Detection of IgG antibodies to *Toxocara vitulorum* soluble larval extract (Ex) by Western Blotting in the colostrum and serum of buffalo cows and calves

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Abstract

Toxocara vitulorum is a nematode parasite of the small intestine of cattle and water buffaloes, particularly buffalo calves between one and three months old, causing high morbidity and mortality. The purpose of this research was the characterization of soluble larval extract (Ex) antigen of *T. vitulorum* by SDS-PAGE and Western blotting (WB) using immune sera and colostrum of buffaloes naturally infected by *T. vitulorum*. The parasitological status of the buffalo calves was also evaluated using sequential fecal examinations. The results showed that this antigen revealed eleven (11, 13, 16, 22, 25, 32, 43, 53, 68, 82 and 96 kDa) polypeptide bands by SDS-PAGE. Five polypeptide bands of higher molecular weight (43, 53, 68, 82 and 96 kDa) were recognized by sera and colostrum of all groups of infected animals when analyzed by WB. But only the fractions of 53, 68, 82, and 96 kDa that were more prominent persisted in the groups of buffalo calves during the beginning of the infection, at the peak of egg output, as well as during the period of rejection and post-rejection of *T. vitulorum* by the feces of the calves. On the other hand, sera of buffalo calves at one day old, after suckling the colostrum and at the beginning of infection, reacted with the same bands detected by serum and by colostrum of the buffalo cows.

Key-words:
Bubalus bubalis.
Toxocara vitulorum.
Buffalo.
Ex antigen.
SDS-PAGE.
Western Blotting.

Introduction

Toxocara vitulorum is a parasite of the small intestine of ruminants, particularly buffalo calves of one to three months of age from tropical countries. It is responsible for high morbidity and mortality rates[1,2,3] resulting in serious economic losses[4]. This parasite is acquired by calves when they suckle colostrum/milk contaminated with infective larvae from infected cows[5,6,7,8].

Antibodies against larval excretory/secretory (ES)[9] and larval soluble extract (Ex)[10] of *T. vitulorum* were detected in serum of buffalo cows and calves naturally infected with *T. vitulorum*, indicating that *T. vitulorum* infection can stimulate the immune system of the buffalo. Similarly, Souza[11] showed by ELISA that the highest level of anti-Ex antibodies of *T vitulorum* were detected in buffalo cow sera during the perinatal period and were maintained at high levels through 300 days after parturition. Colostrum antibody concentration was the highest on the first day post-parturition, but decreased sharply during the first fifteen days. On the other hand, calves passively acquired antibodies from colostrum that were kept on the high concentrations from the birth to approximately 45 days, coincidentally with the peak of *T. vitulorum* infection. Then, the rejection of the worms by the calves occurred simultaneously with the decline of antibody levels, which reached their lowest levels between 76 and 150 days. Thereafter, the antibodies started slightly to increase,

possible due acquired active immunity and remained stable on a plateau between[11].

On the other hand, immunizations of mice with ES antigen of larvae and perienteric fluid (Pe) antigen of adult *T. vitulorum* induced protection superior to 92% against larval migration in their tissues[12]. Later, Paula[13] showed a rate of 86% of larval migration inhibition from the gut to the liver of mice immunized against *T. vitulorum*-Ex antigen.

Based on the hypothesis that *T. vitulorum* infection can stimulate the humoral immune system of buffaloes and Ex antigen may be a protective antigen, the objective of the present study waas to characterize Ex antigen by SDS-PAGE and Western blot (WB), using immune sera and colostrum of buffaloes naturally infected by *T. vitulorum*. In addition, the parasitic status of the buffalo calves naturally infected with *T. vitulorum* during their first year was evaluated using sequential fecal examinations.

Materials and Methods

Buffalo housing

Water buffaloes naturally infected with *T. vitulorum* were kept for about 12 months on a 12 ha pasture of *Brachiaria decumbens* grass with a pond as the source of water. The cows were not milked and the calves were grazed together with the dams in this area.

Fecal, serum and colostrum/milk samples from buffalo calves and cows

Rectal fecal samples were collected from the buffalo calves (n = 10) according to the following schedule: weekly (from birth to 90 days) and fortnightly (from day 91 to 192 days). Fecal examinations were performed according to Whitlock[14] and results expressed as eggs per gram (EPG) of feces.

