Research Article

First Report of Trypanosoma sp. in Spectacled Caiman (Caiman crocodilus): Morphological and Phylogenetic Relationships

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In Crocodylidae family three trypanosomes species were described, T. grayi in African crocodilian and T. cecili and Trypanosoma sp. in Caimans species from Brazil. T. grayi was transmitted by tsetse flies and the vector of Brazilian caimans trypanosomes is unknown. We characterized first Brazilian trypanosome isolated in spectacled caiman (Caiman crocodilus) from Mato Grosso State in Brazil. Morphological findings in epimastigotes forms from axenic culture showed high similarity with Trypanosoma sp. described in Caiman yacare from Brazilian Pantanal. Phylogenetic studies performed with SSU rDNA and gGAPDH (glyceraldehydes-3-phosphato dehydrogenase glycosomal) clustering in T. grayi Clade and together to genotype Cay 01 from Trypanosoma unnamed species isolated in C. yacare. This is the first isolate of Trypanosoma sp. from C. crocodilus and the phylogenetic position with isolates in C. yacare from Pantanal region and demonstrates the low host specificity of cayman trypanosomes in Brazil.

1. Introduction

The order Crocodylia includes 23 living species and three families are recognized, Crocodyliidae, Gavialidae, and Alligatoridae [1]. Most species of families Crocodyliidae and Gavialidae occur in Africa and Asia. Only Alligatoridae occurs in South America and is composed of six species, Paleosuchus palpebrosus, P. trigonatus, Melanosuchus niger, Caiman yacare, C. latirostris, and C. crocodilus [2].

The spectacled caiman (Caiman crocodilus) has the widest distribution of the New World crocodilians with geographic range from southern Mexico to Peru and Brazil. This geographic variability enabled a segregation of this species into four subspecies [3, 4]. A single subspecies occur in North and Central Brazil, Caiman crocodilus crocodilus [5].

The crocodilians are host to a wide variety of parasites, like intestinal parasites (nematodes and trematodes) [6–10] and hemoparasites (haemogregarines and trypanosomes) [10–13].

The species of genus Trypanosoma are parasites of all vertebrate classes (fish, amphibians, reptiles, birds, and mammals) with life cycles alternating between vertebrates and invertebrates hosts. Most species develop in arthropod vectors, which may belong to different orders and families, while fish, amphibian, and reptiles parasites are transmitted by leeches or insects. Other species are only mechanically transmitted. This genus has several stages, present in different combinations, in blood and/or tissues in the vertebrate and invertebrate hosts [14–19].

In reptiles are described about 80 species of Trypanosoma parasites, including 42 in lizards, 14 in turtles, 21 in snakes, and 3 in crocodilians. The species descriptions are based on morphology of blood forms, host, and geographic origin [20]. In Africa, T. grayi has been described in Osteolaemus tetraspis...
and Crocodylus niloticus, both in family Crocodylidae [21]. In Brazil, Trypanosoma unnamed species was described in Caiman yacare [22] and T. cecili in Caiman crocodilus [13].

Phylogenetic studies performed with a large number of isolates from Africa (T. grayi) and Brazil (Trypanosoma sp.) positioned all sequences from whole SSU rDNA and gGAPDH in a unique monophyletic branch named T. grayi Clade [19, 23, 24]. The Brazilian isolates are segregated in two genotypes, named Cay01 and Cay02, and are different from T. grayi isolates [19]. The host of Brazilian trypanosomes isolates is Caiman yacare captured in Pantanal region.

In the present study, we described a first record of Trypanosoma sp. in spectacled caiman (Caiman crocodilus) from Mato Grosso State and addressed the phylogenetic relationships with other crocodilian trypanosomases.

2. Materials and Methods

2.1. Study Areas and Capture of Spectacled Caiman. The sample was collected from a single animal captured by ethnic Indians Tapirapé within the area of their Indian reserve in Mato Grosso State, county of Confresa (10°38'22'' S, 51°34'08'' W), composed of a mosaic of Amazonian Rain Forest and Cerrado biomes. The animal included in this study was obtained from legal hunting activity, authorized by IBAMA, held by the indigenous population. Three specimens of C. crocodilus, after being handed and immobilized, were anaesthetized and the blood samples and tissue samples (spleen, heart, kidney, lung, and liver) were collected.

