

Evaluation of CD4⁺CD25⁺ T lymphocyte response time kinetics in patients with chronic Chagas disease after *in vitro* stimulation with recombinant *Trypanosoma cruzi* antigens

Suellen Carvalho de Moura Braz^[1], Virginia Maria Barros de Lorena^{[1],[2]}, Adriene Siqueira Melo^[1], Maria da Glória Aureliano Melo Cavalcanti^[3] and Yara de Miranda Gomes^{[1],[2]}

[1]. Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, PE. [2]. Programa Integrado de Doença de Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ. [3]. Pronto-socorro Cardiológico de Pernambuco, Universidade de Pernambuco, Recife, PE.

ABSTRACT

Introduction: CD4⁺CD25⁺ T lymphocytes have been implicated in the regulation of host inflammatory response against *Trypanosoma cruzi*, and may be involved in the clinical course of the disease. **Methods:** Peripheral blood mononuclear cells from patients with chronic Chagas disease were cultured in the presence of *T. cruzi* recombinant antigens and assayed for lymphocytes at distinct time points. **Results:** It was possible to differentiate clinical forms of chronic Chagas disease at days 3 and 5 according to presence of CD4⁺CD25⁺ T cells in cell cultures. **Conclusions:** Longer periods of cell culture proved to be potentially valuable for prospective evaluations of CD4⁺CD25⁺ T lymphocytes in patients with chronic Chagas disease.

Keywords: Chronic Chagas disease. Recombinant antigens. CD4⁺CD25⁺ T lymphocytes.

The clinical course of chronic Chagas disease (ChD) varies from absence of symptoms (indeterminate form - IND) to severe manifestations with cardiovascular (cardiac form - CARD) and/or digestive (digestive form) impairment¹. It has been widely accepted in the scientific community that symptomatic forms of chronic ChD are associated with the establishment of an exacerbated cytotoxic cellular immune response that leads to inflammatory tissue damage and development of symptoms^{2,3}. Nevertheless, there is evidence of a specific CD4⁺ T lymphocyte lineage, characterized by expression of CD25 surface markers and the FoxP3 transcription factor, that is involved in the regulation of the cytotoxic response in chronic ChD^{4,6}. Furthermore, developmental or functional disturbances in these cells have been linked to autoimmune and inflammatory diseases in humans and animals⁷. Higher percentages of CD4⁺CD25⁺ T lymphocytes are observed in peripheral blood from patients with IND forms of ChD^{4,6}, which indicates that these cells have a role in controlling development of the morbidities common to human ChD. These lymphocytes produce cellular factors such as interleukin 10 (IL10) and transforming growth factor beta (TGF-β), which are among the host immunosuppression

mechanisms employed during the host immune response to *Trypanosoma cruzi*⁴. While the role of these cytokines in immune modulation guided by CD4⁺CD25⁺FoxP3⁺ T cells has been reported^{4,8,9}, there is still no consensus about their mechanisms of action.

The development of specific recombinant antigens of *T. cruzi* represents an important step in better understanding the role of the cellular responses in ChD¹⁰. Our group has been evaluating the use of the recombinant antigens CRA (cytoplasmic repetitive antigen) and FRA (flagellar repetitive antigen) as immunological markers of the clinical evolution of ChD¹¹⁻¹³, demonstrating significant results for the differentiation of distinct clinical manifestations of the chronic phase of ChD.

This study aimed to evaluate detection of CD4⁺CD25⁺FoxP3⁺, CD4⁺CD25⁺IL10⁺ T cells, and TGF-β in patients with chronic ChD at different cell culture time points after stimulation with CRA and FRA antigens. Eleven patients from the Ambulatório de Doença de Chagas e Insuficiência Cardíaca, PROCAPE-Universidade de Pernambuco/UPE who were diagnosed with chronic ChD (IND=06/CARD=05) were selected. Forty milliliters of blood were collected in heparin tubes (Vacurette) to obtain the peripheral blood mononuclear cells (PBMC) through a concentration gradient technique (Ficoll-PaqueTM Plus - Amersham Biosciences). Approximately 5 × 10⁶ cells/mL were cultured (RPMI 1640 medium/Sigma with 10% of fetal bovine serum/Sigma) in the presence of Phytohemagglutinin (PHA - GIBCOTM/Invitrogen Corporation) (5μg/mL) (positive control), CRA, or FRA (2μg/mL), or with no stimulatory agent (negative control). Culture flasks were then incubated

Address to: Dra. Yara de Miranda Gomes. CPqAM/FIOCRUZ. Caixa Postal 7472. Av. Moraes Rego s/n, 50670-420 Recife, PE, Brasil.

