

Genetic profile of two isolates of *Trypanosoma evansi* from southern Brazil with different parasitaemias

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Resumo

Perfil genético de dois isolados de *Trypanosoma evansi* do Sul do Brasil com diferentes parasitemias. *Trypanosoma evansi* é o mais difundido dos tripanossomas patogênicos, devido à sua capacidade de ser transmitido mecanicamente por moscas hematófagas. Alguns estudos têm demonstrado que *T. evansi* possui uma homologia genética entre populações de todo o mundo, mas não descreveram as relações entre os padrões de infecção no hospedeiro. Neste estudo, foram utilizadas técnicas moleculares para determinar as variabilidades genéticas de dois isolados de *T. evansi* do Sul do Brasil que mostraram diferentes padrões de infecção em modelo murino. Os marcadores de variabilidade genética foram amplificados utilizando as técnicas descritas, bem como a presença de DNA do cinetoplasto também foi verificado. Foi encontrado um perfil genético muito próximo entre os isolados de *T. evansi* utilizando *random amplified polymorphic DNA* (RAPD) e marcadores *inter-simple sequence repeats* (ISSR). Os iniciadores derivados da sequência de maxicírculos de *Trypanosoma brucei* que codificam a subunidade 5 da NADH desidrogenase (*nad5*) foram usados para demonstrar a ausência de maxicírculos, enquanto imunofluorescência foi usada para verificar a ausência de DNA do cinetoplasto. Todos os métodos demonstram a ausência de kDNA, como ocorre nos isolados americanos de *T. evansi*. As diferenças notadas nos padrões fenotípicos dos animais que foram observadas durante a infecção não estão, provavelmente, relacionados com as alterações moleculares, mas à adaptação a diferentes hospedeiros, como descrito para outros tripanosomatídeos.

Palavras-chave: Cinetoplasto; RAPD; *Trypanosoma evansi*; Variabilidade genética

Abstract

Trypanosoma evansi is the most widespread pathogenic trypanosome, due to its ability to be mechanically transmitted by hematophagous flies. Some studies have shown that *T. evansi* has a high genetic homology among populations around the world, but they have not described the relationships among infection patterns in the host. In this study, we used molecular techniques to determine the genetic variability of two isolates of *T. evansi* from southern Brazil showing different infection patterns in a murine model. Genetic variability markers were amplified using the techniques described, as well as the presence of DNA from kinetoplast was also checked. We found a very close genetic profile between the isolates of *T. evansi* using random amplified polymorphic DNA (RAPD) and markers *inter-simple sequence repeats* (ISSR). Primers derived from the sequence of *Trypanosoma brucei* maxicircle encoding the subunit 5 of NADH dehydrogenase (*nad5*) were used to demonstrate the absence of maxicircles, while immunofluorescence was used to check the lack of DNA from kinetoplast. All methods reveal the absence of kDNA, as occurring in American isolates of *T. evansi*. Perhaps, differences noticed in the phenotypic patterns of animals that were observed during infection are not associated with the molecular changes, but with the adaptation to different hosts, as described for other trypanosomatids.

Key words: Genetic variability; Kinetoplast; RAPD; *Trypanosoma evansi*

Introduction

Trypanosoma evansi is the most widespread pathogenic trypanosome, causing significant morbidity and mortality in breeding and wild animals around the world. The first human case of trypanosomiasis caused by *T. evansi* was recently reported in India (JOSHI et al., 2005). This disease is commonly known as “surra” or “mal de cadeiras” and it results in low productivity in animal breeding, which commonly occurs in endemic areas (SEIDL et al., 2001). *Trypanosoma evansi* infection has been included into the list of notifiable diseases by the World Organization for Animal Health (OIE). Transmission is mechanical and it occurs mainly through fly bites (BRUN et al., 1998).