The sera of buffalo calves (n = 10) previously assayed by ELISA[11] were pooled (n = 10) and sampled as follows: 1) at one day of age before suckling the colostrum (negative reference serum); 2) at one day after suckling the colostrum; 3) at the beginning of *T. vitulorum* infection; 4) at the peak of the infection; 5) at the parasite-rejection period, and 6) after parasite-rejection period.

Colostrum and serum samples of buffalo cows were collected on day of parturition (n = 10). Sera of the cows were considered as positive reference serum. The samples of colostrum were centrifuged at 4°C in a refrigerated centrifuge at 460g for 15 minutes. After removal of solidified fat, the samples were left in an incubator at 37° C for one hour for casein precipitation with one percent rennin. Then the colostrum/milk serum was separated by centrifugation for 15 minutes at 460g at 4°C. Serum and colostrum/milk samples were separated, aliquoted and stored at –70ºC.

T. vitulorum Ex antigen preparation

T. vitulorum adults were recovered by expulsion of this parasite through the feces of naturally infected water buffalo calves by administration of 100 mg/kg of piperazine. Mature females were dissected and the uteri and eggs removed. The eggs were incubated in PBS solution (phosphate-buffered saline, 0.1 M; 7.5 pH) with several drops of commercial sodium hypochlorite solution (1% available chlorine) in Petri dishes for 20-45 days at room temperature. The dishes with the egg suspension were gently stirred for daily aeration while the development of eggs was observed daily with an optical microscope until the development of infective third-stage larvae (L_3). After that, the egg suspension was transferred to tubes (15 ml) and washed in distilled water by centrifugation. Sediment from eggs was collected then combined with an equal volume of sodium hypochlorite solution (14% available chlorine) and incubated for 20 min at room temperature until the eggs were completely decorticated. PBS was added to this solution to increase the volume to 15 ml after which the mixture was centrifuged 10 times at 460g for 2 minutes or until chlorine odor could not be detected The

decoated eggs were suspended in PBS and placed in a water bath at 37°C for one hour while air was bubbled with a Pasteur pipette through the suspension until the L_3 were hatched (about 5 minutes).

Ex antigen was obtained from infective L_3 as described previously by Starke-Buzetti, Machado and Zocoller-Seno[10] using an ultrasonic homogenization in ice. To the larvae suspension, a protease inhibitor solution (Protease inhibitor cocktail, Sigma containing: AEBSF, E-64, sodium EDTA, bestatin, leupeptin and aprotinin) was added. After centrifugation in a refrigerated centrifuge (3700g for 10 minutes at 10°C), the supernatant was filtered through a membrane (Gelman Science, membrane filter, pore size 0.22 mg) and dehydrated in a vacuum centrifuge (Vacufuge Concentrator 5301, Eppendorf) and stored at –70°C.

Protein concentration of each antigen was measured with a Protein Assay Kit (Sigma® P-5656) using Lowry's reagent. The protein concentration of Ex was 950mg/ml.

Polyacrylamide gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis (PAGE) was carried out in 12% gels of acrylamide/bis ratio of 36.5:1 in the presence of 10% sodium dodecyl sulphate (SDS) supplemented with Temed (Sigma, T-9281) and amonium persulphate in TRIS-HCL buffer pH 8.8, according to Laemmli[15]. Molecular weight standard mixtures (M.W. 15,000 – 150,000, Sigma M-0671) were used for calibrating the gel. The antigen diluted in a TRIS (pH 6.8) sample buffer (0.1M Tris-HCL, 2% SDS, 10% glycerol, 0.2 M 2-mercaptoethanol and 0.1% bromophenol blue) was loaded in the gel with 20 mg/lane (4 lanes with the same concentration). The electrophoresis was monitored using 0.1% bromophenol blue and the current was set at 30 mA. The protein fractions were visualized by staining with 0.10% Coomassie Brilliant Blue R 250 (Sigma, B-0149).

The relative molecular weights were calculated using prestained protein molecular weight standard according to the relative electrophoretical mobility (RM), using the following equation:

$$RM = \frac{\text{Distance of the protein migration}}{\text{Distance of bromophend blue migration}}$$

The RM values (ordinate) were related to known molecular weights of the standard proteins (abscissa) in a semi-logarithmic graph giving base for interpolation of the data of proteins of the Ex-antigen.

