IMMUNOREACTIVE PROTEINS OF *Trypanosoma vivax*

Proteínas Inmunorreactivas de Trypanosoma vivax

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ABSTRACT

Bovine trypanosomosis, caused by *Trypanosoma vivax* has a significant negative impact on livestock. This research was performed with the aim of determining the immunoreactive proteins present in *T. vivax*. Thus, five sheep were experimentally infected with *T. vivax* TvZC1 isolate. Animal number 1 was used as the source of the trypanosomes and to prepare the soluble extract of parasites. Sheep numbers 2 to 5 were monitored for eight weeks and sera was obtained every two weeks for immunodetection. Parasites obtained from animal 1 were analyzed for *T. vivax* proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (WB). The WB analysis showed three immunodominant proteins with a molecular mass of 42, 64 and 72 kDa, approximately. The 64 kDa protein was recognized by every animal during the complete infection period. The 72 kDa protein only was detected by animals 2, 3 and 5 during the infection course, whereas in animal 4 it was only detected during the 6th and 8th weeks post infection. Moreover, the 42 kDa polypeptide was slightly immunorecognized by animals 2, 3 and 4 during the complete infection period, but in animal 5 only it was identified during the 2nd week post infection. It is assumed that the 42 kDa protein is the VSG of *T. vivax*, which resulted in a low antigenic capacity, contrary to the protein of 64 kDa which showed a high antigenic capacity and cross-reactivity with *Trypanosoma evansi*.

Key words: *Trypanosoma vivax*; Antigens; Proteins; trypanosomosis.

RESUMEN

La tripanosomiasis bovina, causada por *Trypanosoma vivax* tiene un impacto negativo significativo en la ganadería. Esta investigación se realizó con el objetivo de determinar las proteínas inmunorreactivas de *T. vivax*. Para esto, cinco ovejas se infectaron experimentalmente con un aislado de *T. vivax*, denominado TvZC1. El animal número 1 se utilizó como fuente de los tripanosomas y para la obtención del extracto de proteínas soluble de parásitos. Los ovinos enumerados del 2 al 5 se mantuvieron infectados durante ocho semanas y se obtuvieron sueros cada dos semanas para realizar la inmunodetección. Los parásitos obtenidos del animal número 1 fueron analizados por electroforesis en geles de poliacrilamida (SDS-PAGE) y Western blot (WB) para obtener el perfil de proteínas antigénicas. El análisis WB mostró tres proteínas inmunodominantes con una masa molecular aproximada de 42; 64 y 72 kDa. La proteína de 64 kDa fue reconocida en todos los animales durante todo el período de infección, mientras que la de 72 kDa sólo fue detectada por los animales 2; 3 y 5 durante el período de infección completa, mientras que en el animal número 4 solo se detectó durante la sexta y octava semanas de la infección. Por otra parte, el polipéptido de 42 kDa fue immunoreconocido ligeramente por los animales 2; 3 y 4 durante el período de infección completa, en el animal 5 solo fue identificada durante la segunda semana post-infección. Se asume que la proteína de 42 kDa es la VSG de *T. vivax*, la cual resultó en una baja capacidad antigénica, contrario a la proteína de 64 kDa que mostró una alta capacidad antigénica, además de demostrar reactividad cruzada con *Trypanosoma evansi*

Palabras clave: *Trypanosoma vivax*; antígenos; proteínas; tripanosomosis.

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INTRODUCTION

Bovine (*Bos taurus* and *Bos indicus*) trypanosomosis caused by *Trypanosoma vivax* is a chronic disease of livestock in Africa and Latin America [14]. Cattle infected with *T. vivax* present fever, anemia, lethargy, loss of physical function, reduced milk yields, abortion and death [11, 14]. In Venezuela, the parasite is present all over the Country, affecting cattle with a general seroprevalence of 33.1% [20]. The diagnosis is mostly based in the detection of the parasites in Giemsa-stained blood smears or by microhaematocrit centrifuge technique (HCT) [8]; however, it is difficult to demonstrate the presence of trypanosomes in this way, since infections are often chronic and few parasites are present [14]. Many serological assays have been developed for the diagnosis of *T. vivax* but few have found practical application in the field [8]. The immunodiagnosis of *T. vivax* is often performed by the ELISA test using soluble antigenic extract of *T. evansi* because of the high antigenic cross-reactivity and the ease of replicating *T. evansi* in rodents to obtain the crude extracts [11, 14, 19], but there have been few attempts to determine the differences in the type of antigen preparation or of the particular isolate from which the antigen is derived. Unfortunately, there is no species-specific ELISA because of cross-reactions between these trypanosomes [19, 23]. However, given that cattle can also be infected by *T. evansi*, and that the clinical and epidemiological importance of this parasite is not clear [16], it would be extremely desirable to use purified antigens to perform *T. vivax*-specific serodiagnosis because the treatment and control of the disease is trypanosome species-dependent [8]. For this reason, this research was undertaken with the aim of determining the immunoreactive proteins of the soluble extract antigens of *Trypanosoma vivax*.

