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Detection of immune complexes and evaluation of alcoholic individuals' serological profile in the diagnosis of strongyloidiasis

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ABSTRACT

Strongyloidiasis is a human parasitosis that is considered a public health problem. Early diagnosis of this infection is extremely important in immunocompromised patients (i.e. subjects with alcoholism). This study aimed to evaluate anti-*Strongyloides* immunoglobulin G (IgG) and immunoglobulin A (IgA), assess levels of circulating immune complexes (IC) and determine IgG avidity in serum samples from alcoholic and nonalcoholic individuals. A total of 140 blood samples were collected from male individuals (70 alcoholic and 70 nonalcoholic subjects). Serum was obtained and analysed by enzyme-linked immunosorbent assay for IgG, IgA, IC detection and avidity determination. Anti-*Strongyloides* IgG was detected in 55.7% of alcoholic subjects and 32.8% nonalcoholics, while IC levels showed frequencies of 38.6% and 17.1% in these groups, respectively. Anti-*Strongyloides* IgA was lower among alcoholics (4.3%) than nonalcoholics (34.3%). Spearman's correlation coefficient reported a positive correlation between IgG, IC and IgA in alcoholic individuals and no correlation in nonalcoholics. The median avidity index was higher in alcoholics (83.8%) than nonalcoholic subjects (73.2%). In conclusion, this study shows that alcoholic subjects produced specific antibodies against *S. stercoralis* regardless of the possible immunosuppression caused by chronic alcoholism. Considering that alcoholics are more susceptible to the severe forms of strongyloidiasis, the implementation of immunological methods as a complementary approach to parasitological diagnostics (i.e. detection of IgG, IC and antibody avidity) appears to be an alternative method for early diagnosis in these individuals.

1. Introduction

Human strongyloidiasis is caused by the intestinal nematode *Strongyloides stercoralis* and is considered to be an underestimated global public health problem [1–3]. The global prevalence of *S. stercoralis* has increased in endemic areas [4], with the prevalence in Brazil reaching 5.5% and thus characterising the country as hyperendemic [5].

Strongyloidiasis is usually asymptomatic in immunocompetent individuals; however, it can cause gastrointestinal and pulmonary symptoms and develop into its severe forms such as hyperinfection or disseminated *S. stercoralis* infection [6]. Severe forms were previously associated with specific groups of patients, such as carriers of the human lymphotropic virus [7], individuals infected with human immunodeficiency virus [8], transplant patients [9] and alcoholic subjects [10–13]. Therefore, early detection of strongyloidiasis in these individuals is extremely important [14].

Some studies showed a higher prevalence of *S. stercoralis* infection among alcoholic subjects compared to nonalcoholic subjects [10–13]. The association between alcoholism and *S. stercoralis* infection may be justified by the suppressive effects that alcohol has on both the innate and adaptive immune responses [15–18], through the breakdown of intestinal protective barriers, and worsening nutritional status [13, 19]. The high endogenous cortisol levels found in alcoholic subjects may also increase the differentiation of rhabditiform larvae into infectious filariform larvae since the metabolites produced by corticosteroids resemble the parasite's ecdysteroid hormone. This, in turn, increases the fertility of *S. stercoralis* female parthenogenetic, thus favouring autoinfection [11, 13].

The diagnosis of strongyloidiasis is performed using parasitological techniques; however, the intermittent release of larvae means that these tests only have a high sensitivity after seven samples have been

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analysed and during the acute phase or disseminated form of infection, which consequently results in the underestimation of the infection's prevalence [4, 20]. Optimisation of *S. stercoralis* diagnosis and greater knowledge of a specific serological response or prevalence profile in immunocompromised individuals is a crucial step in the fight against strongyloidiasis so that future complications or fatalities caused by this parasite may be avoided [14]. In this scenario, serological tests for the detection of parasite-specific antibodies may be a useful alternative method [1, 5, 21, 22]. Due to their high sensitivity and specificity for detecting antigens, antibodies and immune complexes (IC) in serum samples, enzyme-linked immunosorbent assays (ELISA) are the most used serological tests [1, 21, 23] and were therefore chosen for this study.

Considering the clinical importance and the need for an early diagnosis of strongyloidiasis in immunocompromised patients, the aims of this study were to evaluate parasite-specific immunoglobulin G (IgG) and immunoglobulin A (IgA) using the detergent fraction from *Strongyloides venezuelensis* third-stage (L3) infective larvae saline extract (SE), assess the levels of circulating IC and determine IgG avidity (ELISAs) in serum samples of alcoholic and nonalcoholic individuals.

2. Materials and methods

2.1. Ethical statement

This cross-sectional study was performed in Uberlândia, Minas Gerais state, Brazil, and data collection occurred between November 2014 and June 2015 after approval from the Federal University of Uberlandia (UFU) Research Ethics Committee (protocol numbers 840.852 and 974.357). All individuals signed an informed consent form before the study.

