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SEROPOSITIVITY FOR ASCARIOSIS AND TOXOCARIOSIS AND CYTOKINE EXPRESSION AMONG THE INDIGENOUS PEOPLE IN THE VENEZUELAN DELTA REGION

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SUMMARY

The present study aimed at measuring seropositivities for infection by *Ascaris suum* and *Toxocara canis* using the excretory/secretory (E/S) antigens from *Ascaris suum* (AES) and *Toxocara canis* (TES) within an indigenous population. In addition, quantification of cytokine expressions in peripheral blood cells was determined. A total of 50 Warao indigenous were included; of which 43 were adults and seven children. In adults, 44.1% were seropositive for both parasites; whereas children had only seropositivity to one or the other helminth. For ascariosis, the percentage of AES seropositivity in adults and children was high; 23.3% and 57.1%, respectively. While that for toxocariosis, the percentage of TES seropositivity in adults and children was low; 9.3% and 14.3%, respectively. The percentage of seronegativity was comparable for AES and TES antigens in adults (27.9%) and children (28.6%). When positive sera were analyzed by Western blotting technique using AES antigens; three bands of 97.2, 193.6 and 200.2 kDas were mostly recognized. When the TES antigens were used, nine major bands were mostly identified; 47.4, 52.2, 84.9, 98.2, 119.1, 131.3, 175.6, 184.4 and 193.6 kDas. Stool examinations showed that *Blastocystis hominis*, *Hymenolepis nana* and *Entamoeba coli* were the most commonly observed intestinal parasites. Quantification of cytokines IFN-γ, IL-2, IL-6, TGF-β, TNF-α, IL-10 and IL-4 expressions showed that there was only a significant increased expression of IL-4 in indigenous with TES seropositivity ($p < 0.002$). *Ascaris* and *Toxocara* seropositivity was prevalent among Warao indigenous.

KEYWORDS: Zoonoses; *Ascaris suum; Toxocara canis;* Warao.

INTRODUCTION

Infections with gastrointestinal nematode parasites are widespread and contribute significantly to both morbidity and mortality among humans, and livestock in developing countries[45]. The most prevalent parasitic helminth in humans, *Ascaris lumbricoides,* is estimated to infect 1.5 billion people globally[4]. *Toxocara canis* and *Ascaris suum* are roundworms of dogs and pigs, respectively; these are the causative agents of important zoonoses such as toxocariosis and ascariosis[2,4]. Humans may accidentally become infected with *T. canis* or *A. suum* after ingestion of embryonated eggs present in soil contaminated with dog or pig feces or after consumption of infected raw or undercooked meat[2,45]. In the accidental hosts, the *T. canis* larvae do not develop to the adult stage but persist in tissues as the larval stage for many years[33,34]. Once the infective eggs are ingested the larvae hatch, penetrate the small intestine and migrate to different tissues in the body inducing inflammatory responses. Migration of larvae can lead to a syndrome known as Visceral Larva Migrans (VLM). Symptoms of VLM include fever, hepatosplenomegaly and respiratory distress such as wheezing, coughing and episodic airflow obstruction[34,38]. Other symptoms include eosinophilic pneumonia (Loeffler's pneumonia) that bears a clinical resemblance to the pulmonary inflammatory responses observed in asthmatic patients. Immunological features of these zoonoses include eosinophilia and increased serum IgE levels[30,36,40].

Diagnosis of these zoonoses depends mostly on serological tests because the eggs are not passed in the feces of the host and biopsies to detect the larvae are usually negative[11,12]. Since the studies made by SAVIGNY[37], the antigens mostly used for the immunodiagnostic tests are excreted products derived from larvae cultivated *in vitro* and are referred to as *Toxocara* excretory/secretory (TES) antigens[35,37]. Nematode excretory/secretory (E/S) antigens are not species or genus specific and serum samples from patients with ascariosis, filariosis and strongyloidiosis show cross-reactivity with ES from *T. canis* and *A. suum* antigens when using enzyme-linked immune assay (ELISA), immunoprecipitation and Western blotting[3,5,18,20,21,29,37]. A major concern is the specificity of the ELISA and WB diagnosis of *T. canis* and *A. suum* in areas where gastrointestinal nematode infections of humans also exist. In this context, in most areas in which *A. lumbricoides* is endemic, which is a common intestinal nematode of children, exposure to *A. suum* and

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T. canis is likely to be sufficiently common to confuse serodiagnosis. In Venezuela, diagnosis of zoonotic infections with *A. suum* and *T. canis* is routinely made by ELISA after absorption of serum samples with *A. lumbricoides* antigens, a nematode antigenically related with *A. suum* and *T. canis*[6,22].

Although parasitic helminth infections generally do not lead to mortality; chronic infections can lead to considerable morbidity[25,31]. Chronic helminth infections are characterized by skewing towards a T helper 2 type response as well as regulatory responses[8,43]. The regulatory network is thought to prevent strong immune responses against parasitic worms, allowing their long-term survival and restricting pathology. A number of parasitic nematodes have also been reported to exert potent immunomodulatory effects also suppressing immune responses to non-parasite antigens and to other infectious agents in a nonspecific manner[30,41]. PATERSON *et al.* have reported that the body fluid from the adult *Ascaris suum* (ABF) has potent immunomodulatory activity and that the effects observed are consistent with skewing towards Th2-type response[32,40]. In addition, the induction of interleukin-10 by ABF also suggests that T regulatory cells may play a role in immunomodulation of immune responses by parasitic helminths[32].

The present study aimed to measure anti-*Ascaris suum* and anti-*Toxocara canis* antibodies in sera through ELISA and Western blotting techniques. In addition parasitological examination from stool samples and the cytokine gene expression of pro-inflammatory and Th1/Th2-type cytokines in blood of Warao indigenous, a population that live closely with pigs and dogs, were performed.