MATERIALS AND METHODS

Trypanosoma vivax isolate

T. vivax isolate (TvZC1) was obtained from a natural infected heifer in a farm localized in Zulia State, Venezuela. Diagnosis was performed by the microhaematocrit centrifuge technique [27] and Giemsa-stained blood smears. Then, blood was collected in tubes containing 1 mg/mL of ethylenediaminetetracetic acid (EDTA), and cryopreserved as previously described [6]. Prior to the study, parasitological diagnosis was confirmed using a *T. vivax*-specific polymerase chain reaction (PCR) assay [12].

Experimental animals

Six mixed Barbados Blackbelly sheep (*Ovis aries*), of approximately one year of age, negative to *T. vivax* infection (determined by serological and molecular tests) were used in the study. Two weeks before and during experimental infection, all animals were maintained under veterinary supervision in order to safeguard health and minimize animal suffering. Protocols used were approved by the Ethical Committee for Laboratory Animal Use (University Simón Rodríguez) under the number 013–11. As well, these ethical procedures were based on Venezuelan law [13].

T. vivax infection

In order to expand the isolate, one sheep was used as a donor which was immunosuppressed with six intravenous injections of 2 mg/kg dexamethasone every other day, as previously described [7] and then, 4 mL of cryopreserved blood was inoculated intravenously. This sheep was monitored every day for haematocrit, temperature and parasitemia values. When the parasitemia reached a value of 10^6 trypanosomes/mL, as determined by the hemocytometer method [25], the other animals (named from 1 to 5) were inoculated intravenously with 2 mL of sheep donor fresh blood (approximately 2 x 10^6 trypanosomes). The donor animal was later treated with 3.5 mg/kg of 7% diminazene aceturate through a deep intramuscular (i.m.) injection. Animal number 1 was used to obtain soluble extract of proteins at the first parasitemia peak and then it was treated similarly to the donor animal. Infected animals 2 through 5 were monitored during eight weeks in order to obtain sera and perform Western blot analysis (WB). The serum samples of each animal were obtained every two weeks (weeks 2, 4, 6 and 8 post infection). In order to safeguard the health of these animals, clinical aspects such as temperature, haematocrit and general condition were monitored daily.

T. vivax purification

At day 5 after infection, animal 1 presented the first parasitemia peak. In this time, *T. vivax* purification was made as described [7]. The final cell pellet was suspended in 1 mL of phosphate buffered saline (PBS) pH 7.2 containing 1% glucose (PBSG) in the presence of protease inhibitors and was kept frozen at -70 ºC (Revco™ ULT-1790, Thermo Scientific™, USA) until further use.

Preparing of soluble extract of parasites and quantitation of total protein

Parasites pellet was thawed and prepared by sonication (Vibra-Cell™ VCX130, Sonics and Materials Inc, USA) three times for 30s on ice and centrifuged at 10.000 *g* for 10 min at 4°C (CRU-5000 Centrifuge, Damon/IEC™, USA) to obtain a supernatant rich in soluble trypanosome protein. The protein concentration of this extract was determined using the micro BCA™ (Bicinchoninic acid) kit with bovine serum albumin (BSA) as standard (Pierce Laboratories, Illinois, USA).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Prior to electrophoresis, 30 µg soluble protein extract was diluted with sample loading buffer (0.06 M Tris-CI, pH 6.8, 5% β-mercaptoethanol, 10% glycerol, 2% SDS, 0.01% bromphenol blue) and heated at 100 ºC for 5 min. For identification of antigenic proteins from soluble extract, electrophoresis was carried out on 12% SDS-PAGE [10]. Standard molecular weight marker protein (BlueRanger® Pierce Laboratories, Illinois, USA) was run simultaneously. Following SDS-PAGE, the proteins were electrotransferred (100 V constant for 2 h at 4 ºC) to a nitrocellulose membrane (0.45 µm pore size) [22]. After the