Phone: 55 81 2101-2500; Fax: 55 81 3453-1911

e-mail: yara@cpqam.fiocruz.br

Received 23 August 2011

Accepted 06 February 2012

at 37°C with 5% CO₂ for 1, 3, and 5 days. Four hours before the end of the respective incubation period, Brefeldin A (Sigma) (10µg/mL) was added to the cultures. Cells were washed by centrifugation (300 × g/5 min) with PBS-Wash (Phosphate-Buffered Saline with 0.5% bovine serum albumin/Sigma and 0.1% sodium azide/Sigma) and incubated for 30 min with surface antibodies (anti-CD4-fluorescein isothiocyanate/Caltag; anti-CD25-allophycocyanin/Invitrogen). The steps that followed were cell fixation (Human FoxP3 Buffer Set/BD Biosciences®; BD Cytotfix™/BD Biosciences®), centrifugation (300 × g/5 min), and permeabilization (Human FoxP3 Buffer Set/BD Biosciences®; PBS-Wash add 0.2% of saponin/Sigma). Samples were washed again with PBS-Wash and incubated for 30 min with intracellular antibodies (FoxP3-PE/BD Pharmingen; IL10-PE/Caltag). After another round of wash by centrifugation (300 × g/5 min), cells were fixed (BD Cytotfix™) and 30,000 events/sample were acquired in a FACScalibur® flow cytometer (Becton Dickinson) and analyzed using the CellQuest Pro Software. Detection of TGF-β in supernatants from days 1 and 5 was performed using the Human TGF-β1 Immunoassay

Kit - Quantikine® (R&D Systems®). Comparison of different culture time points within each group (IND and CARD) was performed through Friedman test followed by Wilcoxon test. To compare groups at each time point, data normality was analyzed by Student's *t*-test. When presumption of homogeneity was not confirmed, Mann-Whitney test was used. A significance level of 5% was considered in all analyses. The software utilized was Excel 2007, GraphPad Prism 5.0, and Statistical R 2.9.0. The procedures described here were approved by the Ethics Committee at Centro de Pesquisas Aggeu Magalhães (CPqAM)/ Fundação Oswaldo Cruz (FIOCRUZ)(Nº: 157/08).

Analysis of CD4⁺CD25⁺ T lymphocyte population in each group (CARD and IND) showed that there was no statistical difference among cell culture time points (1, 3, and 5 culture days) after stimulation with either recombinant antigen (**Figures 1A and 1B**). However, an increase in the mean number of CD4⁺CD25⁺ T cells in IND patients was observed over the course of the cultures, mainly after FRA stimulation, while in the CARD group, there was a decrease in the mean number of CD4⁺CD25⁺ T cells from the first to the fifth day of culture.