Recently, a study demonstrated that *T. evansi* is phylogenetically related to *T. brucei*, but the difference lies on the fact that *T. evansi* lacks some parts of kinetoplasmic DNA (LAI et al., 2008), locking the trypanosome at the bloodstream stage, as also shown by Timms et al. (2002). Although genetic exchange of *T. brucei* occurs in the laboratory (JENNI et al., 1986), this process remains unclear, because there are different models suggested for the population structure of trypanosomatids, e.g. clonal population structure and the expansion of few genotypes in a short time frame when genetic exchanges take place (MACLEOD et al., 1999). Thus, the absence of vector phases in *T. evansi* disables genetic recombination (GIBSON; STEVES, 1999; SCHNAUFER et al., 2002) and restricts establishing different genotypes.

The characterization of genotypes has practical implications, such as the establishment of various disease patterns, which make diagnosis more difficult, as well as understanding on parasite spread in areas with low occurrence. *Trypanosoma evansi* stocks isolated from various world regions (America, Asia, Africa) and animal species (dogs, horses, coatis) have shown that these organisms constitute a genetically homogeneous group. Studies using isoenzymes and analysis of ribosomal internal transcribed DNA (QUEIROZ et al., 2000; LIMA et al., 2008) evidenced this connection. Similar results have been found by using random amplified polymorphic DNA (RAPD) (VENTURA et al., 2002; LUN et al., 2004), amplified fragment length polymorphism (AFLP) (MASIGA et al., 2006), *inter-simple sequence repeats* (ISSR), isoenzymes (QUEIROZ et al., 2000), ribosomes (HUGHES; PIONTKIVSKA, 2003), minisatellite (MACLEOD et al., 1999; NJIRU; CONSTANTINE, 2007; NJIRU et al., 2007), and analysis of expression-site-associated-genes (ESAGs) (WITOLA et al., 2005; MEKATA et al., 2009).

Our laboratory has already noticed different infection patterns in two different isolates of *T. evansi* in a murine model. In this study, we conducted a multifactorial characterization of these recent isolates to check the relationships between genotypes and the various phenotypes. Additionally, the absence of kDNA was verified.

Material and Methods

Two *T. evansi* stocks confirmed through species specific Polymerase chain reaction (CLAES et al., 2004) were used in this study: one was isolated from a dog (COLPO et al., 2005) and designated as TeD, and the stock designated as TeH was isolated from a horse, in 2009. Both natural cases occurred in the state of Rio Grande do Sul, southern Brazil. A *T. brucei* stock was used as control in this study.

Two Wistar rats (*Rattus norvegicus*) weighing ~220 g were kept at controlled temperature (23°C), humidity, and light cycle (12 h/12 h light/dark). All animals received water and food *ad libitum*. The procedures involving animals were approved by the Animal Welfare Committee of the State University of Santa Catarina (UDESC), under the Protocol 1.42.10, in accordance with the Brazilian legislation and the ethical principles published by the Brazilian College of Animal Experimentation (COBEA).

To obtain the parasites, Wistar rats were infected and their blood was collected at the peak of parasitaemia. After a Percoll® gradient (NDAO et al., 2004), the samples underwent ion exchange chromatography on DEAE-cellulose (LANHAM; GODFREY, 1970). Genomic DNA was obtained using the phenol-chloroform method (SAMBROOK; RUSSEL, 2001).

RAPD reactions were performed using six primers from a Ready-to-go™ RAPD Analysis Beads Kit (GE Healthcare, UK), according to manufacturer's instructions. The similarity coefficient was calculated as described by Nei and Li (1979).

A microsatellite analysis of the marker MORF2-CA was performed using the primers described by Bringaud et al. (1998). In short, the primers were used to amplify a region between RAB1 and ORF2, flanking genes of glucose transporters. Amplicons were sequenced in a Megabace 1000 DNA Analysis System (ACT-gene). All high quality DNA sequences (Phred P20) were analyzed using the Phred/Phrap/Consed package (EWING; GREEN, 1998) The identity of sequences was confirmed by the BLAST tool (www.ncbi.nlm.nih.gov/blast) (ALTSCHUL et al., 1997).

