites with CAL ≥ 3 mm, indicating a moderate periodontitis, according to the American Academy of Periodontology[16] Clinical attachment loss does not affect all subjects in the same manner,[22] but is the only consistent factor correlated with bone loss.[23] In addition, McArthur et al.[24] noted that serum IgG levels to *P. gingivalis* were positively correlated with loss of alveolar bone in an elderly population. Thus, the correlation between the criterion 2 and the levels of IgG and IgG2 reactive to *P. gingivalis* provide compelling evidence for the causative contribution of this microorganism to periodontitis.

It was interesting to note that there was no correlation between the IgG4 levels and the different strata of periodontal status referred in this study, although individuals with periodontal disease presented high serum titers of this immunoglobulin. It can be speculative that the production of high levels of IgG4 against *P. gingivalis* starts at the beginning of the disease.

There was statistically significant positive correlation between the criterion 3 and levels of total IgG ($r = 0,499$; $p < 0,01$ – Figure 4A), IgG2 ($r = 0,518$; $p < 0,01$ – Figure 4B) and IgG3 ($r = 0,370$; $p < 0,05$ – Figure 4C). The criterion 4 correlated positively with levels of IgA ($r = 0,339$; $p < 0,05$ – Figure 5A), IgG ($r = 0,607$; $p < 0,001$ – Figure 5B), IgG1 ($r = 0,345$; $p < 0,05$ – Figure 5C) and IgG2 ($r = 0,487$; $p < 0,01$ – Figure 5D). The humoral immune response has been suggested as a means of differentiating between distinct periodontal disease status. According to Kinane et al.,[25] the characteristics of periodontitis (that is, classification, prognosis, treatment success) are generally subjectively determined. The results of this study indicate that the more accurate is the clinical diagnosis criterion employed in the periodontal disease determination, the higher are serum levels of immunoglobulins. These findings support that antibody may prove to be a marker of disease activity. However, this data are consistent with systemic antibody as a reflection of the host response to a local infectious agent, and the translation of this research to clinical practice and patient management is still reserved.

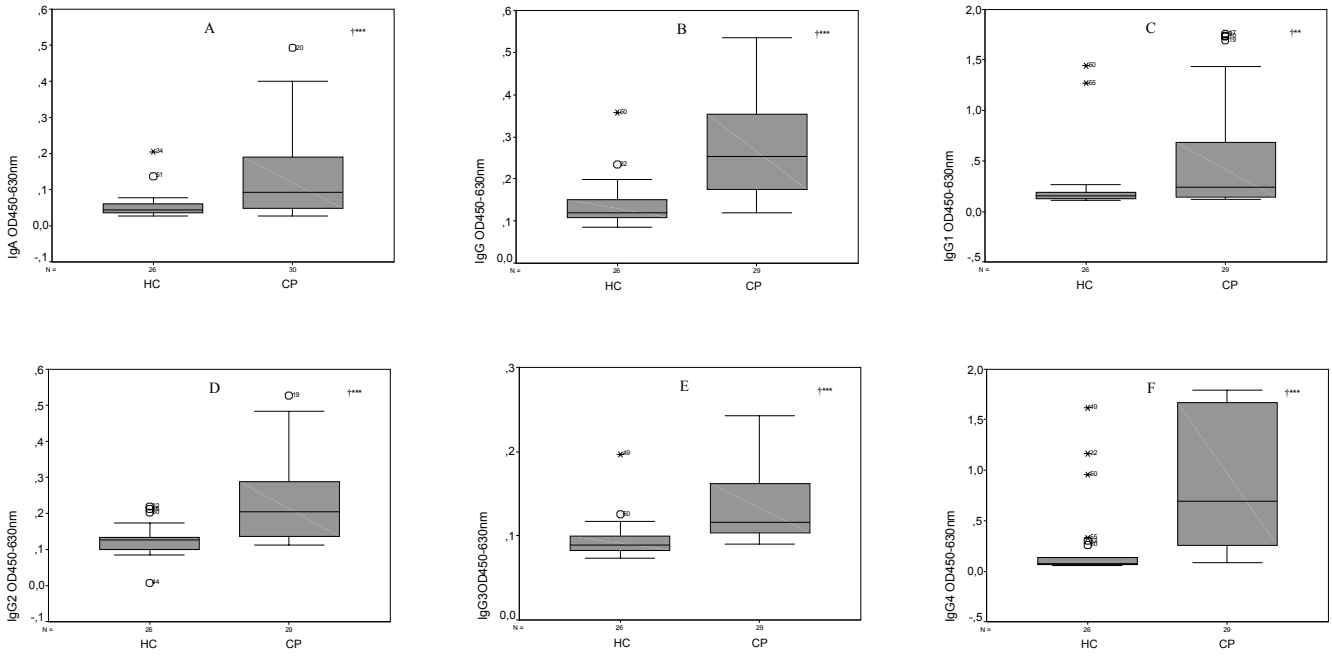

Figure 2 – Serum antibody levels reactive to crude fraction from *Pg* ATCC33277 evaluated in Chronic Periodontitis (CP) patients and Healthy Control (HC) by indirect ELISA optical density (OD) at 450-630nm: levels of IgA (A), IgG (B), IgG1 (C), IgG2 (D), IgG3 (E) and IgG4 (F).