MATERIALS AND METHODS

The study was conducted in Warao indigenous communities from the Antonio Díaz and Pedernales municipalities, in the Venezuelan Delta, two parasitic-endemic rural regions of the Delta Amacuro State. The Warao indigenous communities are settled in remote rural areas. The houses are constructed on wooden stilts on the River Orinoco. Most inhabitants do not have access to reliable potable water for drinking nor adequate sanitation systems. Within this indigenous population is not easy to carry out research projects because invasive procedures cannot be used to take samples due to ethical considerations. Adults and children aged 15 to 70 and four to 14, respectively, were studied, of whom according to ELISA reactivity were grouped as: 1) Sera positive for the excretory/secretory (E/S) antigens of *Ascaris suum* (AES), 2) Sera positive for the E/S antigens of *Toxocara canis* (TES), 3) Sera positive for both, AES and TES antigens and 4) Sera negative for both AES and TES antigens which were used as a negative reference (control group).

Blood samples were collected in vacutainers with and without EDTA as anticoagulant. Serum was separated and stored at -20 °C until use. Individuals were included in the study taking into account inclusion and non inclusion criteria. Inclusion criteria: 1) The volunteers are healthy individuals without evidence of clinical symptoms suggesting pulmonary infection. Non inclusion criteria: 1) Individuals who were HIV positive, 2) Patients taking immunosuppressive drugs (e.g., corticosteroids, azathioprine and cyclophosphamide), 3) Participants who did not sign an informed consent agreement. This study was approved by the Ethical Committee of the Biomedicine Institute (protocol number PG-09-8007/2011). All the inhabitants who participated were included after obtaining a free and informed consent statement from them.

The excretory-secretory (E/S) antigen derived from *Toxocara canis* and *Ascaris suum* was prepared as previously described[33]. Briefly, adults of *T. canis* and *A. suum* worms were collected from the feces of naturally infected dogs and pigs, after routine deworming using antihelminthic treatment. Eggs were collected from the uteri of female worms and were allowed to embryonate in 0.05 M H2SO4 in the dark at room temperature for 4-6 weeks. Embryonated eggs were stored in 0.05 M H2SO4 at 4 °C until use. Larvae were freed from the egg shells and allowed to migrate through cotton wool contained in Pasteur pipettes that were placed in tubes filled with medium at 37 °C overnight. The migrating larvae were collected and counted. A suspension of 150 larvae per mL medium was incubated at 37 °C. Fresh medium was added and after a week, the harvested medium was used as the E/S antigens.

Detection of anti-*Toxocara* and anti-*Ascaris* IgG antibodies was performed using an ELISA and the excretory/secretory (E/S) antigens derived either from *T. canis* or *A. suum* larvae as previously reported[33]. Medium binding ELISA microtiter plates (Nunc, Roskilde, Denmark) were used for the *Toxocara* ELISA and high binding plates (Greiner, Frickenhausen, Germany) were used for the *Ascaris* ELISA. The plates were coated with E/S antigens (10 µg/mL) diluted in 0.1 M sodium carbonate (Na2CO3), pH 9.6. The plates were incubated overnight (without lids) at 37 °C to allow the E/S antigens to dry onto the wells. They were then washed three times with phosphate-buffered saline (pH 7.2) containing 0.05% v/v Tween-20 (PBS/Tween). For the *Ascaris* ELISA an additional blocking step was performed by adding 2% bovine serum albumin (BSA) (Boehringer Mannheim, GmbH, Germany) solution in PBS/Tween to every well. The plates were incubated for 30 min at 37 °C and thereafter washed three times with PBS/Tween. Serum samples were diluted 1:40 in 2% BSA/PBS/Tween and added to the plates. After one hour incubation at 37 °C, the plates were washed and anti-human IgG conjugated to alkaline phosphatase (DAKO, Glostrup, Denmark) diluted in 4% BSA/PBS/Tween was added for one hour at 37 °C. After the plates were washed, substrate, H_2O_2, 0.05% and 5-ASA was added for one hour at room temperature after which the absorbance was read at 450 nm. The extinction value of the tested serum and of the cut-off serum was used to calculate a ratio. A ratio higher or equal to 1.0 was considered positive. The cut-off value was defined as the mean absorbance of 20 serum samples from healthy blood donors plus three times the standard deviation[33].

Electrophoresis procedure was performed; TES and AES antigens were fractionated by polyacrylamide gel electrophoresis with dodecil sulfate (SDS-PAGE) according to LAEMMLI[17] 10% running gel and a 4% acrylamide stacking gel were used. 1.2 µg of TES or AES antigens was mixed with sample buffer (Tris 0.5 M, pH 6.8; 10% SDS, 0.4 mL of 2-mercaptoethanol, 0.3% bromophenol blue, 2 mL of glycerol) for one min and applied to a polyacrylamide gel. The sample was electrophoresed with constant voltage (100 V) until bromophenol blue had entered the running gel when it was increased to 120 V. Transfer buffer, pH 8.3, contained three g of Tris base, 14.4 g of glycine and one g of SDS. Molecular weight standards (Sigma SDS-200) were included to calculate molecular weights.

Transfer was performed according to TOWBIN *et al.*[42] in a Mini-protean II cell (Bio-Rad Laboratories, CS, US) using 180 mAmp

applied over two hours to a nitrocellulose membrane in transfer buffer. Nitrocellulose strips containing transferred proteins were rinsed with PBS and incubated for one hour with PBS-Tween and 5% skimmed milk to block remaining free sites and test sera diluted 1:100 overnight. Following three washes with PBS-Tween to remove unbound antibody, strips were incubated for one hour in anti-human IgG conjugated with peroxidase diluted 1:2000 (Vector lab, Inc). After three washes with PBS-Tween and 5% skimmed milk, TMB substrate KIT containing two drops of buffer stock solution, three drops of tetramethylbenzidine (TMB), two drops of stabilization solution and two drops of hydrogen peroxide was added and bands were visible within 5-15 min (peroxidase substrate kit TMB SK-4400, Vector lab., Inc). To prevent cross-reactions, sera tested in the *Toxocara*-ELISA and *Ascaris*-ELISA were pre-absorbed with *A. suum* or *A. lumbricoides* extracts diluted 1:50 in PBS with 5% skimmed milk, at room temperature.

