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Highly specific and sensitive anti-*Strongyloides venezuelensis* IgY antibodies applied to the human strongyloidiasis immunodiagnosis

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ABSTRACT

Due to the epidemiological problem of the neglected condition of human strongyloidiasis, rapid and effective diagnosis is extremely important, with the development of new diagnostic tools being essential to reduce infections and chronic cases. Avian immunoglobulin Y (IgY) technology is an alternative for antibody production that has high specificity and profitability. This study aimed to produce and fractionate IgY antibodies from the egg yolks of hens that were immunized with the total antigenic extracts of *Strongyloides venezuelensis* infectious filariform larvae (iL3) and parthenogenetic females (pF). IgY antibodies were then evaluated by their recognition of antigenic proteins, evolutive helminth forms, and serological diagnosis of human strongyloidiasis by the detection of immune complexes in serum samples. Egg yolks were fractionated to obtain IgY antibodies by thiophilic interaction chromatography. Immune complex detection in serum samples showed diagnostic values for anti-iL3 IgY and anti-pF IgY antibodies at 95.56% and 88.89% sensitivity and 95.56% and 91.11% specificity, respectively. Therefore, IgY technology is a promising tool for the detection of blood circulating *Strongyloides* antigens, with possible application as a serological diagnostic method.

1. Introduction

Strongyloidiasis is a soil-transmitted nematode infection that affects > 300 million people worldwide, mainly in tropical and subtropical areas [1–3]. It is a parasitosis in the conditions of negligence and social disadvantage, which are strongly associated with areas of low socioeconomic status and poor sanitation and infrastructure [4–6].

Due to the parasitological diagnosis of strongyloidiasis presenting low sensitivity because of intermittent larval shedding [7,8], several studies have focused on serological diagnosis. In these studies, they have achieved higher rates of sensitivity and specificity; however, there remain limitations due to cross reactivity with other parasites. In addition, due to the difficulty of obtaining larvae or females of *Strongyloides stercoralis* for antigen preparation, heterologous antigens derived from *Strongyloides venezuelensis* have been used as an alternative in a number of serological diagnostic platforms. [9–13].

Avian immunoglobulins Y (IgY) exhibit functional similarity to mammalian IgG. However, IgY antibodies have a number of advantages when compared to IgG, including the reduction of animals used for antibody production, low cost, a large mass of antibodies produced in the eggs, having no cross-reactions with mammalian Fc receptors, and not activating the complementing system [14,15]. IgY antibodies have already been produced against a number of parasites and pathogenic microorganisms, such as *Taenia solium* [16], *Schistosoma japonicum* [17], *Trichinella spiralis* [18], *Toxoplasma gondii* [19], *Plasmodium falciparum* [20], the influenza virus [21], and the hepatitis A virus [22]. Furthermore, these antibodies have been shown to be useful in antigenic target recognition, diagnostic tests, passive immunizations of humans and animals, and the neutralization of snake venom toxins [23–30].

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To develop a new biotechnological tool in the study of strongyloidiasis, with possible application in the immunodiagnostics of disease in humans, this study aimed to produce specific IgY antibodies against two *S. venezuelensis* life forms. The selected antibodies were fractionated and evaluated for their ability to recognize variable antigenic and life stages of the parasite, and their applicability to the serological diagnosis of human strongyloidiasis by their ability to recognize immune complexes.

2. Material and methods

2.1. Ethics statement

All procedures using experimental animals (*Rattus norvegicus*, *Wistar* rats and *Gallus Gallus domesticus*, hens) were conducted after being approved by the *Comitê de Ética na Utilização de Animais* (CEUA) – *Universidade Federal de Uberlândia* (n° 097/14). The evaluation of the serological diagnosis was performed according to the ethical guidelines of the Brazilian Health Ministry and was approved by the *Comitê de Ética em Pesquisas com Seres Humanos* (CEP) of the *Universidade Federal de Uberlândia* (CAAE: 48492315.8.0000.5152).

2.2. Serum samples

A panel of 135 serum samples were divided into three groups (positives, negatives and other parasites), which contained 45 subjects, which were each from previously parasitologically characterized immunocompetent patients by Baermann-Moraes and Lutz methods [31–33]. All serum samples were selected from the Biological Samples Bank of Laboratório de Parasitologia from Universidade Federal de Uberlândia, Minas Gerais state, Brazil.

Patients with strongyloidiasis (positives) and with other parasitic infections were diagnosed with hookworm ($n = 7$), *Ascaris lumbricoides* ($n = 6$), *Schistosoma mansoni* (n = 6), *Enterobius vermicularis* (n = 6), *Taenia* sp. (n = 6), *Hymenolepis nana* ($n = 4$), *Giardia lamblia* (n = 6), and *Entamoeba histolytica/dispar* (n = 4), were ensured to be infected with only a single parasite. The healthy volunteers (negatives) lived in endemic areas of strongyloidiasis but had no clinical signs and were found to be negative under the Baermann-Moraes and Lutz methods [31–33] of the three separate fecal samples.

2.3. Antigenic preparation

Filariform third-stage larvae (iL3) of *S. venezuelensis* (300,000) were obtained from charcoal cultures of feces from experimentally infected rats (*Rattus norvegicus*). After 3 days at 28 °C, iL3 were recovered as previously described [34] and were centrifuged at 1500 × *g* for 5 min in 0.01 M phosphate-buffered saline (PBS) at pH 7.2. Parthenogenetic females (pF) of *S. venezuelensis* (2500) were obtained after of experimentally infected rats with strongyloidiasis were euthanized by the administration of an anesthetic overdose,. The small intestine was subsequently removed, washed with PBS, longitudinally sectioned, and incubated at 37 °C for 2 h in a Petri dish with PBS [11]. The recovered females were counted as previously described [35].