2.2. Isolation in Culture of Trypanosoma sp. from Spectacled Caiman. For Trypanosoma isolation, blood samples from spectacled caiman (Caiman crocodilus) were inoculated in vacutainer tubes with a biphasic medium containing 15% of sheep red blood cells with 4% Blood Agar Base and overlaid with liquid LIT medium supplemented with 20% of FBS as before [19]. The culture was incubated at 28°C and expanded in LIT (Liver Infusion Tryptose) medium for DNA preparation. The isolate (CBT 02) was cryopreserved in liquid nitrogen in the Department of Preventive Veterinary Medicine and Animal Health, Faculty of Veterinary Medicine, University of São Paulo. Samples from cultures were smeared on glass slides and fixed with methanol and stained with Giemsa and photographed.

2.3. Molecular Study. DNA sample was extracted from parasite mass of trypanosome culture using the phenol-chloroform method and primary samples were purified using the Wizard DNA Clean-Up System (Promega, Fitchburg, Wisconsin). Extracted DNA samples were subjected to conventional polymerase chain reaction (PCR) targeting a fragment of approximately 900 base pairs (bp) of the V7V8 SSU rDNA [18, 19] and approximately 800 bp of the gGAPDH (glyceraldehydes-3-phosphate dehydrogenase glycosomal) as previously described [23]. PCR products of the expected sizes were purified and sequenced in an automatic sequencer (Applied Biosystems/PerkinElmer, model ABI Prism 310 Genetic, Foster City, CA) according to the manufacturer’s recommendations. The nucleotide sequences generated were deposited in GenBank under the accession numbers JQ768791 and JQ768792, respectively, for V7V8 SSU rDNA and gGAPDH genes. These sequences were concatenated and aligned using ClustalX [25] and adjusted manually using GeneDoc software [26] with sequences previously determined from other trypanosomatids species available in Genbank (Table 1). The V7V8 region of SSU rDNA of crocodilian isolates was used to construct a dendogram for intraspecific analysis. The phylogenetic tree was inferred by the maximum parsimony (MP) method using PAUP version 4.0b10 [27] with 500 replicates of random addition taxa and TBR branch swapping. Bayesian analysis (B) was performed by Mrs. Bayes v3.1.2 [28] and 1.000.000 generations were employed as using GTR substitution model and four categories range proportion of invariant sites. Posteriori probabilities were used to support branches.

3. Results

The positive hemoculture obtained from a single spectacled caiman captured generated one isolate maintained in culture (33%). Cultures were maintained in axenic medium LIT and used for morphological analysis. Unfortunately, no smears were obtained from the blood of the spectacled caiman. All tissues, including the positive animal, were negative to trypanosome DNA barcoding.

The morphology consisted of epimastigotes with a large kinetoplast positioned near the nucleus and small and narrow undulating membrane (Figure 1) similar to the morphotype 1 in C. yacare trypanosomes previously described [19].

We determined sequences from gGAPDH and SSU rDNA (V7-V8 region) and these sequences were aligned with sequences from different trypanosomatids species retrieved from GenBank (Table 1).

Phylogenetic relationships based on gGAPDH and SSU rDNA sequences inferred by parsimony and Bayesian clustered this new isolate together to the major branch, which corresponds to crocodilian trypanosomes isolates (T. grayi Clade) (Figures 2(a) and 2(b)). The T. grayi Clade included both African crocodilians trypanosomes and American caymans trypanosomes. The isolates from Brazilian caimans were segregated into genotypes assigned Cay01 and Cay02 previously described [19].

Despite distinct host and different geographic origins, the isolate from C. crocodilus and Cay01 genotype from C. yacare shared high similarity (~0.1% for gGAPDH and identical SSU rDNA V7-V8 region). Phylogenetic analysis using the concatenated data set of gGAPDH and SSU rDNA genes generated very similar phylogenetic topologies (data not show).