For coproparasitological tests, two stool samples per individual were taken and collected fresh or preserved in Railliet-Henry solution at room temperature. Stool samples collected were analyzed using the gravitational sedimentation, Kato-Katz techniques and two fecal smears per individual were analyzed by direct microscopic observation to detect eggs of helminth and also protozoan cysts. The results of the stool examinations were provided to patients and the parents, and appropriate treatment for parasite infections was given.

As regards relative quantification of cytokine expressions, total RNA was extracted from peripheral blood cells by using a Total RNA Isolation System kit (Promega Corporation, WI, US) following the instructions of the supplier, and the RNA content was measured in a spectrophotometer at 260 nm. cDNA was made from five micrograms RNA using a Reverses Transcription System kit (Promega Corporation, WI US). The RNA was incubated with a one µL of oligo dT primer (50 µM), made up to 12 µL with sterile and RNaseOut-free water, and incubated at 70 ºC for 10 min, after which it was quickly cooled on ice. A total of two µL 10X first-strand buffer (100 mMTris-HCl, pH 8.8 at 25 ºC; 500 mMKCl; 1% Triton X-100), two µL $MgCl_2$ (25 mM), two µL deoxynucleoside trisphosphate mix (10 mM of each dATP, dGTP, dCTP and dTTP) and one µL of RNaseOutRNase inhibitor (40 U/µL) were added. The mix was incubated at 42 ºC for two min after which it was further incubated at 42 ºC for 50 min and 70 ºC for 10 min with one µL of AMV Reverse Transcriptase. The cDNA content was measured in a spectrophotometer at 280/260 nm. The cDNA samples were stored at -80 °C until use.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed, 25 nanograms of cDNA generate as above was amplified and made up to 10.5 µL with sterile and nuclease-free water. Twelve and a half µL of the master-mix containing the PCR buffer (50 mMTris-HCl, pH 9; 50 mMNaCl; 5 mM $MgCl_2$; 200 µM of each deoxynucleoside trisphosphate, dATP, dGTP, dCTP and dTTP) and the Taq DNA polymerase (50 U/µL) and one µL of each primer (five mM) was added in a final volume of 25 µL. Sequences of the used primer pair are shown in Figure 1. Mixtures with cDNA were placed in a MJ mini Personal Thermal Cycler (BioRad Laboratories, CA, US) preheated to 95 ºC for 10 min. Cycling parameters were 40 cycles of denaturation at 95 ºC for 15 sec, annealing at 60 ºC for one min, extension at 70ºC for one min and a final extension for seven min at 72 ºC. For IL-2 and β-actin the annealing was carried out at 58 ºC and 55 ºC respectively. Amplified products were separated by 2% agarose gel electrophoresis, stained with SYBR Green I (Sigma-Aldrich Co, St. Louis MO, US) and visualized by a Benchtop UV transilluminator, MultiDoc-It Digital Imaging System camera combination.

IL-2	Forward: 5'-AAGTTTTACATGCCCAAGAAGG-3' Reverse: 5'-AAGTGAAGTTTTTGCTTTGAGC-3'
IL-4	Forward: 5'-CACCGAGTTGACCGTAACAG-3' Reverse: 5'-GCCCTGCAGAAGGTTTCC-3'
IL-6	Forward, 5'-ATGTAGCCGCCCACACAGA-3' Reverse, 5'-CATCCATCTTTTTCAGCCAT-3'
IL-10	Forward, 5'-ACAGGGAAGAAATCGATGACA-3' Reverse, 5'-TGGGGGAGAACCTGAAGAC-3'
IL-12p35	Forward, 5'-CACTCCCAAAACCTGCTGAG-3' Reverse, 5'-TCTCTTCAGAAGTGCAAGGGTA-3'
IFN-γ	Forward, 5'-TTTGGATGCTCTGGTCATCTT-3' Reverse, 5'-TTTGGATGCTCTGGTCATCTT-3'
TGF-β	Forward, 5'-CAGCCGGTTGCTGAGGTA-3' Reverse, 5'-GCAGCACGTGGAGCTGTA-3'
TNF-α	Forward, 5'-GCCAGAGGGCTGATTAGAGA-3' Reverse, 5'-CAGCCTCTTCTCCTTCCTGAT-3'
β-actin	Forward, 5'-GTGGGGCGCCCCAGGCACCA-3' Reverse, 5'-CTCCTTAATGTCACGCACGATTTC-3'

Fig. 1 - Primers sequences. The following primer pairs were used: IL-2, IL-4, IL-6, IL-10, IL-12p35, IFN-γ, TGF-β, TNF-α and β-actin as internal control.

Statistical analysis was carried out using the software Epi-Info 6.0. Chi-square test was used to compare the significance of the differences according to the percentage values of seropositivities for infection by *Ascaris suum* (AES) and *Toxocara canis* (TES). A logistical regression method was used to compare independent variables including cytokine expression in peripheral blood cells from Warao indigenous with or without antibodies against the AES and TES antigens. A probability value $p < 0.05$ was considered statistically significant.

RESULTS

Study population. Forty three adults aged 40 ± 16 years old (25 females and 18 males) and seven children aged 9 ± 2.6 years old (four females and three males) were studied (data not shown).