Total saline antigenic extracts of iL3 and pF *S. venezuelensis* were both produced as previously described [10]. The parasites were resuspended in PBS containing protease inhibitors (complete ULTRA Tablets, Roche, Mannheim, Germany), which was disrupted by 10 cycles of freezing (1 min, −196 °C) and thawing. The suspensions were then centrifuged at 12,400 x*g* for 30 min at 4 °C, and the supernatants were recovered. The protein concentration of the supernatant was then determined by using the previously described Lowry method [36] and was characterized by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (12% SDS-PAGE) [37].

2.4. Hen immunization

Six laying hens of the Isa Brown lineage (*Gallus gallus domesticus*) at 25 weeks of age were used in this study. Hens were kept in individual suspended cages and received commercial poultry feed and water *ad libitum*. They were immunized for IgY polyclonal antibody production, with two groups of immunizations performed with total antigenic extracts of varying *S. venezuelensis* stages (iL3 and pF), and one with 0.01 M PBS at pH 7.2 (control group). All testing groups contained two hens each.

Five intramuscular immunizations in the pectoral muscle were conducted at 14-day intervals. The primary immunization was performed with 100 µg antigenic extracts in 250 µL PBS and an equal volume of Freund's complete adjuvant (Sigma-Aldrich Co., USA). The remaining immunizations were executed with the same antigenic extracts with Freund's incomplete adjuvant (Sigma-Aldrich Co., USA). Hens were monitored daily for adverse effects and inflammatory processes. Blood samples were collected to obtain serum at 14-day intervals, alternating with the immunizations. Eggs of all postures were pooled weekly, and the eggshells were sanitized with 70% ethanol and stored at 4 °C until further processing.

2.5. Fractionation of IgY antibodies

Egg collection started one week before the first immunization and was performed for 10 weeks. Egg yolks from each immunization group were separated from the egg whites and were pooled weekly before the purification of IgY antibodies. The fractionation of IgY antibodies was conducted using the water-dilution method with modifications [38]. Briefly, the egg yolks of each week were diluted 15-fold in ultrapure water adjusted to pH 5.5 with sodium acetate buffer 0.06 M (pH 4.8) and were homogenized overnight at 4 °C. Then, after centrifugation at 800 x*g* for 45 min at 4 °C, the lipid-free supernatant (S1) was collected and adjusted to pH 7.4 with 0.1 M sodium hydroxide. Then, S1 was precipitated with 20% ammonium sulphate (Sigma-Aldrich Co., USA) under slow stirring for 45 min at 4 °C, and subsequently centrifuged at 2000 x*g* for 25 min at 4 °C. The IgY-enriched pellet (P2) was recovered and resuspended in PBS (1:10 initial volume).

The IgY-antibody-enriched samples (P2) were fractionated by affinity liquid chromatography following the manufacturer's instructions. In brief, the P2 samples were washed with 0.02 M sodium phosphate buffer in an ultrafiltration system (Amicon YM 30, Sigma-Aldrich Co., USA) with a 30 kDa cut-off membrane. They were then dialyzed against a 0.5 M K_2SO_4 solution (pH 7.4) and fractionated using an affinity chromatography column (HiTrap IgY Purification HP 5 mL, GE Healthcare, USA) at a flow rate of 1 mL/min. IgY antibodies were eluted using a linear gradient of 0.02 M sodium phosphate buffer (pH 7.2) in an ÄKTA Prime liquid chromatography system (GE Healthcare, USA). The final volume obtained from the fractionated samples was approximately 15 mL (15 fractions of 1 mL each). The eluted fractions were dialyzed with ultrapure water to eliminate the residual salt, and the protein concentration was determined at 280 nm (BioDrop, UK). Samples were lyophilized and then stored at −20 °C until required.

The quality of the fractionation process was assessed by 12% SDS-PAGE under reducing conditions with 5% 2-mercaptoethanol (Sigma-Aldrich Co., USA) and staining with Coomassie Brilliant Blue 250R (Sigma-Aldrich Co., USA).

2.6. ELISA for detection of specific anti-iL3 and anti-pF IgY

The production kinetics and avidity of the fractionated anti-iL3 and anti-pF IgY antibodies from the egg yolks were evaluated by indirect ELISA assays. Alongside IgY detection in egg yolks, the seroconversion of specific IgY antibodies produced by the immunized hens was also evaluated. Low-affinity polystyrene 96-well microplates (Greiner Bio-One, Kremsmünster, Austria) were coated with iL3 and pF antigenic

extracts (10 μg/mL) in 0.06 M carbonate bicarbonate buffer (pH 9.6) and were incubated overnight at 4 °C. Plates were washed 3 times (5 min each) with PBS containing 0.05% Tween 20 (PBS-T) and were blocked with PBS-T plus 1% skimmed milk powder (PBS-T-M 1%) for 1 h at 37 °C. Fractionated samples of anti-iL3, anti-pF IgY (2 μg/mL) and serum samples from the hens (1:200) were diluted in PBS-T-M 1%, added in duplicate, and incubated for 1 h at 37 °C. After 3 washes with PBS-T, the secondary antibody, anti-chicken IgY, produced in rabbit and peroxidase-conjugated (Sigma-Aldrich Co., USA), was diluted 1:15,000 in PBS-T-M 1%, added, and incubated for 1 h at 37 °C. After 3 washes (5 min), the reaction was developed by adding *O*-phenylenediamine (Sigma-Aldrich Co., USA) with 0.03% hydrogen peroxide (Merck, Brazil) diluted in 0.1 M citrate phosphate buffer (pH 5.5) for 15 min. The reaction was then stopped by adding 25 μL of 2 M H_2SO_4 (Vetec, Brazil). The optical density (OD) was determined at 492 nm by an ELISA reader (Thermo Plate, China). Data were expressed as the ELISA index (EI) = OD/cut-off of negative controls plus three standard deviations. To establish the cut-off values for the negative egg yolk controls, the OD from the first six weeks of the control group hens (immunized with PBS) was used and, to establish those for serum, the OD of the first five weeks of the control group (immunized with PBS) was used. Values of EI > 1.0 were considered to be positive.

