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Immune complex detection in saliva samples: an innovative proposal for the diagnosis of human strongyloidiasis

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Research Article

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Abstract

Human strongyloidiasis is caused by helminth *Strongyloides stercoralis*. It has a worldwide distribution, often neglected and cause of severe morbidity. The parasitological diagnosis is hindered by the low and irregular amount of larvae in feces. The goal of the present study was to detect IgG and IgG immune complex using conventional serum samples and saliva as alternative samples. We collected samples from 60 individuals, namely: group I composed of 30 healthy individuals; and group II composed of 30 individuals eliminating *S. stercoralis* larvae in feces. We calculated the area under the curve, general index of diagnostic accuracy, Kappa index and determined the correlations between different diagnostic tests. The detection of IgG levels was performed by an immunoenzymatic assay with alkaline extract of *S. venezuelensis* larvae as antigen. Positivity of anti-*S. stercoralis* IgG in serum samples from group I was 3·3%, and from group II 93·3%. The detection of immune complex indicated that group I exhibited 3·3% and group II 56·7%. In the saliva samples, IgG detection was 26·7% for group I and 43·3% for group II. Immune complex was detected in 20% of group I, and 30% of group II. IgG immune complex in conventional serum samples and saliva as alternative samples can be considered biomarkers for the diagnosis of active strongyloidiasis.

Introduction

Strongyloidiasis is an infection caused by helminth *Strongyloides stercoralis* (Bavay, 1876) and considered to be neglected. It has heterogeneous worldwide distribution, mainly in tropical and subtropical regions (Olsen *et al.* 2009; Paula and Costa-Cruz, 2011). Usually, strongyloidiasis evolves in an asymptomatic manner; however, in some cases, the presence of eggs, larvae and adult parasites in the mucosa of the small intestine can result in inflammatory reaction, malabsorption syndrome and, consequently, chronic diarrhoea with protein loss, abdominal pain and bleeding with development of hypoalbuminemia and anaemia (Grove, 1996; Fardet *et al.* 2007). In addition, in immunocompromised individuals, extra intestinal manifestations can promote severe clinical conditions, such as hyperinfection and potentially fatal conditions, such as disseminated infection (Da Silva *et al.* 2014; Levenhagen and Costa-Cruz, 2014). These clinical conditions can cause mortality ranging from 15 to 87% when the disease is not diagnosed early (Agrawal *et al.* 2009).

The diagnosis of strongyloidiasis is based on the identification of larvae in fecal samples. However, the parasitological diagnosis becomes complex due to the low and irregular amount of larvae released in feces (Uparanukraw *et al.* 1999; Schär *et al.* 2013; Mendes *et al.* 2017). In order to assist the parasitological diagnosis, immunological tests such as immunoenzymatic assay (ELISA) are indicated for the diagnosis of human strongyloidiasis due to their high sensitivity. These tests are considered useful in serological screening (Gonzaga *et al.* 2011; Gonçalves *et al.* 2012; Bosqui *et al.* 2015).

In addition to the use of conventional serum samples for immunological diagnosis of human strongyloidiasis, the recent years have been characterized by the gradual increase in the number of studies on specific salivary antibodies as biomarkers in epidemiology and toxicology (Kaufman and Lamster, 2002; Ribeiro *et al.* 2010). They represent an alternative tool in the diagnosis of different protozoa (Del Muro *et al.* 1990; Loyola *et al.* 1997; Pinto *et al.* 1999; Borges and Figueiredo, 2004) and also human strongyloidiasis (Costa *et al.* 2003; Ribeiro *et al.* 2010; Bosqui *et al.* 2015). These studies have pointed out saliva as an alternative tool for assessing specific antibodies for diagnosis.

Infectious diseases result not only in the production of antibodies but also imply the formation of antigen-antibody complexes. These immune complexes are important because they bind to the phagocytic cells, where they are destroyed and eliminated. Moreover, they can be used as biomarkers in the course of the disease (Frank and Hester, 2009; De Carvalho *et al.* 2013). Therefore, the detection of antibodies and immune complexes through immunological assays contributes to the diagnosis in humans, especially when there is a high risk of developing severe forms of the disease (Gonçalves *et al.* 2016). In this context, the detection of

immune complexes using saliva samples represents an innovative proposal for the diagnosis of strongyloidiasis in order to observe active forms of the disease.

Considering the severity of disseminated strongyloidiasis and the importance of identifying active infections, the present study proposes the detection of IgG immune complexes in the diagnosis of strongyloidiasis using conventional serum samples and alternative saliva samples. The procedure is carried out with the ELISA method using alkaline extracts of *S. venezuelensis* larvae as heterologous antigen. These are innovative approaches for the diagnosis of this potentially fatal infection when not detected and treated early in some cases.