Comparable seropositivity for ascariosis and toxocariasis. The percentage of individuals with AES and TES seropositivity is shown in Table 1. Findings indicate that in adults, 19/43 (44.1%) were seropositive for both parasites, whereas children had only seropositivity for one or the other helminth, 0/7 (0%), there was significant difference between adult and children groups, $p < 0.03$ (Table 1). For ascariosis, the percentage of AES seropositivity in adults and children was 10/43 (23.3%) and 4/7 (57.1%), respectively (Table 1). While that for toxocariasis, the percentage of TES seropositivity in adults and children was low; 4/43 (9.3%) and 1/7 (14.3%), respectively, there was no significant difference among groups. The percentage of seronegativity was comparable for AES and TES antigens in adults 12/43 (27.9%) and children 2/7 (28.6%) (Table 1).

Table 1
Comparable seropositivity for *Toxocara canis* and *Ascaris suum*

Marker	*A. suum* Positive	*T. canis* Positive	*A.suum/T. canis* Positive	*A.suum/T. canis* Negative
Female (%)	57.1	20.0	42.1	80.0[(c)]
Male (%)	42.9	80.0	57.9	20.0[(d)]
Adults (%)	23.3	9.3	44.1[(a)]	27.9
Children (%)	57.1	14.3	0[(b)]	28.6

For the AES and TES seropositivity group there was significant difference between (a) and (b), $p < 0.03$. Within the seronegativity group there was significant difference between females (c) and males (d), $p < 0.002$.

Gender difference in ascariosis and toxocariosis. When the possible gender effect on seropositivity for *A. suum* and *T. canis* was analyzed, findings showed that gender has no influence on either AES seropositivity; 8/14 (57.1%) and 6/14 (42.9%) for females and males, respectively, or AES/TES seropositivity; 8/19 (42.1%) and 11/19 (57.9%) for females and males, respectively (Table 1). In contrast, a high percentage of males showed TES seropositivity 4/5 (80.0%) as compared to females 1/5 (20.0%), there was not a significant difference, probably due to the low number of individuals that composed these groups (Table 1). Within the seronegative group there was a significant difference between females 12/15 (80.0%) and males 3/15 (20.0%), $p < 0.002$ (Table 1).

Age distribution and seropositivity for *Ascaris* and *Toxocara*. The possible age effect on seropositivity for *A. suum* and *T. canis* was also analyzed. The distribution of the age group was: 0-10, 11-20, 21-40 and 41-60 years old and the age group of 61 years and older. The percentage of *A. suum* seropositivity ranged from 7.7% for the age group of 0-10 years old to 30.8% for the age group of 21-40 years old. The latter showed a higher percentage of *A. suum* seropositivity statistically significant as compared to the age groups of 0-10 (7.7%), 11-20 (10.3%) years old and 61 years and older (7.9%), $0.01 < p < 0.04$. There was no significant difference between the age groups of 21-40 (30.8%) and 41-60 (17.9%) years old (data not shown).

The percentage of *T. canis* seropositivity ranged from 2.6% for the age group 0-10 years old to 25.6% for the age group of 21-40 years old. The latter showed a higher percentage of *T. canis* seropositivity statistically significant as compared to the age groups of 0-10 (2.6%), 11-20 (5.1%) years old and the age group of 61 (6.7%) years and older, $0.006 < p < 0.02$. There was no significant difference between the age groups of 21-40 (25.6%) and 41-60 (10.3%) years old. The seropositivity thereafter clearly decreases for the age group of 61 years and older (data not shown).

Western blotting and band patterns. In order to identify the pattern of bands displayed by sera from Warao; AES and TES antigens were analyzed by Western blotting technique. For *A. suum*, the immunoblotting showed a pattern of bands ranging from 52 to 206.9 kDas, 12 bands of 206.9, 200.2, 193.6, 149, 114.6, 97.2, 94.1, 82.5, 67.8, 65.6, 55.6 and 52.2, kDas from *A. suum* were detected; of these, three bands of 193.6 kDa (33.3%), 200.2 kDa and 97.2 kDa (22.2%) were principally recognized. Sera from healthy individuals did not show any IgG reactivity (data not shown). Western blotting procedures showing the obtained bands and the WB profile according to AES seropositivity are shown in Figure 2. Western blotting analysis using AES antigens showed the AES pattern composed by bands ranging from 52 kDa to 206.9 kDa (some of the most frequently recognized bands are shown in Figure 2A). The WB profile could be divided in the following groups according IgG reactivity to AES antigens; the group composed of bands between 50 kDa and 100 kDa was the most reactive (50%), followed by 151-207 kDas (37.5%) and 101-150 kDas (12.5%) (Fig. 2B).