Western Blotting (WB)

Gels with *T. vitulorum*-Ex antigen were electrophoretically transferred to nitrocellulose sheets (0.22 mm) for immunoblotting according to the procedure described by Towbin, Staehelin and Gorodon[16]. The transfer was performed in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad apparatus) for 12 hours in a constant current of 35V in a transfer buffer (Tris-Glycine-Methanol). The nitrocellulose papers were blocked in a blocking solution with 5% non-fat dried milk in TBS-Tween (0.01M Tris, 0.15M NaCl, 0.05% Tween-20) and incubated for 90 minutes with primary antibodies (serum of buffalo cows and calves) diluted 1/50 in the blocking solution and 5% normal rabbit serum in a rotating homogenizer. After that, the nitrocellulose was washed three times (15 minutes each) in TBS-Tween and milk solution. Specifically bound antibodies in all filters were detected with anti-bovine alkaline phosphatase conjugate (Sigma, A-7914) diluted 1:30.000 in the blocking solution for 90 minutes. After rinsing three times in TBS-Tween and milk solution, the blots were incubated at room temperature for about 10 minutes in enzymatic substrate (5 -Bromo-4-Chloro-3-Indolyl Phoshate/Nitro Blue Tetrazolium-BCIP/NBT, Calbiochem, 203790) until developing the color, as described by Blake et al.[17] and adapted by Machado et al.[18]. All incubations were done at room temperature (about 28° C) and under rotating homogenization.

Results and Discussion

Parasitic Status of Buffalo Calves

Parasitic status of the buffalo calves for *T. vitulorum* infection was evaluated by sequentially fecal examination (EPG) from the birth to 192 days of age. This parasitic infection was represented by a curve of four periods: beginning (period 1) occurring between 16 and 39 days; peak of maximum EPG counts (period 2) between 40 and 47 days; rejection of the parasite (period 3) between 48 and 117 days, and absence or post-rejection (period 4) after a one-month absence of eggs in the feces (Figure 1).

The calves of the present work showed only moderate symptoms of diarrhea during the period of the rejection of the worms. After rejection, eggs of *T. vitulorum* were no longer seen in feces of the buffalo calves for a period of eight months (period of feces collection) after the worm rejection, indicating that the buffalo calves acquired an immunological resistance against intestinal reinfection.

SDS-PAGE and WB

As shown in figure 2, the SDS-PAGE pattern of larval *T. vitulorum*-Ex antigen indicated the presence of polypeptides of 11, 13, 16, 22, 25, 32, 43, 53, 68, 82 and 96 kDa. The most prominent band were at approximately 68 kDa. Table 1 and figure 3 show the results of WB. It was possible to identify by WB polypeptide bands ranging from 43 to 92 kDa that reacted with the antibodies present in serum and colostrum from buffalo cows and serum from buffalo calves collected from different times of *T. vitulorum* infection. The most prominent antigenic bands were observed in the range between 68 and 96 kDa. The bands detected by serological antibodies from buffalo cows (bands of 43, 53, 68, 82 and 96 kDa) were very similar to those detected by antibodies