Method

Subjects

Biological specimens were collected from 60 individuals divided into two groups: Group I: 30 apparently healthy individuals based on their clinical observation, without evidence of contact with *S. stercoralis* infection, no previous history of strongyloidiasis and three fecal samples tested negative. Group II: 30 patients attending the outpatient departments or admitted to the Clinical Hospital of the State University of Londrina, Paraná state, Brazil, harbouring *S. stercoralis* larvae, coprologically detected.

The study received approval from the Research Ethics Committee of the Universidade Estadual de Londrina, Paraná state, Brazil, n° 1494·2013-56.

Serum and saliva samples

Feces, blood and saliva samples were collected from all subjects. Three fecal samples were analysed according to Hoffmann *et al.* (1934) and Rugai *et al.* (1954). Serum was separated from blood and stored at −20 °C until use. Unstimulated whole saliva samples were collected by using clean cotton swabs after washing oral cavity with water; distributed in aliquots and conserved until use; slowly thawed and centrifuged at 12 400 ***g*** for 15 min.

Parasites

Infective third stage larvae (L3) of *Strongyloides venezuelensis* were obtained from cultures of rat feces coal *Rattus norvegicus* (Wistar). The cultures were stored at 28 °C for 48 h, the infective larvae were collected and concentrated using the method of Rugai *et al.* (1954). The number of larvae recovered was washed 5 times with phosphate buffered saline (PBS, 0·01 mol L^{-1}, pH 7·2) and stored at −20 °C in PBS until use.

Alkaline extract from S. venezuelensis

To alkaline extracts were prepared according to Machado *et al.* (2003) 1 mL of 0·15 mol L^{-1} NaOH was added to 300 000 *S. venezuelensis* larvae and kept under gentle shaking for 6 h at 4 °C. Subsequently, 0·3 mol L^{-1} HCl was added until a pH of 7·0 was reached. These preparations were then centrifuged at 12 400 ***g*** for 30 min at 4 °C. Supernatants (alkaline extracts) were analysed for protein content according to Lowry *et al.* (1951), subdivided into aliquots and stored at −20 °C until use.

IgG-specific measurement in serum and saliva samples by ELISA

Preliminary experiments were carried out in order to determine the optimal conditions for ELISA through block titration of reagents (antigens, control serum and saliva samples, and conjugate). Briefly, polystyrene microplates (BioAgency Laboratories, São Paulo, Brazil) were coated overnight at 4 °C with 5 μg mL^{-1} of *S. venezuelensis* larvae (L3), in 0·06 mol L^{-1} carbonate-bicarbonate buffer (pH 9·6). Plates were washed 3 times with PBS containing 0·05% Tween 20 (PBST). After washing, plates were incubated with serum sample (50 μL $well^{-1}$) with diluition 1 : 80 (for IgG detection) and incubated with saliva sample 50 (μL $well^{-1}$) 1 : 20 (for IgG detection) for 45 min at 37 °C and subsequently with the secondary antibody consisting of peroxidase-labelled goat anti-human IgG conjugate (Sigma, St Louis, MO, USA) at the ideal dilution of 1 : 2000 for 45 min at 37 °C in low affinity plate for sera and high affinity plate in saliva. The reagents were used for a final assay volume of 50 μL $well^{-1}$. The reaction was revealed by adding the enzyme substrate (5 μL 30% H2O2 Merck (Darmstadt, Germany) and 5 mg *o*-phenylenediamine [OPD] in 12·5 mL of 0·1 M citrate-phosphate buffer, pH 5·0) and incubation for 15 min at room temperature. Optical densities (OD) were determined at 492 nm in an ELISA reader (Titertek Plus, Flow Laboratories, USA).