To evaluate the avidity of the IgY antibodies, microplates were coated and blocked as previously described. After the IgY incubation step, one duplicate was rinsed with 6 M urea (Synth, Brazil) in PBS-T, and the other was rinsed with PBS-T only for 5 min at room temperature. The reaction was developed, stopped, and measured as described for the regular indirect ELISA assay. The avidity results were calculated as the avidity index (AI), which was the ratio between the OD obtained from the wells treated with urea (U^+), and those without urea (U^-) according to the following formula: AI% = OD (U^+/OD U^-) x 100.

2.7. Immunoblotting

Antigenic recognition patterns of fractionated specific IgY antibodies were investigated by immunoblotting assays. The antigenic extracts of *S. venezuelensis* (iL3 and pF) were submitted to 12% SDS-PAGE under nonreducing conditions and were transferred to 0.45 μm nitrocellulose membranes (GE-Osmonics Inc., USA). Membrane strips were blocked with PBS-T plus 5% skimmed milk powder (PBS-T-M 5%) for 2 h at room temperature. Samples of fractionated anti-iL3 and anti-pF IgY (30 μg/mL) from the eighth week were diluted in PBS-T-M 1% and incubated with respective strips overnight under agitation at 4 °C. After 6 cycles of washing, 5 min each, with PBS-T, the strips were incubated with rabbit anti-chicken IgY conjugated with peroxidase (Sigma-Aldrich Co., USA) diluted (1:15,000) in PBS-T-M 1% for 2 h at room temperature. The reactions were revealed by the addition of 10 mg 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB-Sigma Fast tablets, Sigma-Aldrich Co., USA) diluted in tris-buffered saline (TBS 0.02 M, pH 7.4) and 30% hydrogen peroxide (H_2O_2, Merck, Brazil). The reaction was stopped by adding distilled water, and positive reactions were determined by the appearance of defined bands. Fractionated IgY antibodies from the control group hens were used to verify the existence of cross reactions in antigenic recognition under the same conditions as that of the specific IgY antibodies. The relative molecular weights of the recognized protein bands were determined by a comparison with the protein standard marker (Real Biotech, RECOM™ Blue Wide Range Prestain Marker, Banqiao, Taiwan) and were analyzed by ImageJ version 1.44 software (National Institutes of Health, Bethesda, USA).

2.8. Immunofluorescence antibody test (IFAT)

Anti-iL3 and anti-pF IgY from the eighth week were applied in recognition of tissue sections of iL3 and pF of *S. venezuelensis* by indirect immunofluorescence. Both parasites were embedded in Tissue-tek (Sakura Finetek, Zoeterwoude, NL), frozen at −70 °C, sectioned at 2 μm with a cryomicrotome and placed on microscope slides. Each life stage (iL3 and pF) sections were incubated with the specific IgY produced (anti-iL3 and anti-pF) (300 μg/mL) and were diluted in PBS for 30 min at 37 °C. Then, the sections were incubated with secondary antibody produced in rabbit anti-chicken IgY conjugated with fluorescein isothiocyanate (FITC, Sigma-Aldrich Co., USA) (1:300) and were counterstained with 3% Evans blue (Vetec, Rio de Janeiro, Brazil) at 37 °C for 45 min. Between each step, 5 washes (5 min) were performed with PBS. To evaluate the cross reactivity of the fractionated specific IgY antibodies, the same conditions were applied in sections of *Taenia solium* metacestode as a negative control. Fractionated IgY antibodies from the control group hens were incubated with iL3 and pF sections as the reaction control under the same conditions as that of the specific IgY antibodies. The slides were mounted with glycerol/PBS (pH 9.0) and coverslips. The reaction was read using an LSM 510 confocal microscope (Meta, Carl Zeiss, Germany).

2.9. Application in human serological diagnosis

Preliminary tests were carried out to determine the optimal conditions for the ELISA (IgY antibodies, human serum and conjugate). ELISA detection of immune complexes with anti-iL3 and anti-pF IgY antibodies in serum samples was performed as previously described, with modifications [11]. In brief, high-binding 96-well microplates (Greiner Bio-One, Kremsmünster, Austria) were coated with the fractionated IgY antibodies (10 μg/mL) from the eighth week in 0.06 M carbonate bicarbonate buffer (pH 9.6) and were incubated overnight at 4 °C. The plates were then blocked with PBS-T plus 3% skimmed milk for 45 min at 37 °C. Subsequently, serum samples were diluted 1:60 in PBS-T-M 1% and were incubated for 45 min at 37 °C. Then, goat anti-human IgG, Fc specific peroxidase-conjugated (Sigma-Aldrich Co., USA) was diluted 1:2000 in PBS-T-M 1%, added, and incubated for 45 min at 37 °C. Between all steps, 3 washes with PBS-T were carried out for 5 min each. The reactions were developed, stopped, and analyzed as described previously for the regular indirect ELISA assay. Data were expressed as an ELISA index (EI) = OD/cut-off (determined by the TG-ROC curve) plus three standard deviations [39]. To establish the cut-off, the OD values of the healthy subjects and the infected-with-other-parasites subjects were used. Values of EI > 1.0 were considered to be positive.