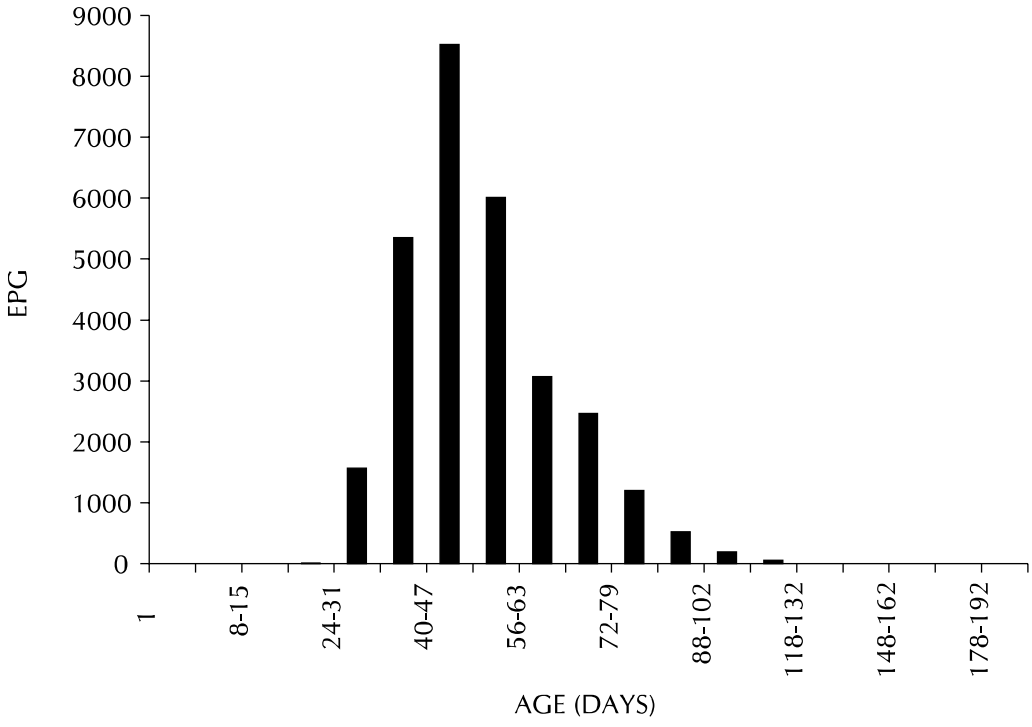

Figure 1 - Eggs/gram (EPG) of feces from buffalo calves (n = 10)

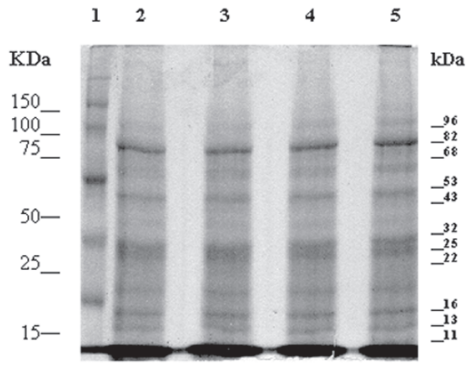

Figure 2 - SDS-PAGE pattern of larval *T. vitulorum*–Ex antigen. Column 1 = molecular size standards; columns 2-5 = Ex run in quadruplicate. - Note that Ex reveals eleven polypeptide bands: 11, 13, 16, 22, 25, 32, 43, 53, 68, 82 and 96 kDa. The gel was stained with Coomassie blue.

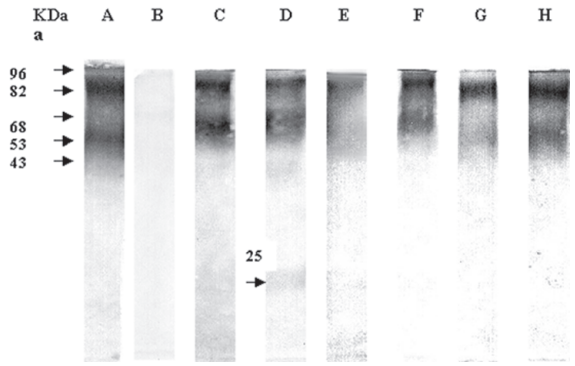

Figure 3 - Characterization of *Toxocara vitulorum*-Ex antigen with pool sera (previously assayed by ELISA) from buffalo sera (n = 10) by WB after SDS-PAGE. Test sera used are from: cows on day of parturition which were considered positive reference sera (lane A); calves before suckling the colostrum which were considered negative reference sera (lane B); colostrum (lane C); buffalo calves at one day of age after suckling the colostrum (lane D); buffalo calves at beginning of infection (lane E); buffalo calves at the peak of infection (lane F); buffalo calves during the period of rejection of the worm (lane G), and buffalo calves during the period of post-rejection of the worm (lane H).

from colostrum and serum of buffalo calves at one day of age after suckling the colostrum (exception for the band 25 that only appeared in buffalo calf serum with one day after suckling the colostrum). However, no bands were seen in the buffalo calves with the same age but without suckling the colostrum, suggesting that IgG antibodies from serum of the cows were transferred to the colostrum and then passively to the calves within 24 hours of birth. Similarly by ELISA, Souza[11] and Starke-Buzetti, Machado and Zocoller-Seno[10] revealed the presence of high levels of antibodies against *T. vitulorum*-Ex antigen in the serum of 100% of the buffalo cows and calves on the first day of calving. In the colostrum, on the other hand, the antibody concentration against this antigen was the highest on the day of parturition, but declined rapidly after the seventh day to reach a very low concentration on day 15[11]. The development of the antibody-mediated immune response in buffalo cows and calves against *T. vitulorum* infection was also reported by other authors[9,10]. Only bands of higher molecular weights (53, 68, 82 and 96 kDa) were detected by anti-Ex antibodies in the sera of the buffalo calves during the beginning of

Table 1 - Characterization of *Toxocara vitulorum*-Ex antigen by SDS-PAGE and WB using sera and colostrum of buffalo cows and sera of buffalo calves naturally infected with *T. vitulorum* during the beginning and at the peak of the infection and during the rejection and post-rejection period