Measurement of immune complex in serum and saliva samples

Preliminary experiments were carried out in order to determine the optimal conditions for ELISA through serial dilution of the reagents (antibody, control serum and saliva, and conjugate). Polystyrene microtitre plates were coated for 18 h at 4 °C with 50 μL $well^{-1}$ of IgG obtained from immunized rabbits (40 μg mL^{-1}) (Gonçalves *et al.* 2010) in 0·06 M carbonate-bicarbonate buffer (pH 9·6). After three washes with PBST, 50 μL $well^{-1}$ of serum samples (1 : 80) or saliva (pure) were added and incubated for 45 min at 37 °C. Positive and negative serum and saliva samples to the immune complex were used as a control. The positive serum and saliva samples consisted of a positive sample for IgG anti-*S. venezuelensis*. After incubation for 45 min at 37 °C and another three washes, the peroxidase – labelled goat anti-human IgG (Sigma, St Louis, MO, USA) was added (50 μL $well^{-1}$) at the ideal dilution of 1 : 2000 for 45 min at 37 °C. The reaction was revealed by adding the enzyme substrate (5 μL 30% H2O2 Merck (Darmstadt, Germany) and 5 mg *o*-phenylenediamine [OPD] in 12·5 mL of 0·1 M citrate-phosphate buffer, pH 5·0) and incubation for 15 min at room temperature.

Statistical analysis

Analyses were performed using the GraphPad software package version 5.0. Optimum point for each condition of ELISA reaction and cut-off points were established using a 2-graph receiver operating characteristic curve (TG-ROC) combined with a ROC curve (Greiner *et al.* 1995). ELISA index (EI) was obtained by the ratio between OD and cut-off. Values of EI greater than the optimum point of reaction for each extract were considered positive. ROC curves were built to describe test indexes (Martinez *et al.* 2003). The area under the ROC curve (AUC), an index of diagnostic accuracy, was calculated and values close to 1 indicate an informative test and values close to 0·5 indicate an uninformative test (Hanley and McNeil, 1982). Likelihood ratio, which indicates how likely patients with strongyloidiasis are going to have a specified test detection compared with patients not infected by the parasite, was estimated as Se/(1 − Sp). Correlations between levels of different diagnostic tests in each sample tested with *S. stercoralis* were determined using Spearman (r) or Spearman rank test (rs). The concordance of the results obtained between the serum and saliva samples was carried out analysis of Kappa

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coefficient (κ) (Landis and Koch, 1977). Probability (P) values of <0·05 were regarded as significant.

Results

The detection of IgG antibodies and immune complexes in serum and saliva samples is illustrated in Fig. 1. The positivity rate of anti-*S. stercoralis* IgG in serum samples of individuals with negative parasitological results (Group I) was 3·3% ($n = 1$), whereas in individuals harbouring *S. stercoralis* larvae (copropositive) (Group II) was of 93·3% ($n = 28$). Regarding the detection of immune complexes in serum samples, positivity in group I was 3·3% ($n = 1$), whereas in group II it was 56·7% ($n = 17$) (Fig. 1A). The analysis of saliva samples for anti-*S. stercoralis* IgG indicated positivity of 26·7% ($n = 8$) in group I and 43·3% ($n = 13$) in group II. The detection levels of immune complexes in saliva samples were 20% ($n = 6$) for group I and 30% ($n = 9$) for group II (Fig. 1B).

In order to observe the correlation between the results, we quantified the combination of data between human IgG antibodies and immune complexes in serum and saliva samples (Fig. 2). Figure 2A and C illustrate the diagnostic correlation between serum and saliva samples from the group with a positive coproparasitological diagnosis for *S. stercoralis*, respectively. Figure 2A shows that the correlation between the results was weak ($r = 0·25$; $P > 0·05$), whereas Fig. 2C shows that the correlation was significantly positive ($r = 1$; $P < 0·05$). On the other hand, positive correlation of the results obtained in the analysis of serum and saliva samples from the group of healthy individuals (Fig. 2B and D, respectively) can only be observed in Fig. 2D ($r = 0·53$; $P < 0·05$). This study showed excellent agreement in the detection of IgG ($\kappa = 0·90$) and moderate for IC ($\kappa = 0·51$) in serum.

Discussion

Currently, immunological diagnosis of strongyloidiasis is mainly performed detecting IgG antibodies, given that there is a predominance of a humoral response (Atkins *et al.* 1997; Bosqui *et al.* 2015).

In our study, it was possible to observe 93·3% of positive cases for IgG in serum samples. IgG antibodies are the largest class of serum immunoglobulins able to provide protective immunity and remain for a long period of time after the cure of the disease (Carvalho *et al.* 1983; Iriemenam *et al.* 2010). In part, these characteristics are responsible for the success and advantages of the diagnosis of strongyloidiasis by means of detecting this class of antibodies in serum samples from infected individuals (Agrawal *et al.* 2009; Ribeiro *et al.* 2010; Bosqui *et al.* 2015). However, the high concentration of circulating antibodies and antigens in serum can result in the formation of immune complexes (Ohyama *et al.* 2016).