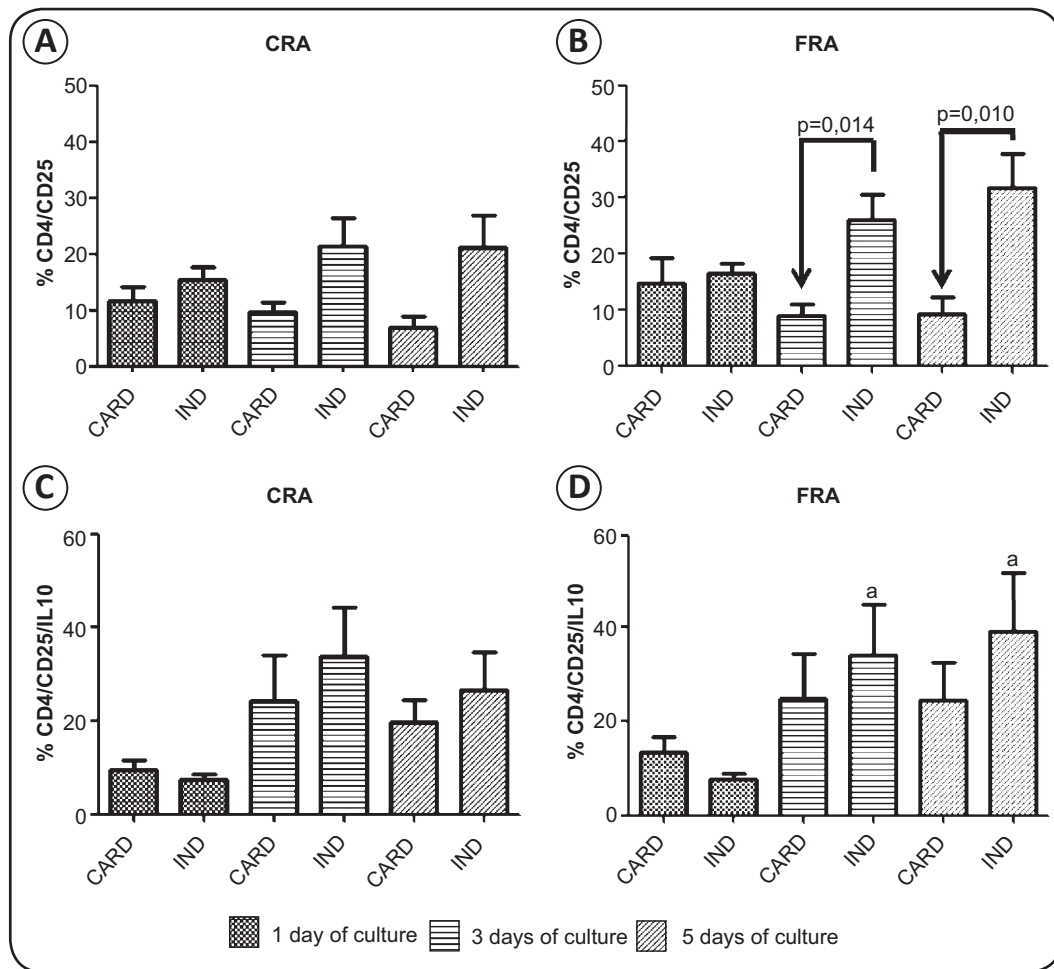


FIGURE 1 - Detection of CD4⁺CD25⁺ (A and B) and CD4⁺CD25⁺IL10⁺ (C and D) T lymphocytes in peripheral blood mononuclear cells of patients with chronic Chagas disease after *in vitro* stimulation with CRA and FRA. Significant differences are indicated in the figures with the respective p-values. An “a” indicates significant differences in comparison with day 1. CRA: cytoplasmic repetitive antigen; FRA: flagellar repetitive antigen; CARD: patients with cardiac clinical form of chronic Chagas disease; IND: patients with indeterminate clinical form of chronic Chagas disease; CD: cluster of differentiation; IL10: interleukin 10.

The means of CD4⁺CD25⁺ T cell counts between the 2 groups (CARD versus IND) for each culture time point (culture day 1, 3, or 5) were also compared. Differences between CARD and IND were statistically significant at days 3 (p = 0.014) and 5 (p = 0.010) after *in vitro* stimulation with FRA (Figure 1B). However, no differences were observed after stimulation with CRA (Figure 1A).

In regard to IL10-producing CD4⁺CD25⁺ T lymphocytes, the mean values of CD4⁺CD25⁺IL10⁺ T cells in the IND group, in the presence of FRA, were statistically different between days 1 and 3 (p = 0.031) and between days 1 and 5 (p = 0.031). Comparison of the CARD and IND forms showed that the mean number of CD4⁺CD25⁺IL10⁺ T cells was higher in the IND group than in the CARD patients after stimulation with CRA and FRA, however, this difference was non-significant (Figures 1C and 1D). With regard to the presence of CD4⁺CD25⁺FoxP3^{total} and CD4⁺CD25⁺FoxP3^{high} T cells, there was no statistical difference observed between the groups or among the cell culture time points within each group (Figure 2).

The presence of TGF-β in supernatants was not significantly different between patients with CARD and IND forms of chronic

ChD. Nevertheless, a reduction in TGF-β production after 5 days was observed upon stimulation with CRA (p = 0.043) (Figure 3).