2.10. Statistical analyses

The data analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, USA). Differences among the groups in the ELISA assay for immune complex detection were analyzed by the Kruskall-Wallis test followed by the Dunn posttest. The threshold for statistical significance was $P < .05$. Diagnostic values of sensitivity (Se), specificity (Sp), and positive and negative likelihood ratios (LR+ and LR-) were calculated according to the formulas: Se (%) = a x 100/(a + c); Sp (%) = d x 100/(b + d); LR+ = Se/(1 – Sp); LR- = 1 – Se/Sp, where “a” is a true positive, “b” is a false positive, “c” is a false negative, and “d” is a true negative. The area under the curve (AUC) was obtained from the ROC curve to determine the diagnostic accuracy of the various methods [40]. 95% confidence intervals (CI) were provided for Se, Sp, and AUC statistical calculations. The ROC curves of the two diagnostic tests were compared as previously described [41].

3. Results

3.1. Fractionation of anti-iL3 and anti-pF IgY antibodies

Hens from each immunization group, despite Freund's protocol, maintained a regular weekly laying pattern throughout the experiment, ranging from 6 to 12 eggs per group. The weekly pooled egg yolks went through three distinct steps of fractionation to obtain the specific IgY

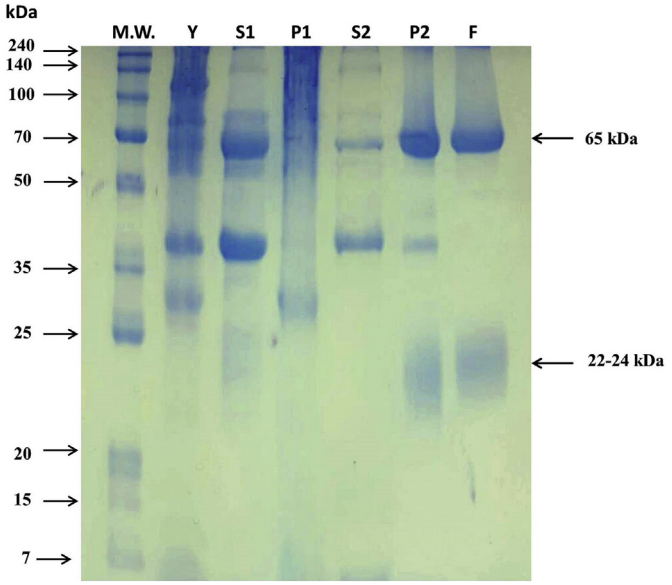

Fig. 1. Representative fractionation steps of IgY antibodies confirmed by electrophoretic profiles in SDS-PAGE 12% (5% 2-mercaptoethanol): crude egg yolk (**Y**); delipidation: **S1**. Water-soluble fraction, **P1.** Lipid-rich precipitate; Protein precipitation: **S2**. Supernatant, **P2**. IgY-enriched fraction; **F**. Fractionation of antibodies on the IgY affinity column in the complete chromatography system. Highlight for bands of heavy (~65 kDa) and light (~22–24 kDa) chains. **M.W.** Molecular weight (kDa).

antibodies. The quality of the antibody fractionation process was observed in the electrophoretic profile of all steps using 12% SDS-PAGE. Furthermore, the addition of 5% 2-mercaptoethanol effectively separated the heavy (~ 65 kDa) and light (~22–24 kDa) chains of IgY molecules (Fig. 1).

Each mL of purified egg yolk yielded approximately 6 mg of the total precipitated protein (P2). Samples were fractionated by thiophilic interaction in liquid chromatography and yielded approximately 3.5–4.5 mg IgY antibodies. Fractions (F) were then pooled, dialyzed, and concentrated for use in subsequent bioassays.

3.2. Monitoring the production of specific IgY antibodies

The specificity and avidity of the IgY antibodies (anti-iL3 and anti-pF) from the fractionated samples were evaluated by indirect ELISA assays. The kinetics of the specific antibodies in the egg yolks and their seroconversion were analyzed from week 0 (pre immunization) until 10 weeks after immunization, and the avidity index (AI) was calculated from the first week, with an EI $>$ 1.0, which was observed until the last week of follow-up.

Polyclonal anti-iL3 IgY antibodies were detected from the third week after the primary immunization (a.p.i) and increased in the following weeks until the peak production, which was observed 7 weeks a.p.i. (Fig. 2A). Anti-pF IgY antibodies were observed from the second week a.p.i. in egg yolks and reached peak production 8 weeks a.p.i. (Fig. 2C). Antibodies from the weeks with higher titers were used in the characterization and applicability tests. Anti-iL3 and anti-pF IgY antibodies presented both high avidity indexes ranging from 68.0% to 82.8% (Fig. 2B) and 74.0% to 95.4% (Fig. 2D), respectively.

3.3. Characterization of IgY antibodies by immunoblotting and IFAT

Immunoblotting assays evidenced the recognition of different polypeptide bands by both antibodies against their specific antigenic target. Anti-iL3 IgY recognized proteins with approximate molecular weights of $>$ 240, $>$ 140, 100, 85, 73, 50, 44, and 25–34 kDa from the antigenic extract of iL3. Antigenic bands of 240, 140, 120, 60 and 32 kDa from the antigen of pF were reactive to anti-pF IgY. No polypeptide bands were observed when the IgY antibodies from the control group hens were applied in iL3 and pF antigenic extracts (Fig. 3). IFAT confirmed the potential of specific polyclonal IgY antibodies in recognition of antigenic targets from different stages of *S. venezuelensis*. Differences in the emitted fluorescence patterns were observed when compared with the negative control (*T. solium* metacestodes) and with nonspecific IgY antibodies (control group hens) in the same iL3 and pF sections. Anti-iL3 and anti-pF IgY antibodies were bound in both the peripheral and internal structures of the parasites (Fig. 4).