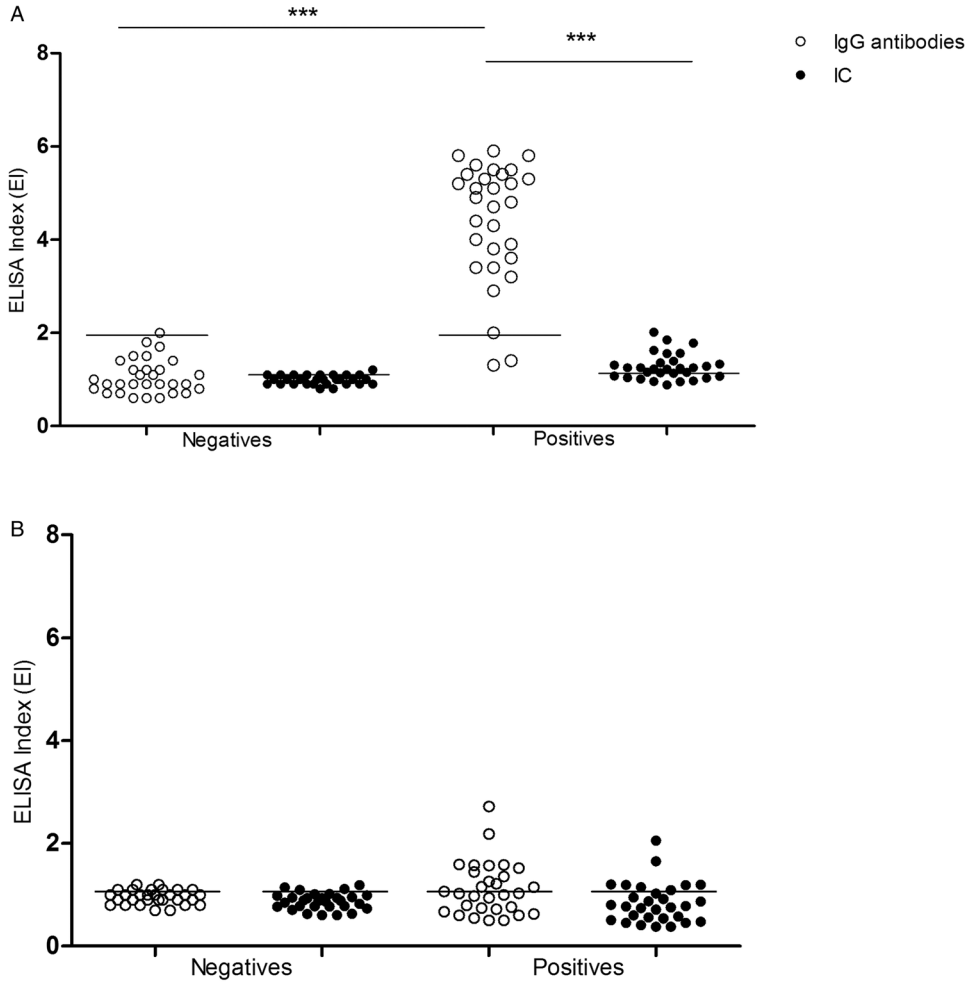

Fig. 1. IgG and immune complex anti-*S. stercoralis* in serum and saliva samples determined by immunoenzymatic assay using alkaline extract of *S. venezuelensis* L3 expressed in EI. The groups were divided into negative (control), apparently healthy individuals (Group 1; $n = 30$) and individuals harbouring *S. stercoralis* larvae (copropositive) (Group II; $n = 30$). (A) Detection of IgG and immune complexes in serum samples from the two groups. (B) Detection of IgG and immune complexes in saliva samples from the two groups. The dashed line represents the threshold for positivity (EI > 1·0); ***$P < 0·001$. EI, ELISA index.