Thus, in the present study we found that *in vitro* culture of PBMC for 3 and 5 days allowed differentiation between CARD or IND forms of ChD through evaluation of CD4⁺CD25⁺ T lymphocyte counts after antigenic stimulation. Previous studies using whole blood (*ex vivo*)^{5,6} and after 22 hours of culture in the presence of epimastigote antigens⁷ were also able to differentiate CARD and IND forms by analyzing the percentage of these cells through flow cytometry. Because CD4⁺CD25⁺ T cells represent a small percentage of total circulating CD4⁺ T lymphocytes⁷, we believe that longer periods of cell culture may improve studies regarding the possible roles of these cells in the clinical course of ChD. In regard to IL10 production by CD4⁺CD25⁺ T lymphocytes in chronic ChD, our data corroborated with those presented by Araújo et al.⁶, in that no difference was found between patients with CARD and IND clinical forms. However, the increased numbers of CD4⁺CD25⁺IL10⁺ T lymphocytes throughout the cell culture period reinforced the importance of considering longer periods of cultivation.

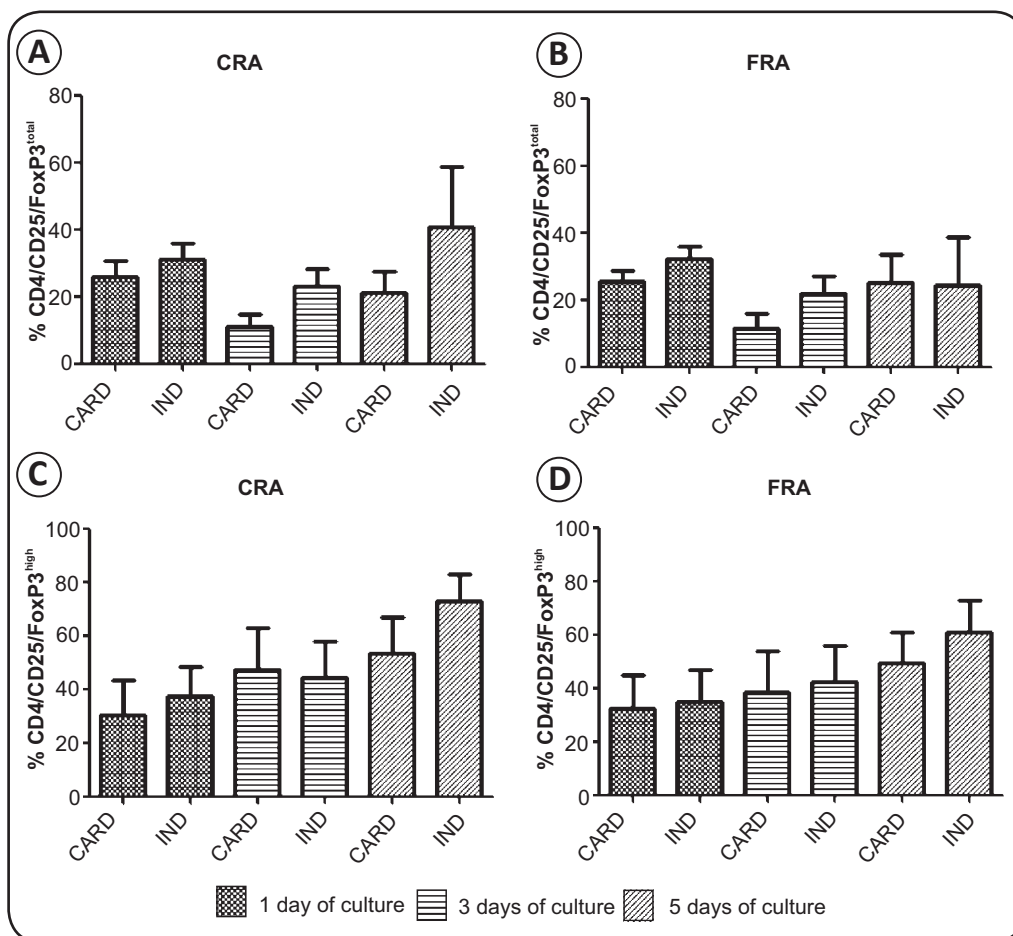


FIGURE 2 - Detection of CD4⁺CD25⁺FoxP3^{total} (A and B) and CD4⁺CD25⁺ FoxP3^{high} (C and D) T lymphocytes in peripheral blood mononuclear cells of patients with chronic Chagas disease after *in vitro* stimulation with CRA and FRA. Significant differences are indicated in the figures with the respective p-values. CRA: cytoplasmic repetitive antigen; FRA: flagellar repetitive antigen; CARD: patients with cardiac clinical form of chronic Chagas disease; IND: patients with indeterminate clinical form of chronic Chagas disease; CD: cluster of differentiation.