3.4. Immune complex detection in human sera

The ELISA indexes of both IgY antibodies (anti-iL3 and anti-pF) showed a significant difference between the sera used ($p < .001$). The Dunn posttest highlighted a significant difference when comparing the EI values from the positive strongyloidiasis patients to both the healthy subjects and patients with other parasites ($p < .001$) in both diagnostic tests (Fig. 5A and C).

Using anti-iL3 IgY in a serological diagnosis, the presence of reactive immune complexes were detected in 95.56% (43/45) of patients with confirmed strongyloidiasis, 6.67% (3/45) of healthy individuals, and 15.56% (7/45) of patients with other parasites, such as 3/45 (6.67%) with hookworm, 1/45 (2.22%) with *S. mansoni*, 1/45 (2.22%) with *Taenia* sp., 1/45 (2.22%) with *A. lumbricoides*, and 1/45 (2.22%) with *E. histolytica/dispar*. Furthermore, the diagnostic values of sensitivity and specificity were 95.56% (95% CI 84.85–99.46%) and 88.89% (95% CI 80.51–94.54%), respectively. Additionally, the test performance that was indicated by the AUC was 0.967 (95% CI 0.942–0.993). In this study, it was found that LR+ was 8.60, which indicated a moderate probability of a true positive strongyloidiasis, whereas LR– was 0.05, which demonstrated a large and conclusive decrease in the probability of the disease (Fig. 5B).

The IgY antibodies of anti-pF detected immune complexes in 95.56% (43/45) of positive patients, 6.67% (3/45) of healthy patients, and 11.11% (5/45) of patients with other parasites, including 2/45 with (4.44%) hookworm, 1/45 (2.22%) with *S. mansoni*, 1/45 (2.22%) with *Taenia* sp., and 1/45 (2.22%) with *E. histolytica/dispar*. The sensitivity, specificity and AUC were 95.56% (95% CI 84.85–99.46%), 91.11% (95% CI 82.23–96.08%) and 0.972 (95% CI 0.948–0.997), respectively. Furthermore, with a calculated LR+ of 10.75, it indicates a large and conclusive probability of a true positive strongyloidiasis, and in addition, an LR- of 0.05 indicates a large and conclusive decrease in the probability of the disease (Fig. 5D). A comparison of the ROC curves of the two different IgY antibodies did not show any significant difference ($z = 1.307$, $p = .1911$) [41].

4. Discussion

Successful immunization of hens with helminthic protein extracts, such as *Trichinella spiralis* [18], *S. japonicum* [42] and recombinant proteins from *Opistorchis viverrini* [27] and *Clonorchis sinensis* [29], were described previously. These studies showed the production of IgY antibodies with high specificity and diagnosis applicability. In addition, they showed an ability to detect circulating antigens in serum and feces samples from humans and experimental animals. In this study, for the first time, the production of specific IgY antibodies against protein extracts from iL3 and the pF of *S. venezuelensis* were applied to experimental tests and the immunodiagnosis of human strongyloidiasis.

The fractionation protocol that was used showed a high yield of 3.5–4.5 mg IgY antibodies from 1 mL egg yolk. These values correspond to the estimates of approximately 2–8 mg/mL IgY antibodies from purified yolks that were passively transferred *via* the ovarian follicle of immunized chickens [43]. Additionally, thiophilic-binding chromatography provided a high level of purity of the samples that were