membrane incubation in blocking solution (5% non-fat dry milk diluted in 20 mM PBS, 150 mM NaCl, pH 7.2 for 30 min), sera from infection of animals 2 through 5 were added to nitrocellulose sheets at dilution 1:100 in PBS and incubated over night at ambient temperature. The antigenically reacting proteins were incubated with peroxidase conjugated secondary antibodies against sheep IgG (Jackson ImmunoResearch® Laboratories, USA), dilution 1:400 in PBS. Finally, the protein bands were visualized by the addition of 4-chloro-1-naphthol (Horseradish Peroxidase-Color Development Reagent 4 CN, Bio-Rad), according to the provider. To assess cross reactivity with other hemoparasites and recognition of other *T. vivax* isolates, sera from sheep naturally and experimentally infected with *T. vivax*, hemoparasites-negative cattle, cattle experimentally infected with *Babesia bigemina* and a horse (*Equus caballus*) naturally infected with *T. evansi* were used, all of them determined by Immunofluorecence antibody test (IFAT) as described previously [15]. Sera from *T. vivax*-positive (experimentally-infected sheep 1 from this study) and *T. vivax*-negative sheep were used as control for WB. The relative molecular weight (*Mr*) of proteins resolved by SDS-PAGE was estimated by Kodak 1D Image Analysis Software [18].

RESULTS AND DISCUSSION

The pattern of *T. vivax* proteins separated by SDS-PAGE showed eleven bands of approximately *Mr* 27, 31, 42, 64, 72, 80, 94, 115, 150, 170 and 215 kDa (FIG. 1), of which the most distinctive bands were: 31, 72, 80 and 64 kDa. Proteins of similar molecular weight have received considerable attention and several research studies have been made in regards to their possible utilization in diagnosing bovine trypanosomosis. Using cytosolic antigens of a different *T. vivax* isolate, other researchers

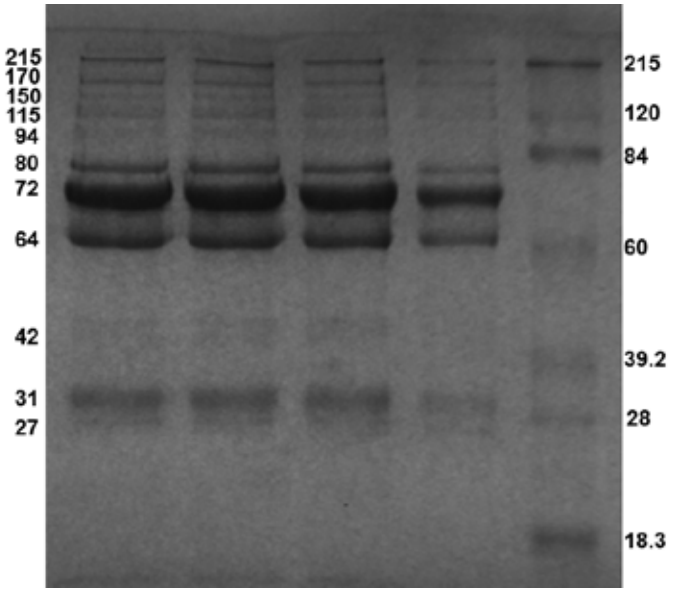

FIGURE 1. SDS-PAGE ANALYSIS OF PROTEIN EXTRACT FROM A VENEZUELAN *Trypanosoma vivax* ISOLATE. M: Molecular weight standard.

[1] observed in 12% SDS-PAGE several proteins with *Mr* between 33.9 and 73 kDa, but only one distinct band of *Mr* approximately 47.5 kDa. Moreover, in a study using the same method of antigen preparation [4], in soluble extract of *T. vivax* were observed several proteins, and the most relevant one having an apparent molecular mass of 39 kDa. Most of these proteins are in close conformity with the polypeptides found in the present study; however, discrepancy between findings might be due to differences in isolates of *T. vivax* used and the methods of antigen preparation.