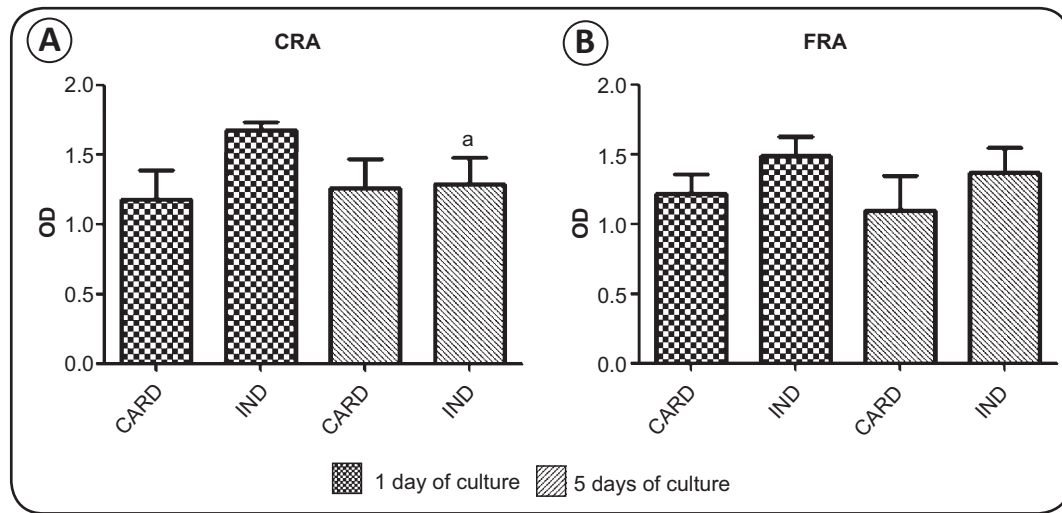


FIGURE 3 - Detection of transforming growth factor- β in supernatant of cultures from patients with chronic Chagas disease after *in vitro* stimulation with CRA (A) and FRA (B). Significant differences are indicated in the figures with the respective p-values. The “a” represents a significant difference in comparison with day 1. CRA: cytoplasmic repetitive antigen; FRA: flagellar repetitive antigen; CARD: patients with cardiac clinical form of chronic Chagas disease; IND: patients with indeterminate clinical form of chronic Chagas disease; OD: optical density.

The observation that there were no differences in TGF- β production among patients with CARD and IND forms of ChD may have been due to the lower sample size evaluated. Moreover, with the chosen method (capture ELISA), the source of cytokine production is not identified. With regard to the reports citing TGF- β as a pathway of immunoregulation by CD4⁺CD25⁺FoxP3⁺ T lymphocytes^{7,8}, the technique reported here did not clarify whether CD4⁺CD25⁺FoxP3⁺ T cells were responsible for TGF- β production. Thus, the evolution of these growth factors under the regulation of CD4⁺CD25⁺FoxP3⁺ T cells in individuals with chronic ChD will only be confirmed upon further study.

We conclude that CD4⁺CD25⁺ T lymphocyte cultures derived from serum of patients chronic ChD that are maintained for longer periods have the potential to be used in prospective studies aimed at understanding the clinical evolution of the disease. Moreover, this approach may also aid in the identification of cellular patterns that can serve as biological markers to monitor patients. Further, it is important to mention that a greater number of individuals should be evaluated to prove the importance of this cell population in immunoregulation in individuals with the IND form of ChD.