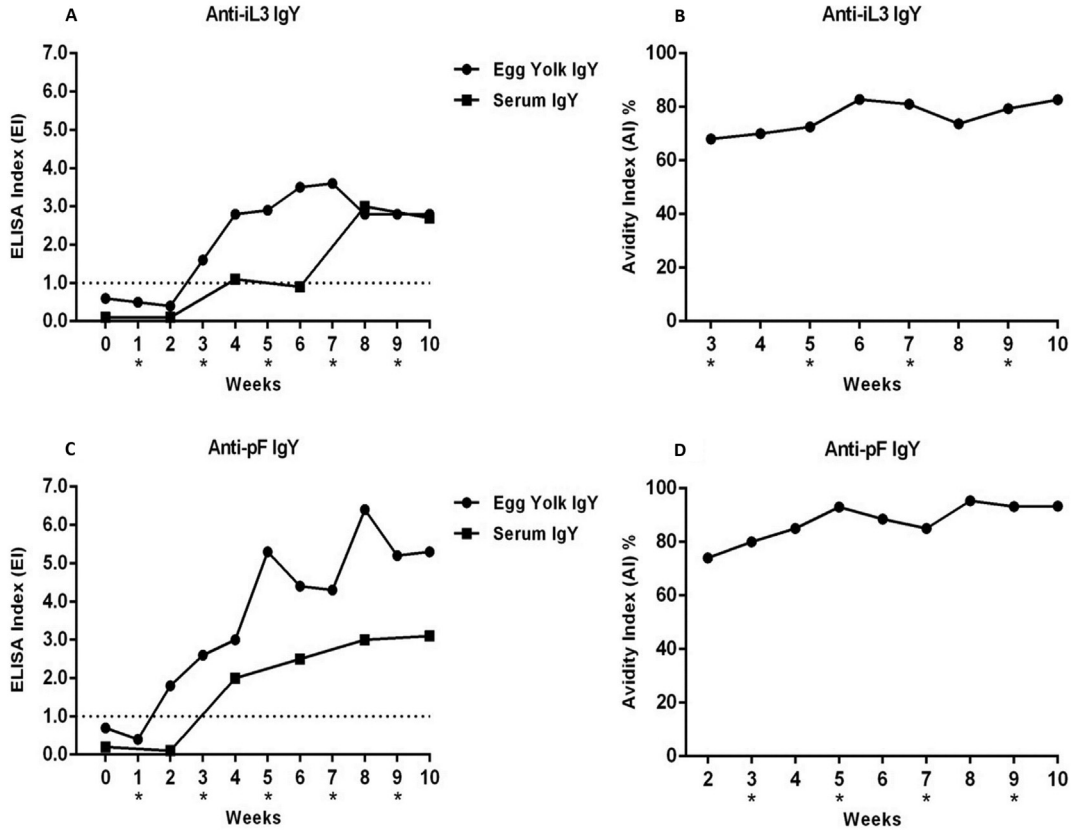

Fig. 2. A, **C**. Indirect ELISA for monitoring the production kinetics of specific IgY antibodies from egg yolks and the serum of immunized hens; **B**, **D**. Indirect ELISA of avidity index (%) of the egg yolk IgY antibodies treated with urea 6 M; **A**, **B**. IgY antibodies specific to *S. venezuelensis* filariform larvae (iL3); **C**, **D**. IgY antibodies specific to *S. venezuelensis* parthenogenetic females (pF). *: Immunizations.

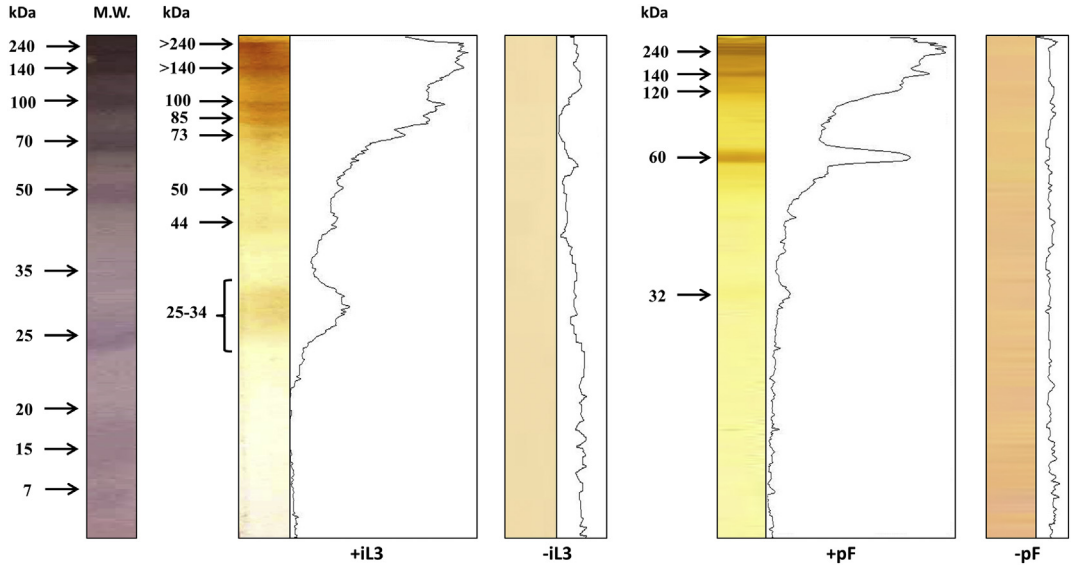

Fig. 3. Immunoblotting presenting the recognition of polypeptides from antigenic extracts of *S. venezuelensis*. **+iL3**. filariform larvae (iL3) antigen incubated with anti-iL3 IgY; **−iL3**. filariform larvae antigen incubated with IgY antibodies from the control group hens; **+pF**. parthenogenetic females (pF) antigen incubated with anti-pF IgY; **-pF**. parthenogenetic females antigen incubated with IgY antibodies from the control group hens. **M.W.** molecular weight. Band intensities were analyzed using ImageJ 1.48.

obtained [44]. This is seen by the absence of variable protein bands of IgY molecules in the samples when they were analyzed by SDS-PAGE. Furthermore, after the third week, the transfer of the specific antibodies to the yolks had already been detected and increased during the following weeks with the application of booster immunizations. The high degree of interaction between IgY antibodies and antigenic extracts was then confirmed by the avidity index [45]. The specificity of the antibodies that were produced was confirmed by recognition of the antigenic targets that were immobilized in different immunological assays. Application in immunoblotting and immunofluorescence assays for *T.*