The WB analysis made with sera from four animals during eight weeks of infection is presented in FIG. 2. As observed, sera recognized only three antigenic proteins with apparent *Mr* 42, 64 and 72 kDa, but some differences were observed among studied animals. The polypeptide of 64 kDa was strongly recognized by all animals during the complete infection period, while the rest of the proteins were weakly recognized in some stages of the infection. The polypeptide with *Mr* 42 kDa was slightly immunorecognized by animals 2, 3 and 4 (FIGS. 2ABC) during the all infection course, but in animal 5 (FIG. 2D) it was only identified during the second week post infection. On the other hand, 72 kDa protein was weakly detected by sera of animals 2, 3 and 5 (FIGS. 2ABD) during the all infection course, but in animal 4 (FIG. 2C) it could only be detected during weeks 6 and 8 post infection. Likewise, to assess cross reactivity with other hemoparasites and recognition of other *T. vivax* isolates, proteins were confronted to several sera, as shown in FIG. 3. The 64 kDa protein was recognized by all *T. vivax* infected sheep and by *T. evansi* naturally infected horse; however, there was no detection of 42 and 72 kDa proteins.

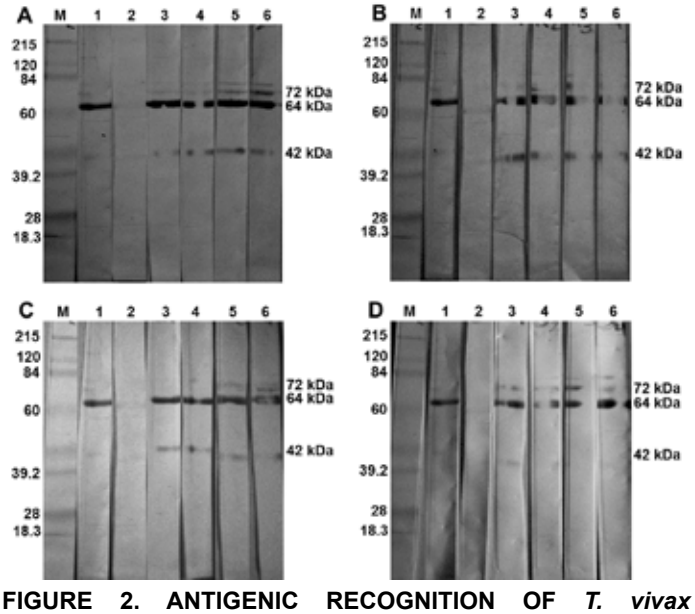

FIGURE 2. ANTIGENIC RECOGNITION OF *T. vivax* POLYPEPTIDES BY SHEEP 2 (A), 3 (B), 4 (C) AND 5 (D) DURING INFECTION PERIOD. Western blot was performed using M: Molecular weight standard; Lane 1: Positive control (*T. vivax* positive serum as assessed by IFAT); Lane 2: Negative control (*T. vivax* negative serum as assessed by IFAT); Lanes 3 to 6: sera from sheep 2 to 5 on weeks 2; 4; 6; and 8 post infection, respectively.

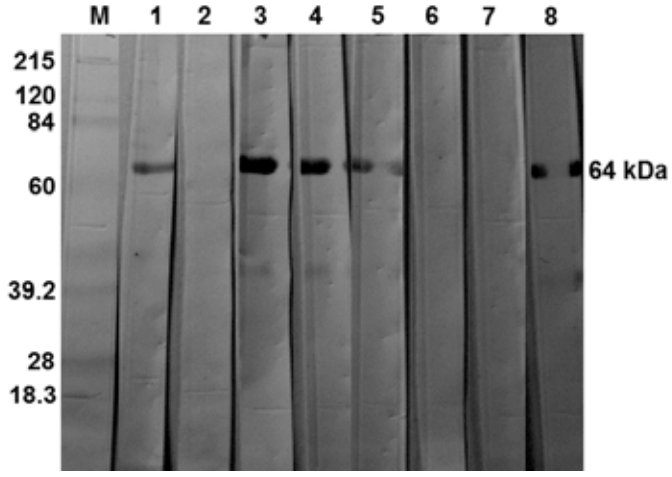

FIGURE 3. ANTIGENIC RECOGNITION OF *T. vivax* POLYPEPTIDES BY SHEEP, CATTLE AND HORSES. M: Molecular weight standard; Lane 1: Positive control (*T. vivax* positive sheep serum as assessed by IFAT); Lane 2: Negative control (*T. vivax* negative sheep serum as assessed by IFAT); Lane 3: serum from a naturally *T. vivax*-infected sheep; Lanes 4 and 5: serum from experimentally infected sheep 1 at the time of the first peak of parasitemia (5 days post infection); Lane 6: serum from a bovine negative to *Anaplasma marginale, Babesia bigemina and T. vivax* as assessed by IFAT; Lane 7: serum from a *B. bigemina*-positive bovine; Lane 8: serum from a naturally *T. evansi*-infected horse (seropositive by IFAT).