ACKNOWLEDGMENTS

We are thankful to the staff of Ambulatório de Doença de Chagas e Insuficiência Cardíaca (PROCAPE/UPE) for assistance in selecting the patients included in this study, to the Programa de Desenvolvimento Tecnológico em Insumos para Saúde (PDTIS/FIOCRUZ) for use of its facilities, to Antônio Ferreira and Edimilson Silva at Biomanguinhos/FIOCRUZ for providing recombinant antigens, to George Diniz at CPqAM/FIOCRUZ for assistance with statistics, and to Mineo Nakazawa at CPqAM/FIOCRUZ for technical assistance.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This research received financial support by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) (Universal Edital n° 478572/2009-3) and *Coordenação de Aperfeiçoamento de pessoal de Nível Superior* (Capes). Y.M. Gomes is a CNPq fellow (number 306427/2006-0). V.M.B. Lorena is a postdoctoral CNPq fellow. S.C.M. Braz and A.S. Melo were candidates for the Master's degree in Public Health (CPqAM-FIOCRUZ) and were CNPq (number 133106/2009-8) and Capes fellows, respectively.

REFERENCES

- Rassi Jr A, Rassi A, Marin-Neto JA. Chagas disease. *Lancet* 2010; 375:1388-1402.
- Dutra OD, Gollob KJ. Current concepts in immunoregulation and pathology of human Chagas disease. *Curr Opin Infect Dis* 2008; 21:287-292.
- Lescure FX, Le Loup G, Freilij H, Develoux M, Paris L, Brutus L, et al. Chagas disease: changes in knowledge and management. *Lancet Infect Dis* 2010; 10: 556-570.
- Vitelli-Avelar DM, Sathler-Avelar R, Dias JCP, Pascoal VPM, Teixeira-Carvalho A, Lage PS, et al. Chagasic patients with indeterminate clinical form of the disease have high frequencies of circulating CD3⁺CD16⁺CD56⁺ natural killer T cells and CD4⁺CD25^{high} regulatory T lymphocytes. *Scand J Immunol* 2005; 62: 297-308.
- Vitelli-Avelar DM, Sathler-Avelar R, Massar RL, Borges JD, Lage PS, Lana M, et al. Are increased frequency of macrophage-like and natural killer (NK) cells, together with high levels of NKT and CD4⁺CD25^{high} T cells balancing activated CD8⁺ T cells, the key to control Chagas' disease morbidity? *Clin Exp Immunol* 2006; 145:81-92.

6. Araujo FF, Gomes JAS, Rocha MOC, Williams-Blangero S, Pinheiro VM, Morato MJS, et al. Potencial role of CD4⁺CD25^{high} regulatory T cells in morbidity in Chagas disease. *Front Biosci* 2007; 12: 2797-806.
7. Sakaguchi S, Tomoyuki Y, Nomura T, Ono M. Regulatory T cells and Immune Tolerance. *The Cell* 2008; 133:775-787.
8. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by cell surface-bound transforming growth factor β . *J Exp Med* 2001; 194: 629-644.
9. Piccirillo CA, Letterio JJ, Thornton AM, McHugh RS, Mamura M, Mizuhara H et al. CD4⁺CD25⁺ regulatory T cells can mediate suppressor function in the absence of transforming growth factor β 1 production and responsiveness. *J Exp Med* 2002; 196:237-245.
10. Lorca M, Gonzalez A, Veloso C, Reyes V, Vergara U. Immunodetection of antibodies in sera from symptomatic and asymptomatic Chilean Chagas' disease patients with *Trypanosoma cruzi* recombinant antigens. *Am J Trop Med Hyg* 1992; 46:44-49.
11. Lorena VMB, Lorena IMB, Braz SCM, Melo AS, Melo MFAD, Melo MGAC, et al. Cytokine levels in serious cardiopathy of Chagas disease after *in vitro* stimulation with recombinant antigens from *Trypanosoma cruzi*. *Scand J Immunol* 2010; 72:529-539.
12. Vasconcelos RHT, Amaral FN, Cavalcanti MGAM, Silva ED, Ferreira AGP, Morais CNL, et al. Increased levels of IgA antibodies against CRA and FRA recombinant antigens of *Trypanosoma cruzi* differentiate digestive forms of Chagas disease. *Hum Immunol* 2010; 71: 964-967.
13. Verçosa AFA, Lorena VMB, Carvalho CL, Melo MFAD, Cavalcanti MGA, Silva ED, et al. Chagas' disease: IgG isotypes against cytoplasmic (CRA) and flagellar (FRA) recombinant repetitive antigens of *Trypanosoma cruzi* in chronic Chagasic patients. *J Clin Lab Anal* 2007; 21:271-276.