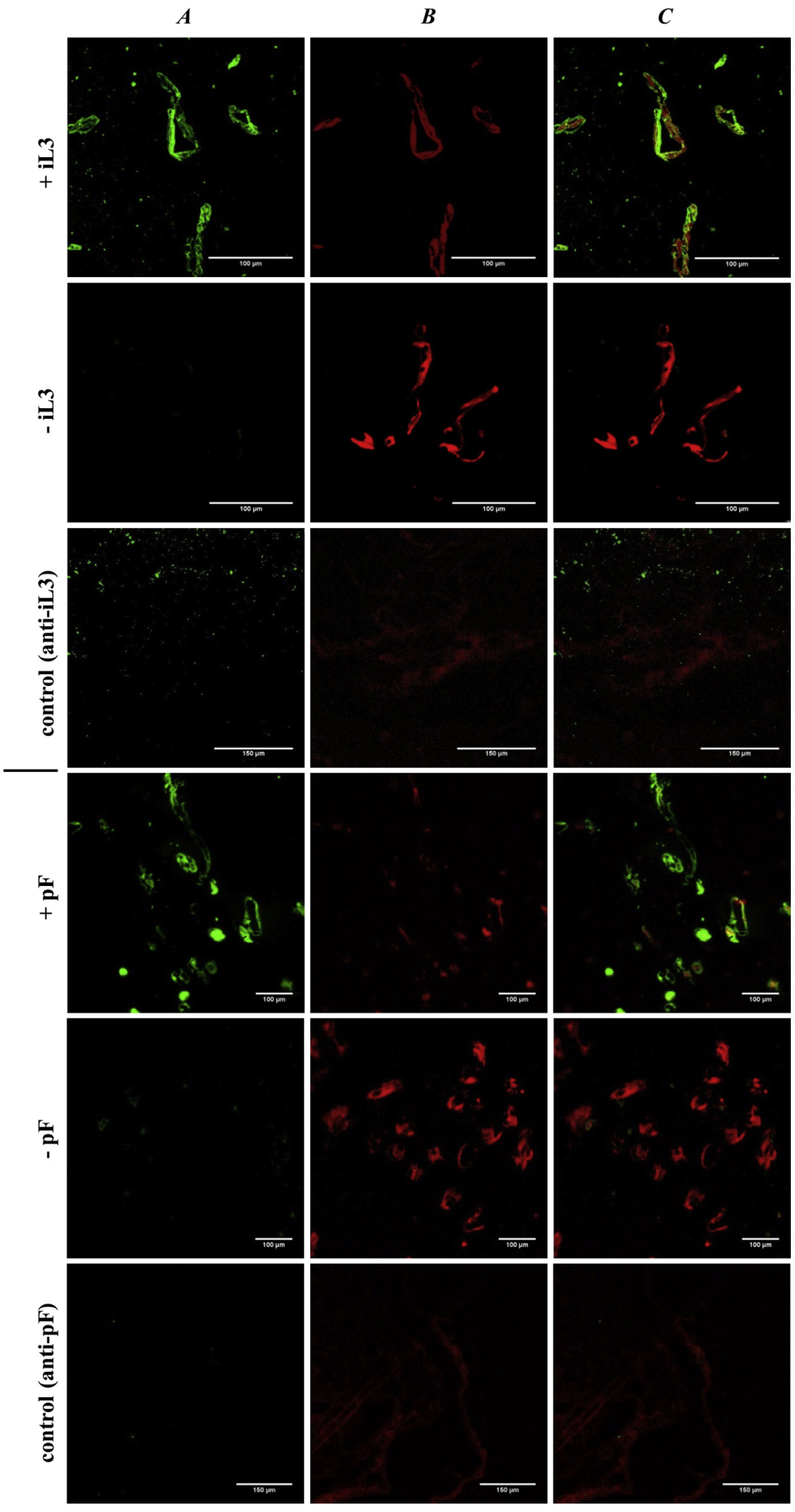

Fig. 4. Immunofluorescence antibody test (IFAT) using fractionated polyclonal IgY antibodies in *Strongyloides venezuelensis* sections (iL3 – filariform larvae; pF – parthenogenetic females) or in the control (*Taenia solium* metacestodes sections). **+iL3**. filariform larvae sections incubated with anti-iL3 IgY; **−iL3**. filariform larvae sections incubated with IgY antibodies from the control group hens; **control (anti-iL3)**. *T. solium* metacestodes sections incubated with anti-iL3 IgY; **+pF**. parthenogenetic female sections incubated with anti-pF IgY; **-pF**. parthenogenetic female sections incubated with IgY antibodies from the control group hens; **control (anti-pF)**. *T. solium* metacestodes sections incubated with anti-pF IgY. Column ***A*** (green): anti-chicken IgY fluorescein isothiocyanate conjugated (FITC); ***B*** (red): Evans blue counterstained; ***C*** (merged): from column 1 to 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