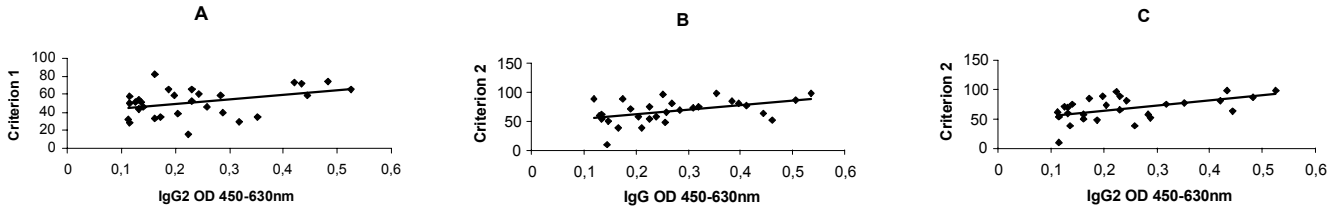

Figure 3 – Correlation between the clinical criterion 1 and serum levels of IgG2 (A), and between the clinical criterion 2 and the serum levels of IgG (B) and IgG2 (C).

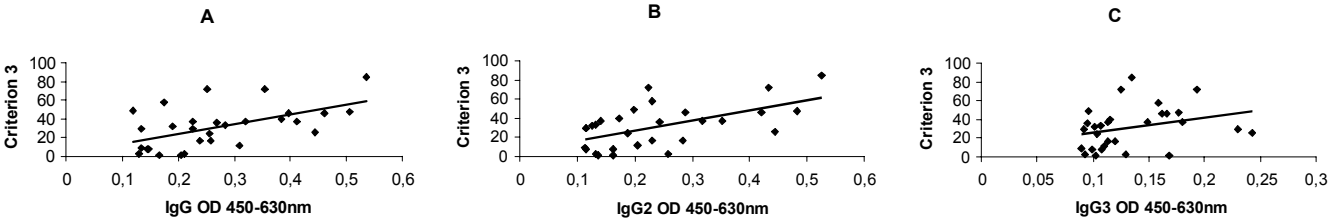

Figure 4 – Correlation between the clinical criterion 3 and serum levels of IgG (A), IgG2 (B) and IgG3 (C).

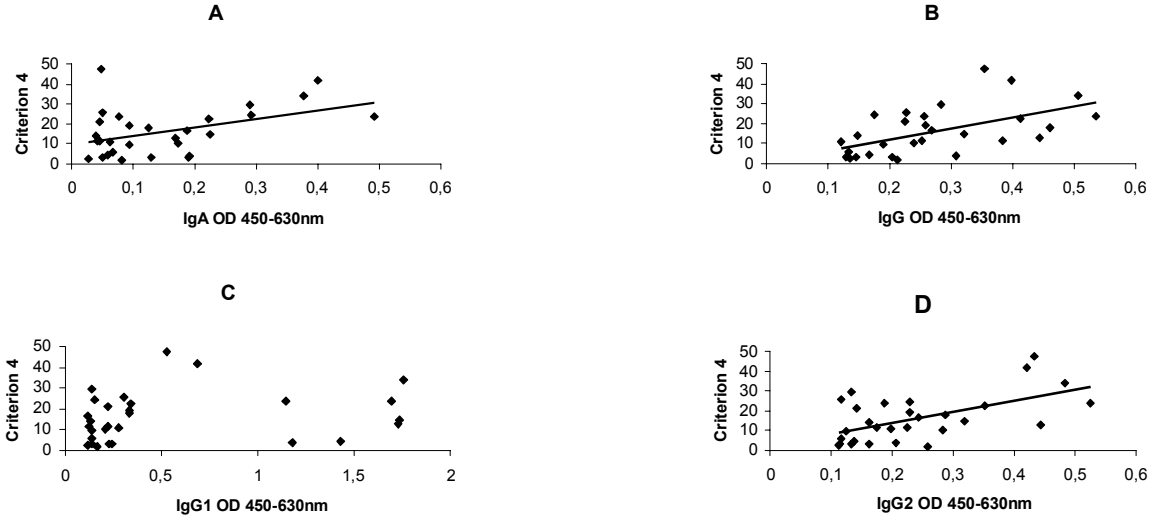

Figure 5 – Correlation between the clinical criterion 4 and serum levels of IgA(A), IgG (B), IgG1 (C) and IgG2 (D).

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