As regards *T. canis*, the immunoblotting showed a pattern of bands,

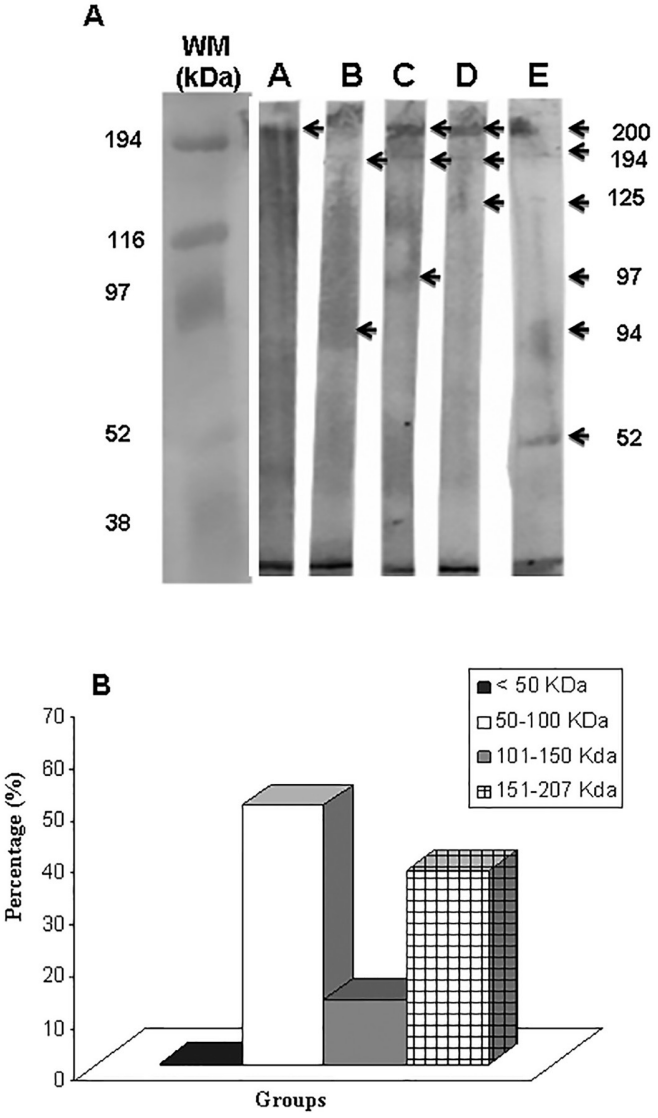

Fig. 2 - Western blotting of *Ascaris suum* antigens. Western blotting procedures showing some of the most frequently recognized bands obtained according to AES seropositivity (2A). Tested sera were assayed in order to identify the bands of *A. suum*. WM: Molecular weight marker (2A). Percentage of individuals who composed the groups of bands according to anti-AES IgG antibodies (2B): Group of bands lower than 50 kDa (■), group of bands between 50 and 100 kDa (□), group of bands between 101-150 kDa (▒), and group of bands between 151- 207 kDa (⊞).

which was ranging from 22.9 kDa to 223 kDas; among these 11 major bands were principally identified; 84.9 kDa (37.7%), 52.2 kDa (33.3%), 131.3 kDa (31.1%), 98.2 kDa (28.8%), 119.1 kDa (26.6%), 41 kDa (26.2%), 184.4 kDa (24.4%), 175.6 and 47.4 kDas (22.2%), 193.6 kDa (15.5%) and 50 kDa (11.9%). Sera from healthy individuals did not show any reactivity (data not shown). Western blotting procedures showing the obtained bands and the WB profile according to TES seropositivity are shown in Figure 3. Western blotting analysis using TES antigens showed the TES pattern composed by bands ranging from 22.9 kDa to 223 kDa (some of the most frequently recognized bands are shown in Figure 3A). The WB profile according to TES seropositivity could be divided into the following groups: the group composed of bands lower than 50 kDa (42.1%) was the most reactive; followed by the group of bands between 50 kDa and 100 kDa (28%), 151 kDa and 207 kDa (18.4%) and 101-150 kDas (11.4%) (Fig. 3B).

Coproparasitological tests and intestinal parasites. The stool examinations showed that intestinal parasites are significantly frequent among Warao indigenous. Among these, protozoas (80.0%) were more frequent than helminths (20.0%). For the children group; *Blastocystis hominis* and *Iodamoeba butschlii* were the most prevalent parasites (85.7%), followed by *Entamoeba coli* (71.4%), *Entamoeba histolytica* and *Hymenolepis nana* (28.5%) and *Giardia duodenalis, Trichuris trichiura* and *Ascaris lumbricoides* (14.2%). For the adult group; *E. coli* was the most prevalent parasite (35.8%), followed by *B. hominis* (28.3%), *I. butschlii* (10.8%), *Endolimax nana* (7.4%), *H. nana* and *T. trichiura* (4.4%), *Chilomastix mesnili* (2.1%) and *Ascaris lumbricoides* (1.5%).

Cytokine expressions and seropositivity for *Ascaris* and *Toxocara*. Figure 4 shows the cytokine expressions when RT-PCR assays were used for the relative quantification of mRNA encoding for IL-2, IL-4, IL-6, IL-10, IL-12p35 (Fig. 4A), and IFN-γ, TGF-β and TNF-α (Fig. 4B) in peripheral blood cells from Warao indigenous. Results indicated that the largest expression of transcript was for IFN-γ (100%) followed by TNF-α (90%), IL-2 (85%), IL-4 (67.5%), IL-12p35 (55%), TGF-β (50%), IL-6 and IL-10 (17.5%) (Fig. 5). When a logistic regression method was used to compare independent variables including cytokine expression in peripheral blood cells from Warao indigenous that had or did not have antibodies against the AES and TES antigens showed that there was only a significant increased expression of the IL-4 in individuals with TES seropositivity ($p < 0.002$), whereas, the expression of all cytokines transcripts were not different to that of individuals with AES seropositivity (Fig. 5).