To check the absence of kDNA, specific primers were used to detect the maxicircle sequence of the subunit 5 (nad5) of NADH dehydrogenase (LI et al., 2007) and a fluorescence staining assay with 4,6-diamidino-2-phenylindole (DAPI) using *T. brucei* as control was performed. The sequences of primers and markers used in the present study are shown in Table 1.

Results

Recently, our laboratory noticed contrasting parasitaemia patterns between two different isolates of

TABLE 1: List of primers used in this study.

| | Sequence | Reference |
|-----------|--|---|
| RAPD | P1: 5'(GGTGCGGGAA) P2: 5'(GTTTCGCTCC) P3: 5'(GTAGACCCGT) P4: 5'(AAGAGCCCGT) P5: 5'(AACGCGCAAC) P6: 5'(CCCGTCAGCA) | Ready-to-go RAPD kit, GE Healthcare (prod. code 27-9502-01) |
| MORF2-CA | For: 5' (TTTATCTCACATTACTCGGCG) Rev: 5' (GCGTCGATCATGTCTACCGTAC) | BRINGAUD et al. (1998) |
| RoTat 1.2 | For: 5' (GCGGGGTGTTTAAAGCAATA) Rev: 5' (ATTAGTGCTGCGTGTGTTTCG) | CLAES et al. (2004) |
| NADH gene | For: 5' (TGGGTTTATATCAGGTTTCATTTATG) Rev: 5' (CCCTAATAATCTCATCCGAGTACG) | LI et al. (2007) |

T. evansi. TeD was kept by multiple passages through murine models and it reached a strict pre-patent period in 3 to 5 days, the peak of parasitaemia occurs and the animal dies. However, the TeH stock, recently isolated, has shown a long and erratic pre-patent period of more than 21 days without a perceptible peak of parasitaemia, then, a regular peak and animal death. To ensure reliability and identity of samples, both isolates were confirmed as being *T. evansi* using the amplification of RoTat 1.2 antigen, which is a specific variable surface glycoprotein gene (data not shown) and, in both, TeD and TeH, DAPI staining suggests a total absence of kDNA (Figure 1)

and the non-amplification of the gene *nad5* indicates the absence of maxicircles, which exist in *T. brucei*.

Then, some techniques were used to check whether different parasitaemia profile of the isolates was due to genetic variability. The RAPD profile (Figure 2) showed a high homology (99%) between TeD and TeH isolates. Regarding *T. brucei*, TeD homology was 99% and TeH was 81%. The marker MORF2-CA of TeD isolate has 50 tandem repetitions, while TeH has 46 repetitions (Figure 3), showing identical alleles to some Brazilian isolates already characterized (BITEAU et al., 2000).

FIGURE 1: Fluorescence staining assay showing the absence of kinetoplast. A: *T. brucei* trypomastigote. B: TeD isolate. C: TeH isolate. Arrow indicates the nucleus and arrowhead indicates the kinetoplast of *T. brucei*.