Out of the 11 bands accounted in this study, one (64 kDa) was recognized as a major antigenic protein and two (42 and 72 kDa) were detected as minor polypeptides. Previous studies related with the antigenic proteins of *T. vivax* indicate that 64 kDa is immunodominant in this species [1, 4]. Indeed, it have been found that serum from *T. vivax*-positive cattle detected two bands with apparent *M*r 32 and 66 kDa [1]. Similarly, other study reported a strong recognition of a 65 kDa protein by antibodies of ovine which had been experimentally infected with *T. vivax* [4]. Both of these both immunodominant proteins, 66 and 65 kDa previously described, should be the same as 64 kDa described in this paper. Since *T. evansi* and *T. vivax* have shown a very high cross-reactivity, some researchers have performed studies addressing the common antigens found between these two hemoflagellates [2, 3, 23, 24, 26]. The results presented here have demonstrated the high antigenic capacity and cross-reactivity of one polypeptide with apparent *M*r of 64 kDa, because it was detected by all infected sheep during the all infection course. It was also recognized by naturally and experimentally *T. vivax*-positive sheep sera and by a horse naturally infected with *T. evansi*, but it was not recognized by the hemoparasites-negative bovine and the *Babesia bigemina*-positive (*T. vivax*-negative) bovine. Therefore, the 64 kDa polypeptide detected in this experiment may correspond to an antigen that share epitopes with proteins from other trypanosomes, such as *T. evansi*, which means that this protein may be a good antigen for diagnosing infections either with *T. evansi* or with *T. vivax*. Recently, it has been studied the *T. vivax* GM6 (TvGM6) antigen which is an invariant antigen associated with the flagellum and partially conserved in *T. brucei*, *T. congolense* and *T. vivax* [17]. These researchers found some cross-reactions with *T. congolense*-infected animals, therefore the TvGM6 ELISA alone cannot be used for species-specific diagnosis. On the other hand, infected animals in the present study recognized two additional proteins with apparent molecular masses of 42 and 72 kDa. Nonetheless, sheep 4 only recognized the 72 kDa polypeptide in weeks 6 and 8 post infection, while sheep 5 detected 42 kDa protein in the second week of infection. In general, the detection of these two proteins was weaker when compared to the reaction against the 64 kDa protein. These discrepancies in antigen recognition could be due to individual differences among host immune systems or the reduced antigenic capacity of these proteins. Since early biochemical studies showed that the *T. vivax* variable surface glycoprotein (VSG), differed from the *T. brucei* and *T. congolense* counterparts in several features, including its size (~40 kDa), pattern of glycosylation, and C-terminal end [5], it is likely that the 42 kDa protein found in this study, would correspond to the *T. vivax* VSG. Although it has been demonstrated that VSG in *T. evansi* is the most immunodominant antigen [2, 23, 24], it appears not to be the case in *T. vivax*. These findings are consistent with those of other researchers [9, 21] that have suggested that the VSG from *T. vivax* is less immunogenic than the VSG from other trypanosomes. In a transcriptome analysis of a different Venezuelan *T. vivax* isolate, LIEM-176, the researchers concluded that the cellular surface of *T. vivax* is substantially different than that of *T. brucei* and very likely different from those of other African trypanosomes [9]. In fact, the VSG represent 55 % of the externally disposed cell-surface proteins in *T. vivax*, a considerably lower figure than the 95–98 % reported for the *T. brucei* VSG, which could explain the lower antigenic capacity of this molecule in *T. vivax* [9].

CONCLUSIONS AND RECOMMENDATIONS

In the present study, it was demonstrated three immunoreactive proteins from *T. vivax*, one major with approximate *M*r of 64 kDa and two slightly immunorecognized proteins with apparent molecular masses of 42 and 72 kDa. These two proteins should be studied and sequenced in order to test them as antigens for *T. vivax*-specific diagnosis. VSG of *T. vivax* seemed not to be the most antigenic protein detected. Besides, WB analysis indicated cross-reactivity of the 64 kDa protein since it was detected by sera of *T. evansi* positive horse.

Additional detailed studies using WB for the characterization of different isolates of *T. vivax* by antibodies of various host species may enhance the understanding of the antigenic proteins and

give some insight about the cross-reactions between *T. vivax* and *T. evansi*. Additional parasite proteins should be further evaluated by 2D-eletrophoresis for identify specific and nonspecific antigens.

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