4. Discussion

In Brazil only two trypanosomes have been reported in Caiman species based on morphological analysis of blood trypomastigote: unnamed species in C. yacare from Pantanal [22] and Trypanosoma cecili in C. crocodilus from Amazonia
| Trypanosomatids species | Isolate code | Host          | Geographic origin | Accession number<sup>a</sup> |
|------------------------|--------------|---------------|-------------------|-----------------------------|
|                        |              |               | gGAPDH            | SSU rDNA                    |
| Trypanosoma sp.        | CLAR         | Clarias angolensis | Africa            | AJ620251                    |
| Trypanosoma sp.        | MARV         | Cyprinus carpio     | Czech Republic    | AJ620248                    |
| T. granulosum          | Portugal     | Anguilla anguilla  | Portugal          | AJ620247                    |
| T. granulosum          | UK           | Anguilla anguilla  | United Kingdom    | AJ620246                    |
| Trypanosoma sp.        | Gecko        | Tarentola annularis | Senegal           | AJ620259                    |
| T. varani              | V54          | Varanus exanthematicus | Senegal          | AJ620261                    |
| Trypanosoma sp.        | T610         | Caiman yacare     | Brazil            | EU596256  EU596252          |
| Trypanosoma sp.        | T624         | Caiman yacare     | Brazil            | EU596257  EU596253          |
| Trypanosoma sp.        | T625         | Caiman yacare     | Brazil            | EU596259                    |
| Trypanosoma sp.        | T1092        | Caiman yacare     | Brazil            | EU596258  EU596254          |
| Trypanosoma sp.        | T1100        | Caiman yacare     | Brazil            | EU596260                    |
| Trypanosoma sp.        | T1101        | Caiman yacare     | Brazil            | EU596261                    |
| Trypanosoma sp.        | T1102        | Caiman yacare     | Brazil            | EU596262                    |
| Trypanosoma sp.        | T1119        | Caiman yacare     | Brazil            | EU596263                    |
| Trypanosoma sp.        | T1120        | Caiman yacare     | Brazil            | EU596255                    |
| Trypanosoma sp.        | CBT02        | Caiman crocodilus | Brazil            | JQ768791  JQ768792          |
| T. avium               | Chaffinch    | Fringilla coelebs | Czech Republic    | AJ620263                    |
| T. avium               | Rook         | Corvus frugilegus | Czech Republic    | AJ620262                    |
| T. cruzi               | USP          | Rattus rattus     | Brazil            | AJ620267                    |
| T. cruzi marinkellei   | B7           | Phyllostomus hastatus | Brazil         | AJ620270                    |
| T. cruzi              | L32          | Rattus rattus     | Brazil            | AJ620272                    |
| T. microti             | TRL132       | Microtus agrestis | England           | AJ620273                    |
| Trypanosoma sp.        | ABF          | Wallabia bicolor  | Australia         | AJ620276                    |
| Trypanosoma sp.        | R5           | Oryctolagus cuniculus | Australia    | AJ620276                    |
| T. theileri            | K127         | Bos taurus        | Germany           | AJ620282                    |
| Trypanosoma sp.        | D30          | Cervus dama       | Germany           | AJ620279                    |
| T. dionisii            | P3           | Pipistrellus pipistrellus | United Kingdom | AJ620271                    |
| T. vespertilionis      | P14          | Pipistrellus pipistrellus | England       | AJ620283                    |
| T. brucei rhodesiense  | 058          | Homo sapiens      | Zambia            | AJ620284                    |
| T. vivax               | Desowitz     | Ovis aries        | Nigeria           | AJ620295                    |
| T. vivax               |              | Microtus agrestis | England           | AJ620273                    |
| T. congolense Forest   | Cam22        | Capra capra       | Cameroon          | AJ620289                    |
| T. congolense Forest   | TSW103       | Sus scrofa        | Liberia           | AJ620286                    |
| T. evansi              |              |                  |                  | AF53743                     |
| T. cyclops             | LV492        | Macaca sp.        | Malaysia          | AJ620265                    |
| T. minasense           | LSTM         | Saimiri boliviensis | South America  | AJ620274                    |
| T. rangeli             |              |                  |                  | AF53742                     |
| *Crithidia fasciculata* |              |                  |                  | AF047493                     |
| T. congolense Forest   | ANR3         | Glossina palfalis | Gambia           | AJ620285                    |
| T. congolense Tsavo    | I14          | Glossina pallidipes | Tanzania     | AJ620291                    |
| T. cruzi              | C8 cl2       | Triatoma infestans | Bolivia         | AJ620268                    |
| T. cruzi              | VINCH 89     | Triatoma infestans | Chile            | AJ620269                    |
Table 1: Continued.