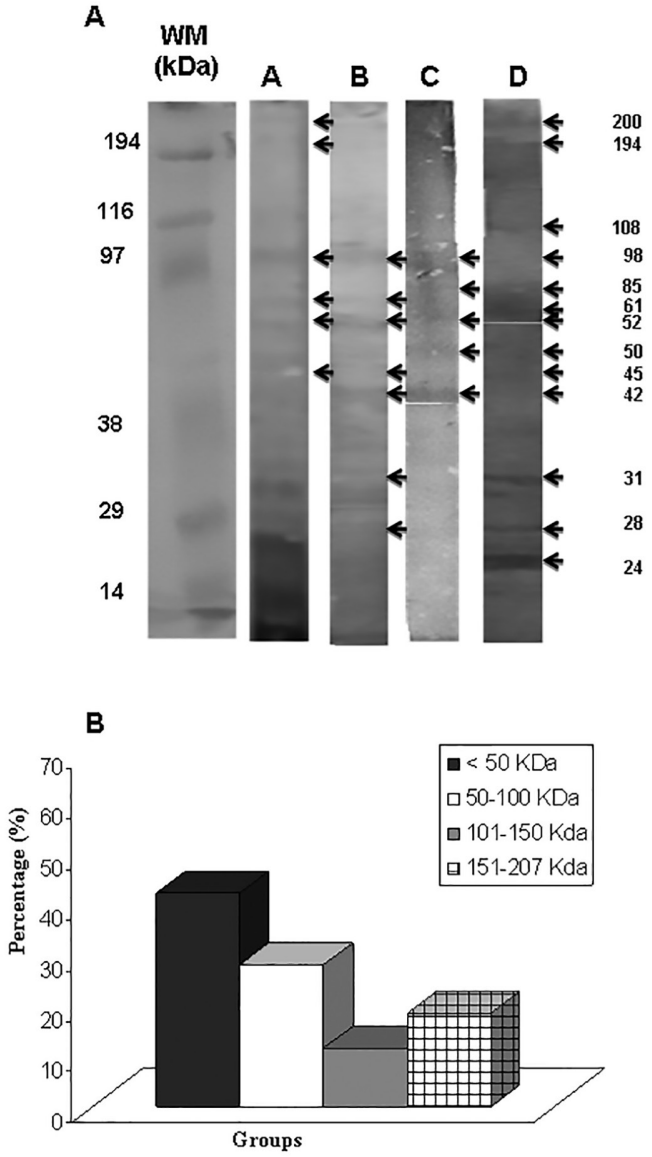

Fig. 3 - Western blotting of *Toxocara canis* antigens. Western blotting procedures showing some of the most frequently obtained bands according to TES seropositivity (3A). Tested sera were assayed in order to identify the bands of T. *canis*. WM: Molecular weight marker. Band (3A). Percentage of individuals who composed the groups according to anti-TES IgG antibodies (3B): Group of bands lower than 50 kDa (■), group of bands between 50 and 100 kDa (□), group of bands between 101-150 kDa (▩), and group of bands between 151- 207 kDa (#).

DISCUSSION

A preliminary assessment of zoonotic helminths such as *A. suum* and *T. canis* among Warao indigenous offered an opportunity to study these two zoonotic infections, namely ascariosis and toxocariosis in some communities that live closely with pigs and dogs. During infection with *A. suum* and *T. canis*, both cellular and humoral immune responses develop. In humans, several studies on the epidemiology, pathology and diagnosis of toxocariasis are available[6,11,28,41] however, much less is known about human infections with *A. suum*. Few studies have reported on *Ascaris suum* being able to mature to the adult stage in the human host[31,38]. NEJSUM *et al.* refer to this infection as zoonotic ascariasis and have recently reported that *A. suum* can also mature to the adult stages in chimpanzees[28]. There are however other studies reporting VLM cases that are suspected to be caused by *Ascaris suum* in which pulmonary and liver lesions have been described[31]. As suggested by ARIZONO *et al.* the pathogenic or physiogenetic factors that determine the course of human infection with pig-derived *Ascaris* remain to be elucidated[2]. In the present study, seropositivity associated with ascariasis and toxocariasis using the indirect-ELISA IgG was evidenced. The findings indicated that both adults and children showed high AES seropositivity, 23.3% and 57.1%, respectively as compared to TES seropositivity, 9.3% and 14.3%, respectively. In the past, antigenic preparations of *T. canis* adults or larvae were used for the immunodiagnosis of toxocariosis[24,39]; however

the latter was improved by SAVIGNY, who obtained secreted antigens called *Toxocara* excretory-secretory antigens (TES-Ag) and used them together with ELISA (TES-ELISA)[37].

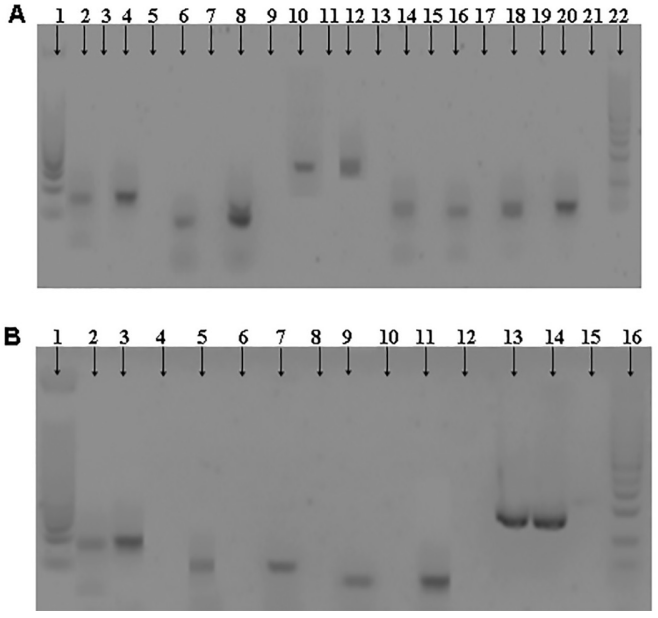

Fig. 4 - Profile of cytokine expression. RT-PCR assays were used for the quantification of mRNA encoding for IL-2, IL-4, IL-6, IL-10, IL-12p35 (3A). 1 = Molecular weight markers (50pb), 2-5 = IL-2 (2: sample positive, 3: sample negative, 4: positive control and 5: negative control), 6-9 = IL-4 (6: sample positive, 7: sample negative, 8: positive control and 9: negative control), 10-13 = IL6 (10: sample positive, 11: sample negative, 12: positive control and 13: negative control), 14-17 = IL-10 (14: sample positive, 15: sample negative, 16: control positive and 17: control negative), 18-21 = IL-12 (18: sample positive, 19: sample negative, 20: control positive and 21: control negative), 22 = Molecular weight markers (100pb). RT-PCR assays were also used for the quantification of mRNA encoding for IFN-γ, TNF-α, TGF-β and β-actin (3B). 1: Molecular weight markers (50pb), 2-4 = IFN-γ, (2: sample positive, 3: control positive and 4: control negative), 5-8 = TNF-α (5: sample positive, 6: sample negative, 7: control positive and 8: control negative), 9-12 = TGF-β (9: sample positive, 10: sample negative, 11: control positive and 12: control negative), 13-15 = β-actin (13: sample positive, 14: control positive and 15: control negative), 16 = Molecular weight markers (100pb).