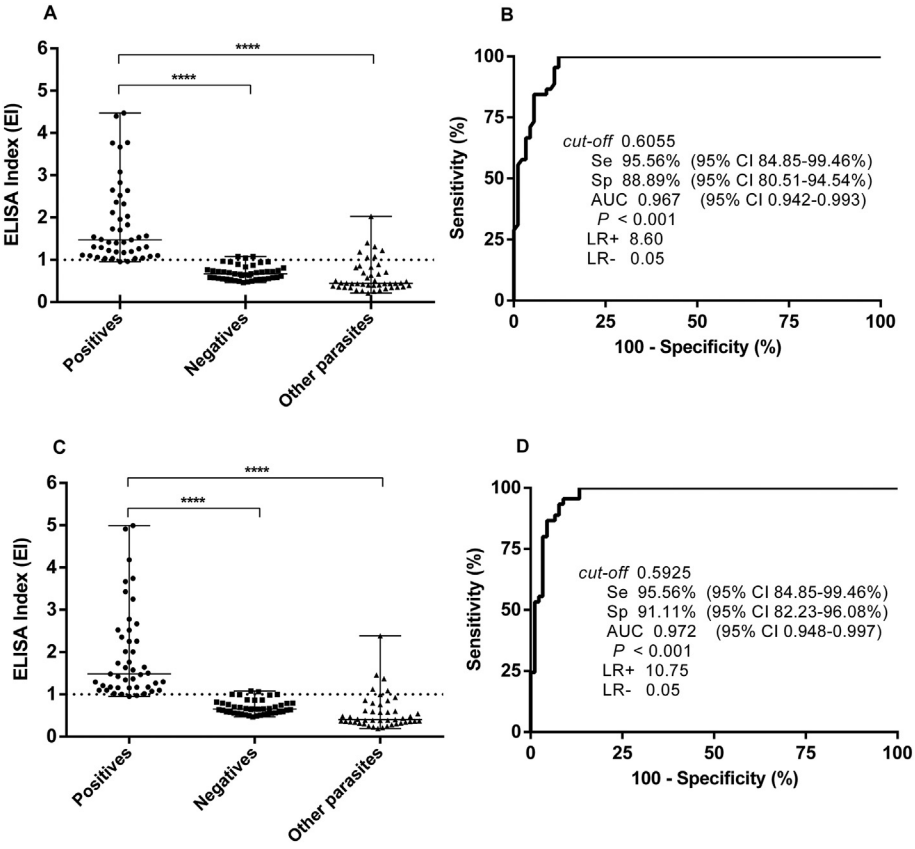

Fig. 5. ELISA for detection of immune complexes using specific IgY antibodies (**A**, **B**. anti-iL3 IgY; **C**, **D**. anti-pF IgY) in serum samples from strongyloidiasis-positive patients ($n = 45$), healthy individuals (n = 45) and individuals with other parasitic diseases (n = 45); **A**, **C**. ****- Kruskal-Wallis, with Dunn posttest ($P < .0001$); **B**, **D**. Receiver operating characteristic curves (ROC) indicating cut-off values, sensitivity (Se), specificity (Sp), area under the curve (AUC), likelihood ratios (LR + and LR-).

gondii has already been applied by Ferreira-Junior et al. [19].

Further studies on the characterization of the major antigenic targets recognized by IgY antibodies should be performed. After sequencing, antigenic proteins can be used for recombinant production and diagnostic application. In this context, recombinant antigens from *S. stercoralis* filariform larvae of 5a and 12a [46], and a 31 kDa (NIE) antigen derived from a cDNA library [47] were produced and evaluated upon immunodiagnostic of human strongyloidiasis.

Given the importance of a rapid and effective diagnosis of strongyloidiasis to avoid hyperinfections and chronic cases and the lack of a gold standard parasitological test, several studies on the immunodiagnostic of strongyloidiasis have been described. In these studies, total, fractionated, and recombinant antigenic preparations were used for the detection of specific circulating antibodies (IgG, IgA, and IgE) in serum and saliva samples [9,10,48]. However, detection of immune complexes in serum samples is another possible alternative for the diagnosis of strongyloidiasis. Therefore, rabbits have already been used for the production and purification of IgG antibodies specific to the antigens, iL3 and pF, of *S. venezuelensis* [11,49]. Rabbits have also been used for the production of specific antibodies against the larvae and adults of *Strongyloides ratti*, which were used for detection of coproantigens in experimentally infected rats [50]. Furthermore, the generation of scFv against proteins from *S. venezuelensis* iL3 highlighted the identity of various immune complexes in human strongyloidiasis [12]. In this study, the potential use of specific polyclonal IgY antibodies in the detection of immune complexes was evaluated in serum samples from patients who are infected with *S. stercoralis*.

The anti-iL3 and anti-pF IgY antibodies presented similar performances in the detection of immune complexes, with both antibodies detecting two false-positive individuals within the group of healthy individuals, and five patients with other parasitic diseases cross-reacted in both diagnostic tests. Additionally, anti-iL3 IgY recognized two more serum samples 7/45 (15.56%) with parasitic infections other than anti-pF IgY, hookworm and *A. lumbricoides.* These results are in contrast to previous research that was conducted by Gonçalves et al. [11], in which 6 out of 30 samples tested with rabbit IgG anti-iL3 were shown to be false positives in individuals with *A. lumbricoides*, *E. vermicularis*, hookworms, and *S. mansoni* and 11 out of 30 false positives in individuals with *E. vermicularis*, *A. lumbricoides*, *G. lamblia*, and *S. mansoni* with rabbit IgG anti-pF. This highlights a lower occurrence of cross-reactivity in serological diagnosis using IgY antibodies compared to that using rabbit IgG antibodies. The serum samples that were used in the study may have come from patients with other parasitic infections that are better detected by using serology, such as filariasis and toxocariasis, which were not evaluated. Since serum samples from patients with these parasites are not available, it was not possible to determine the cross-reactivity indexes for filariasis and toxocariasis. Furthermore, the diagnostic values of sensitivity and specificity for both IgY antibodies and rabbit IgG against the antigenic extracts of iL3 of *S. venezuelensis* presented few differences; however, the hen IgY antibodies against extracts of pF showed higher values (Se: 95.56%; Sp: 91.11%) compared to the rabbit IgG antibodies (Se: 66.6%; Sp: 72.3%). However, several studies have considered antigen preparations derived from the iL3 of *Strongyloides* sp. to be more relevant to the serological diagnosis of strongyloidiasis compared to the preparations that were derived from pF [48,51,52].

5. Conclusion

In this study, hens that were immunized with antigenic extracts of *S. venezuelensis* were able to produce IgY antibodies with high yield and specificity. This technology proved once again its methodological consistency and applicability in several experimental models. Moreover, polyclonal IgY antibodies from egg yolks should also be considered to be another important tool for the study of experimental strongyloidiasis, epidemiological surveys, and the serological diagnosis of the disease. In addition, the results of this study demonstrate the importance of more studies on proteins derived from parthenogenetic

females, which is a persistent life stage of the infection, that established in the intestinal mucosa of the hosts. Future studies should evaluate the potential of IgY antibodies to select mimetic peptides to the parasite and in therapeutic trials of strongyloidiasis.

Declaration of interest

All authors disclose that there was no conflict of interest of any type from the beginning of the work to the submission of manuscript.

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