SDS-PAGE (kDa)	*Toxocara vitulorum*–Ex antigen/polypeptide bands/kDa							
	Positive Serum[a]	Negative Serum[b]	Colostrum[c]	Sera of buffalo calves				
				One day of age[d]	Period 1[e]	Period 2[f]	Period 3[g]	Period 4[h]
96	96	-	96	96	96	96	96	96
82	82	-	82	82	82	82	82	82
68	68	-	68	68	68	68	68	68
53	53	-	53	53	53	53	53	53
43	43	-	43	43	-	-	-	-
32	-	-	-	-	-	-	-	-
25	-	-	-	25	-	-	-	-
22	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-

(-) Without band (negative); a: buffalo cow serum on the parturition day (positive reference serum) ; b: buffalo calf serum before suckling the colostrum (negative reference serum); c: colostrum on the parturition day; d: buffalo calf serum one day after suckling the colostrum; e: at the beginning of infection; f: at the peak of infection; g: period of rejection of the parasite; h: post-rejection period (7 months of age)

the infection (period 1), at the peak (period 2), during the period of rejection (period 3) and post-rejection of the worm (period 4). From these polypeptide bands only the bands of 68 and 96 kDa were very prominent (Table 1 and Figure 3).

Souza[11] observed by ELISA that the high concentration of serological anti-Ex-antibodies passively acquired by calves maintained high levels until the peak of *T. vitulorum* infection, declined after 45 days of age, reached the lowest levels between days 76 and 150, but started to increase slightly to remain at a plateau level between days 211 and 365 (the end of experimental period). These results suggest that at the peak and during the decline of eggs from the feces of buffalo calves, colostral antibodies might still be present in the serum of buffalo calves and might only recognize antigens of higher molecular weight (68 to 96). However, when the calves were 210 to 223 days of age, during the period of post-rejection of the worms, the remaining antibodies that continued reacting with antigens of 68, 82 and 96 kDa might be actively produced by the calves. However, during this period the animals had no adult worms present in the intestines, but the immune system of the calves might be stimulated by the infective larvae migrating from the intestinal mucosa to other tissues such as those of the liver and lungs.

Mice immunization with antigens from *T. vitulorum* larvae and adults has induced protection against larval migration in the tissues[12,13]. Based on this information it is possible to consider some protective mechanism which would reduce larval gut penetration and contribute to the larval migration inhibition, particularly larval migration to the mammary gland of buffalo cows in order to avoid or reduce the potential transmission of the parasite through the colostrum.

The current findings feature Ex antigens of 68 to 92 kDa of *T. vitulorum* as possible components of a vaccine that could be used in buffalo vaccination against *T. vitulorum* infection.

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Detecção de anticorpos IgG aos antígenos extratos solúveis (Ex) de larvas de *Toxocara vitulorum* pelo Western Blotting no colostro e no soro de vacas e bezerros búfalos

Resumo

Toxacara vitulorum é um nematódeo que acomete principalmente bezerros búfalos na faixa etária de um a três meses de vida, causando grande morbidade ou mortalidade quando não tratados. A pesquisa objetivou a obtenção do antígeno extrato larval solúvel bruto (Ex) de larvas infectantes de *T. vitulorum*, bem como a separação das frações antigênicas pelo SDS-PAGE e pelo "Western blotting" (WB), utilizando-se soros imunes e colostros de búfalos naturalmente infectados com *T. vitulorum*. O acompanhamento do quadro parasitário dos bezerros búfalos também foi realizado. Pôde-se verificar que o antígeno revelou 11 (11, 13, 16, 22, 25, 32, 43, 53, 68, 82 e 96 kDa) bandas protéicas. Quando analisadas pelo WB, cinco dessas bandas (43, 53, 68, 82 e 96 kDa) foram reconhecidas pelos anticorpos presentes nas amostras de soros e de colostros das búfalas e de soros dos bezerros búfalos com um de vida após mamarem o colostro. No entanto, somente as bandas de 53 a 96 kDa que foram as mais evidentes persistiram nos grupos de bezerros búfalos que se encontravam tanto no pico da infecção como no período de expulsão ou pós-expulsão dos helmintos adultos do intestino. As bandas antigênicas de 68 e de 92 kDa, por serem as mais proeminentes podem ser consideradas componentes para futuras vacinas contra *T. vitulorum* em búfalos.

Palavras-chave:
Bubalus bubalis.
Toxocara vitulorum.
Búfalos.
Antígeno Ex.
SDS-PAGE.
Western Blotting.

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