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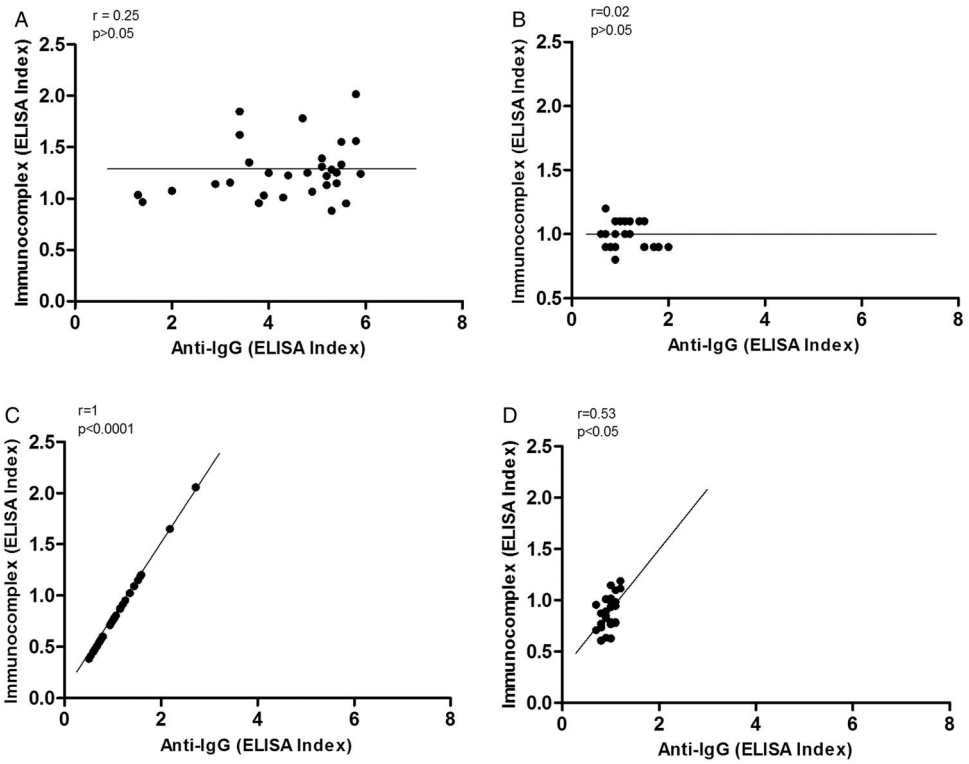

Fig. 2. Use of the correlation between human anti-IgG and immune complexes in paired samples for diagnosis. (A) and (C): correlation between circulating antibodies and antibodies complexed to antigen in serum and saliva samples, respectively, in patients with positive coproparasitological diagnosis for *S. stercoralis*; (B) and (D): correlation between circulating antibodies and antibodies complexed to antigen in serum and saliva samples, respectively, in patients with negative coproparasitological diagnosis for *S. stercoralis*.

Our study revealed that the detection of circulating IgG immune complexes in serum was significant (70%). Immune complexes play an important role in neutralizing the pathogenesis of the disease, being rapidly eliminated from the bloodstream by the innate immune system. In addition, circulating immune complexes have been reported with increased frequency in parasitic diseases (Ohyama *et al.* 2016).

Our assay proved to be effective in the detection of IgG anti-*Strongyloides* spp., both in serum and saliva samples, showing acceptable levels of reliability and being able to minimize false positive results. The same result can be observed in different diagnoses, both experimental and in humans, given that the detection of immune complexes has also been used and the results were promising (Patil *et al.* 1996; Nezlin, 2009; Madhusudana *et al.* 2014; Gonçalves *et al.* 2016; Ohyama *et al.* 2016).

The few positive cases for detecting immunocomplex in the negative group can be explained by the persistence of IgG in an undiagnosed infection due to low release of larvae in feces and/or individuals with prior exposure living in areas with high prevalence of strongyloidiasis (Santos *et al.* 2007; Iriemenam *et al.* 2010; Ahmad *et al.* 2013).

The choice of an appropriate immunological method that complements the parasitological diagnosis is essential for an accurate estimate of strongyloidiasis. Therefore, detecting the levels of circulating immune complexes by an immunoenzymatic assay can be used as complementary screening in the diagnosis of strongyloidiasis. Immune complexes have an important participation in the physiopathology of the disease and, therefore, may reflect the immune response exerted by each individual, thus facilitating the control of this infection (Gonzaga *et al.* 2011; Gonçalves *et al.* 2012).

In addition to the use of serum samples for the immunological diagnosis of strongyloidiasis, saliva samples have been investigated in recent years as an alternative tool. These samples offer advantages such as easy obtainment, noninvasive sampling and low cost, playing an important role in the local and systemic assessment of the immune response against the parasite (Costa *et al.* 2003; Ribeiro *et al.* 2010; Bosqui *et al.* 2015).

The analysis of saliva samples has become important to assess physiological and pathological conditions in humans (Sawangsoda *et al.* 2012). This is a pioneering study assessing patients with strongyloidiasis and presents an innovative proposal for using saliva samples to detect immune complexes.

Concluding remarks

In conclusion, the detection of IgG immune complexes in conventional serum samples and saliva the alternative samples can be considered a promising strategy for the diagnosis of active strongyloidiasis.

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Conflict of Interest. The authors declare that there are no conflicts of interest.

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