2.2. Patients and samples

A total of 140 male individuals were divided into two groups: the first group contained 70 alcoholic individuals (31–63 years) who were receiving treatment at the Psychosocial Attention Center – Alcohol and Other Drugs, while the second group contained 70 nonalcoholic individuals (28–63 years) from a Basic Health Unit. Subjects were separated according to the Alcohol Use Disorders Identification Test [24] and the Cut-Down, Annoyed by Criticism, Guilty, Eye-Opener [25] alcohol screening tests which were used to confirm or exclude alcohol abuse or dependence. The AUDIT consists of 10 questions, it identifies alcohol use in the previous 12 months, and it is recommended that all individuals with an AUDIT score ≥ 8 should be considered as a hazardous alcohol drinker; score < 8 classifies individuals as to alcohol use in low risk or abstinence. The CAGE questionnaire was positive when two or more affirmative responses were given to its four questions. With regard to alcohol consumption, the following aspects were also assessed: daily intake, frequency, duration of lifetime consumption and time of abstinence before the beginning of this present study. Among the alcoholic subjects, 0% had clinically evident liver cirrhosis, 94.3% had consumed alcohol for > 20 years and 0% had been abstinent for > 40 days. Individuals from both groups had the same socioeconomic background.

Three stool samples were collected from individuals on alternate days. The samples were processed using the parasitological methods of Hoffman, Pons and Janer [26] and agar plate culture [27] at the Parasitosis Diagnosis Laboratory, Institute of Biomedical Sciences, UFU. The samples were subsequently analysed by three technical experts for the presence of *S. stercoralis* larvae. Blood (5 mL) was collected from each individual, centrifuged to obtain the serum and stored at −20 °C. Serological tests were performed in duplicate to detect anti-*Strongyloides* IgG, IgA and IC.

2.3. Obtaining and preparing total SE from Strongyloides venezuelensis infective larvae

Strongyloides venezuelensis L3 infective larvae were obtained from the faeces of infected Wistar rats (*Rattus norvegicus*) and maintained in charcoal culture at 28 °C for 72 h. Larvae were recovered, concentrated using the method of Rugai et al. [28] and stored at −20 °C until used in antigen preparation.

The production of total SE from *S. venezuelensis* L3 infective larvae was carried out according to Gonzaga et al. [21] Approximately 300,000 *S. venezuelensis* infective larvae were resuspended in 5 mL of phosphate buffered saline (PBS; 0.01 mol/L, pH 7.2) containing protease inhibitors (cOmplete ULTRA mini, Roche, Mannheim, Germany) and ruptured using five cycles of manual maceration with liquid nitrogen. The suspension was centrifuged (12,400 × *g*, 30 min, 4 °C) and the supernatant collected. Total SE was quantified according to Lowry et al. [29] and stored at −20 °C.

2.4. Fractionation of total SE using triton X-114

Total SE from *S. venezuelensis* was subjected to protein fractionation using Triton X-114 according to Feliciano et al. [30] with modifications. Briefly, 200 µL of Tris-buffered saline (TBS; 10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) was added to 1000 µg of total protein along with 1% Triton X-114. The mixture was incubated in an ice bath for 10 min. Subsequently, 6% (w/v) sucrose in TBS (pH 7.4) and 0.06% Triton X-114 were added and the solution was incubated at 37°C for 10 min. The mixture was centrifuged at 3000 x g for 10 min at room temperature (RT). The pellet consisting of the detergent phase was collected and precipitated (1:2, v/v) in acetone at 4 °C before being resuspended in TBS buffer. Protein was quantified according to Lowry et al. [29] and stored at −20 °C.

2.5. Production of anti-S. venezuelensis IgG antibodies

For immune serum production, two rabbits were immunised with *S. venezuelensis* infective larvae alkaline extract. Alkaline extracts were prepared as described previously by Gonçalves et al. [23]. Briefly, 1 mL of 0.15 M NaOH was added to 300,000 *S. venezuelensis* larvae and kept under gentle shaking for 6 h at 4 °C. Subsequently, 0.3 M HCl was added until a pH of 7.0 was reached. The preparation was 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. [29].

The rabbits were immunised with 500 µg/mL of *S. venezuelensis* L3 infective larvae alkaline extract emulsified in Freund's complete adjuvant. After the first immunisation, inoculations were conducted every 2 weeks using Freund's incomplete adjuvant. When the antibody level reached 15,000 µg/mL, 20 mL of blood was collected and parasite-specific IgG antibodies were purified as described by Gonçalves et al. [31].

2.6. Indirect ELISA for the detection of IgG and IgA antibodies

Preliminary experiments were performed to determine the optimal conditions for ELISAs using reagent titration. Briefly, low-affinity polystyrene microtiter plates (Greiner, Bio-One, Frickenhausen, Germany) were coated with 10 µg/mL of the *S. venezuelensis* detergent fraction and incubated overnight at 4 °C in 0.06 M carbonate–bicarbonate buffer (pH 9.6). The plates were subsequently washed three times for 5 min using PBS plus 0.05% Tween 20 (PBS-T) and blocked with PBS-T plus 3% skimmed milk at 37 °C for 30 min. After washing, blocked plates were incubated with serum samples (50 µL/well) at a 1:80 (IgG detection) or 1:20 dilution (IgA detection) and incubated at 37 °C for 45 min. Plates were then coated with either peroxidase-conjugated goat anti-human IgG secondary antibody (Sigma-Aldrich Co., St. Louis, USA) at a dilution of 1:2000 or peroxidase-labelled goat anti-