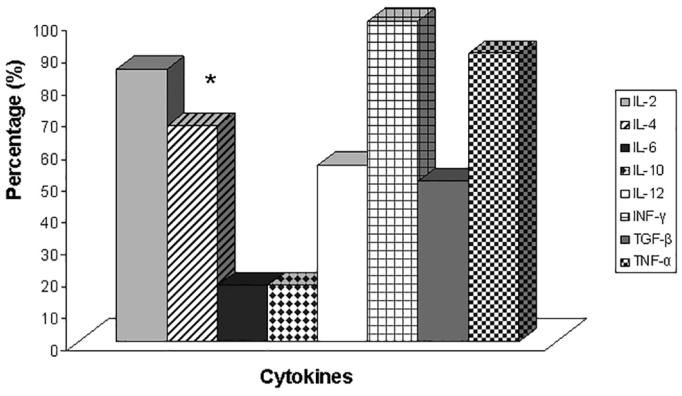

Fig. 5 - Percentage of individuals showing positive expression of cytokine. The percentage of Warao indigenous with positive expression of cytokine: IL-2 (▫), IL-4 (▨), IL-6 (■), IL-10 (⠿), IL-12 (▫), IFN-γ(▦), TGF-β (▬) and TNF-α (▩). (*)There was a significant increased expression of the IL-4 in individuals with reactivity IgG to TES antigens ($p < 0.002$).

A serological follow up was carried out in 27 children with toxocariasis, the results showed, that the highest sensitivity of 100% was reached when the avidity of *T. canis* antibodies IgG was evaluated using ELISA and WB[12,20,23,42]. In addition, a study determined the *T. canis* (TES) seropositivity rate among healthy people with eosinophilia over 10%; the results showed that 67% were positive to bands sizes of 66, 56, 32 and 13 kDas by WB; while that in ELISA, 65% of sera were positive to TES antigen[15]. In the present study, 22.2% and 8.8% of serum were positive to bands size of 32 kDa and 66 kDa, respectively by Western Blot, while that the TES seropositivity obtained by using indirect-ELISA IgG, shows 9.3% and 14.3% in indigenous adults and children, respectively. Several reports describe high and low sensitivity of anti-TES IgG method; these different findings can be explained since it has been reported that serum immunoglobulin G antibodies are produced against a variety of epitopes on the antigen surface and also the number and the species of serologically reactive antigens varied greatly from individual to individual or population to population and the level of specific antibodies could also vary with the age of individuals[6,15,33,39]. In this context, the possible age effect on seropositivity for both of these zoonotic infections was studied. The findings in relation to the age distribution according *A. suum* and *T. canis* seropositivity showed that the trend of seropositivity is similar for both pathogens; however, the *A. suum* seropositivity is slightly higher (30.8%) than *T. canis* seropositivity (25.6%), especially for the age group of 21-40 years old, both seropositivities were significantly increased as compared to the age groups of 0-10, 11-20 years old and 61 years and older, it was observed that the seropositivity thereafter clearly increases with age, but it decreased for the age group of 61 years and older. Whether *A. suum* and *T. canis* transmission for this population is by direct ingestion of contaminated soil remains to be investigated.

On the other hand, in the adult group, 44.1% were seropositive for both parasites; these results suggest that infection by *Toxocara* is essentially as common as that by *Ascaris*; however AES seropositivity in adults and children was shown to be higher; 23.3% and 57.1%, than TES seropositivity, 9.3% and 14.3%, respectively. A high *Toxocara* seropositivity in slum areas of Caracas and El Mojan, Venezuela[10,21,22] has been reported. Since it is more common to see pigs than dogs in these indigenous communities, a low exposure of children to *T. canis*, could explain the present findings.

As mentioned above, the ELISA based on the use of excretory/secretory antigens produced by the larvae of the *A. suum* and *T. canis* is the most common approach for serodiagnosis; however, the specificity of which can be inadequate in regions of endemic helminthiasis[14,32]. In this context, it was reported that reactivity of sera to AES antigens using the ELISA test was reduced by pre-absorption with extracts of *A. lumbricoides,* a nematode antigenically related to *A. suum* and although this topic is still controversial, the most recent research reports that molecular biology has shown it to be a single species, authors concluded that *A. lumbricoides* and *A. suum* are a single species and that the name *A. lumbricoides* Linnaeus 1758 has taxonomic priority; therefore *A. suum* Goeze 1782 should be considered a synonym of *A. lumbricoides*[19], the latter is a common intestinal nematode of Venezuelan children[10,21]. Since Western blotting has been proposed as a confirmatory test for the diagnosis of toxocariosis[20], this method was performed using AES and TES antigens. For AES antigens, the immunoblotting showed a pattern of bands ranging from 52 to 206.9 kDas, with the predominance of the three of these; while that for TES antigens; this pattern of bands was ranging

from 22.9 to 223 kDas; among these 11 major bands were principally identified**.** It has been reported that bands of high molecular weight principally are responsible for the cross-reactivity between *T. canis* and *A. suum*; however, authors reported that a band with molecular weight around 55 to 66 kDa is also, at least, responsible for the cross-reactivity between both parasites[17,28]. Concerning, the existence of cross-reactivity among *A. suum* and *T. canis*, Western blotting was performed using serum samples from patients with positive result by ELISA. The findings showed the existence of cross-reactivity among *A. suum* and *T. canis,* four major bands were principally identified 52.2, 94.1 kDa, 149 kDa, and 193.6 kDa. In experimental animals, cross-reactivity between *T. canis* and *A. suum* has also been reported, so the Western blotting showed that the rat IgG recognized three proteins of 190, 160 and 33 kDas in the antigens from *F. hepatica*, *T. canis* and *A. suum*; the author suggests that the existence of cross-reactivity among these antigens seems to also demonstrate the presence of structural similarities, such as tegumental proteins[35].