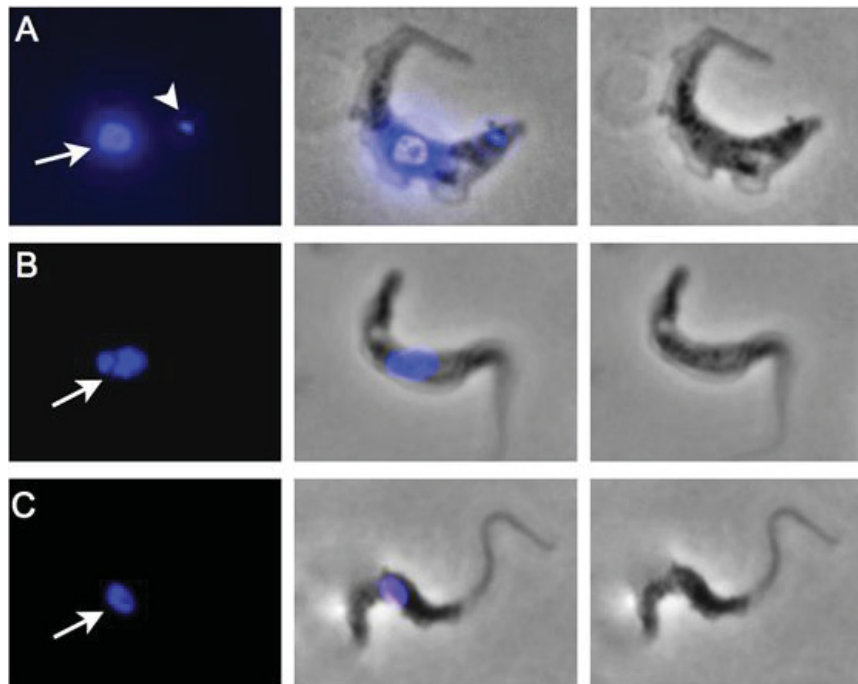
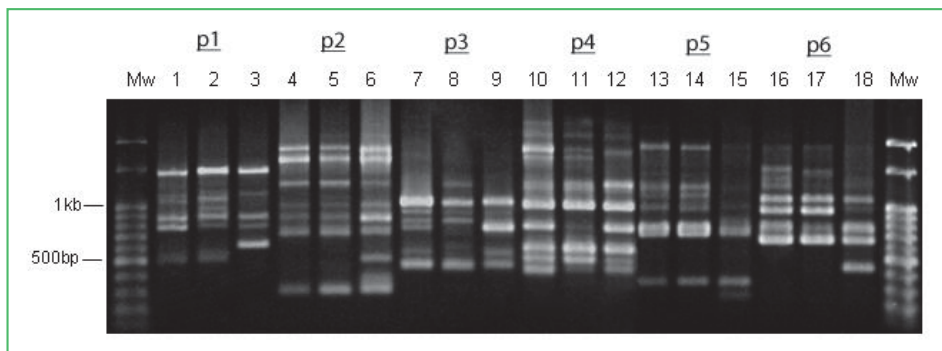


FIGURE 2: RAPD profiles of *T. evansi* (TeD and TeH) stocks and *T. brucei* using six primers from RAPD analysis beads kit. Lanes TeD: 1, 4, 7, 10, 13, 16; TeH: 2, 5, 8, 11, 14, 17; *T. brucei*: 3, 6, 9, 12, 15, 18; M: 100 bp ladder. P1-P6 correspond to RAPD primers.



The genetic variability and its relationship to host pathology have been reported for other trypanosomatids (MACEDO et al., 2002; MORRISON et al., 2009). Despite the complex nature of the phenotypes, Morrison et al. (2009) identified a quantitative trait locus in *T. brucei* that may be related to the pathologic patterns during the infection process. Simultaneously, epigenetic studies of protozoan parasites have revealed that there are correlations between epigenetic mechanisms and virulence factors (LOPEZ-RUBIO et al., 2007; RUDENKO, 2010). In *T. brucei*, the molecular control of antigenic variation is poorly understood, since it is mainly post-transcriptional and there are epigenetic mechanisms that may control differential and stage-specific expression, unlike other trypanosomatids (LOPEZ-RUBIO et al., 2007).

Other recent studies suggested that the variability in the *T. evansi* transferrin receptor genes could be related to the several different hosts affected by the disease (ISOBE et al., 2003; WITOLA et al., 2005) in which American *T. evansi* transferrin receptor genes showed more diversity than in Asian *T. evansi* stocks (MEKATA et al., 2009). The isoenzyme profiles of Brazilian *T. evansi* stocks showed high biochemical homology while remarkable differences in virulence were observed in these stocks (QUEIROZ et al., 2000). The same characteristic was observed in our isolates when TeD was kept by several passages in rodents and showed increased pathogenicity in the murine model. As described in other recent studies, *T. evansi* has exceptional genetic homology throughout the world, as also observed in TeD and TeH stocks. However, by using this standardized molecular markers, we did not notice significant genetic differences that could be linked to the pathogenic patterns between the parasite and the hosts. Further studies on the molecular characterization of these isolates have been developed.

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