Findings about pre-absorption treatment performing Western blotting technique and using serum samples from individuals with *A. suum* seropositivity showed five bands principally identified; 159.4, 137.8, 125.1, 29.2, and 24 kDas; whereas when using serum from individuals with *T. canis* seropositivity, four bands were principally identified; 203.1, 152, 144.7, and 131.3 kDas. The available seroprevalence data about AES and TES antigens here analyzed and results about the microscopic examination of stool samples showing that a high prevalence of intestinal parasites among indigenous exist, especially parasites such as *Blastocystis hominis*, *Hymenolepis nana* and *Entamoeba coli* suggesting that further studies must be performed to improve the sensitivity and specificity of the *Ascaris* and *Toxocara* ELISA test by pre-absorption with extracts of prevalent parasites among Warao indigenous and more serum samples.

On the other hand, relative quantification of cytokine expressions, Th1 and Th2 in peripheral blood cells from indigenous studied was also evidenced. In Venezuela there are 28 different ethnic groups, Warao are one of them (no persons of mixed race or indigenous), whose socio-economic status is low; they do not have access to health care compared with the Creole people from the urban areas, Warao individuals also have recurrent or overwhelming parasite infections[1]. Helminth infections are among the most potent stimulators of Th2-type immune responses and have been widely demonstrated to modify responsiveness to both non parasite antigens and other infectious agents in a nonspecific manner in infected animals; the balance of Th1 and Th2 immune responses is known to be crucial for determining both the protective and pathological responses to infections with a variety of pathogens[13,16,8]. In the case of a number of gastrointestinal nematode infections, Th2 responses are generally associated with protection, while Th1 responses are associated with susceptibility[8,43].

A study showed that *A. lumbricoides* infections in endemic regions are associated with a highly polarized type 2 cytokine response[8]. In addition, a significant association between intestinal helminthic infections and mycobacterial diseases, such as pulmonary tuberculosis and multi-bacillary leprosy, has been demonstrated by several authors[1,7]. It has been reported that concomitant helminthic infection in patients with diagnosed tuberculosis skews their cytokine profile toward a T helper 2 response[7]. Since that, official data on the tuberculosis situation in Venezuela showed that between 1997 and 2001 the tuberculosis rate was between 93.2 and 81.0 among Warao indigenous population[26,27]. Based on this notion and the significant association between intestinal helminthic infections and mycobacterial diseases mentioned above, studies of relative quantification of cytokine expression were performed; the findings showed that there was only a significant increased expression of the IL-4 in individuals with TES seropositivity ($p < 0.002$), whereas, the expression of the IFN-γ, IL-2, IL-6, TGF-β, TNF-α, IL-10 and IL-4 transcripts were not different to that of individuals with AES seropositivity. The results of the present study suggest that *T. canis* antigens have a potent immunomodulatory activity and that the effects observed are consistent with skewing towards a Th2-type response rather than induction of Th1-type response. The latter probably due to two syndromes that have been identified, which remain for a long time in the host, the visceral larva migrans syndrome (VLM) and the ocular larva migrans syndrome (OLM)[9]. Importantly, the induction of interleukin-4 by *T. canis* antigens also suggests that T cells may play a role in immunomodulation of immune responses by parasitic helminths that result in a dominant Th2 type of the immune response. While this presumably promotes parasite survival, it may markedly impair protective immune responses to *Mycobacterium tuberculosis* infection. Further studies are needed to understand the association between helminthic infections such as *A. suum* and *T. canis* and a dominant Th2 cytokine profile in Warao indigenous, which could favor persistent *M. tuberculosis* infection in this population. Finally, the immunoblotting and ELISA techniques may constitute useful methods for the diagnosis of the zoonoses infections like ascariosis or toxocariosis, which are prevalent among Warao indigenous. In addition, measures to control these helminthic infections are recommended.

RESUMEN

Seropositividad para ascariosis y toxocariosis y expresión de citocinas entre la población indígena de la región del delta Venezolano

El objetivo del presente estudio fue determinar la seropositividad de infección por *Ascaris suum* y *Toxocara canis*, utilizando antígenos de excreción/secreción (E/S) de *Ascaris suum* (AES) y *Toxocara canis* (TES) en una población indígena. Adicionalmente, se cuantificó la expresión de citocinas a partir de células de sangre periférica. Un total de 50 indígenas Warao se incluyeron en el estudio; 43 fueron adultos y 7 niños. Entre los adultos, 44,1% fueron seropositivos para ambos parásitos; mientras que los niños sólo mostraron seropositividad a uno u otro de los helmintos. Para ascariosis, el porcentaje de seropositividad para los antígenos AES fue alto tanto en adultos como en niños; 23,3% y 57,1%, respectivamente. Para toxocariosis, el porcentaje de seropositividad para los antígenos TES fue bajo en adultos así como en niños; 9,3% y 14,3%, respectivamente. El porcentaje de seronegatividad fue similar tanto para los antígenos AES como para TES en adultos (27,9%) y niños (28,6%). Cuando la seropositividad fue analizada a través de la técnica de Western blotting utilizando los antígenos AES; 3 bandas de 97,2, 193,6 y 200,2 kDas fueron principalmente reconocidas. Para los antígenos TES, 9 bandas fueron mayormente identificadas; 47,4, 52,2, 84,9, 98,2, 119,1, 131,3, 175,6, 184,4 y 193,6 kDas. Los análisis coproparasitológicos mostraron que los parásitos *Blastocystis hominis*, *Hymenolepis nana* y *Entamoeba coli* fueron los parásitos intestinales más comúnmente observados. La cuantificación de la expresión de las citocinas IFN-γ, IL-2, IL-6, TGF-β, TNF-α, IL-10 e IL-4 mostró que hubo un significante incremento de la expresión de IL-4 entre los indígenas con seropositividad para los

antígenos TES ($p < 0.002$). La seropositividad para *Ascaris* y *Toxocara* fue prevalente entre los indígenas Warao.

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