| Trypanosomatid species | Isolate code | Host | Geographic origin | Accession number<sup>a</sup> |
|------------------------|--------------|------|-------------------|-----------------------------|
| *T. grayi*             | ANR4         | *Glossina palpalis* | Gambia            | AJ620257; AJ005278          |
| *T. grayi*             | BAN1         | *Glossina palpalis* | Gambia            | AJ620258; AJ620546          |
| *T. simiae*            | Ken 2        | *Glossina morsitans* | Gambia            | AJ620293                   |
| *T. godfreyi*          | Ken 7        | *Glossina morsitans* | Gambia            | AJ620292                   |
| *T. simiae* Tsavo      | Ketri 1864   | *Glossina pallidipes* | Kenya             | AJ620294                   |
| *Trypanosoma* sp.     | TL_AQ.22     | *Philaeon* sp.      | Australia         | AJ620280                   |

<sup>a</sup>Sequences determined in this study and deposited in GenBank are underlined and bold.

The prevalence of *Trypanosoma* sp. in *C. yacare* varies from 35 to 46% in different studies performed in Pantanal region from Mato Grosso do Sul state [19, 22]. Only one spectacled caiman captured in Mato Grosso was positive in hemoculture (33%), but the prevalence in this species has not been established and a further study involving a large number of individuals (*C. crocodilus*) is necessary to determine the prevalence of *Trypanosoma* sp. and *T. cecili*.

Most studies of caiman trypanosomes were performed in the Pantanal region. The only study in Amazon with *C. crocodilus* and *Paleosuchus trigonatus* found a very low prevalence with rare trypomastigote forms in blood or tissue. This species was designated *T. cecili* and isolation has not yet been possible [13].

The epimastigotes forms in axenic culture of spectacled caiman isolate are very similar to *Trypanosoma* sp. from *C. yacare* from Pantanal (Figure 1). The tissue imprint forms of *T. cecili* and blood forms of *Trypanosoma* sp. from Pantanal are distinguishable, but similar tissue forms are detected in *C. yacare* and *C. crocodilus* [13, 19, 22].

The variable V7-V8 region of SSU rDNA has been used for DNA barcoding of trypanosomatids and is able to distinguish all species, polymorphisms, and genetic relationships among closely related taxa [18, 19, 29–33]. In addition, the gGAPDH sequences were used to improve the phylogenetic analysis [19, 23, 24, 33]. In all phylogenetic analyses inferred by V7-V8 SSU rDNA and gGAPDH the isolated CBT 02 was included in monophyletic branch called *T. grayi* Clade, together with *Trypanosoma* sp. from Brazilian caiman. The V7-V8 region segregated the Brazilian caimans isolates in two branches: Cay01 and Cay02. Most isolates were comprised of Cay01 genotype, probably the most prevalent in Pantanal region. The new isolate from *C. crocodilus* (CBT 02) nested in Cay01 genotype.

The phylogenetic positions of CBT02 isolate show that *Trypanosoma* sp. from Cay01 genotype I is able to infect two species of *Caiman* genus in Brazil. These findings clarify the morphological approaches in tissues imprints which have trypomastigote similar morphology in *C. yacare* and *C. crocodilus*.

The distribution of Cay01 genotype is not restricted to Pantanal region of Mato Grosso do Sul state and vertebrate host. The Confresa county is located in north of Mato Grosso state and comprises a mosaic of Amazonia and Cerrado biomes and is irrigated by Tocantins-Araguaia basin. The C.
C. yacare occurred in upper Madeira system of the Amazon basin and Paraguay River and lower Paraná system including Brazil, Bolivia, Paraguay, and Argentina. C. crocodiles occur in both Amazon and Orinoco drainage [2, 5]. According to Brazaitis et al. [34], sympatric areas occur in Mato Grosso and Rondônia states. However, in Tocantins-Araguaia basin only C. crocodilus occurs.

The presence of the same trypanosome species in C. yacare and C. crocodilus indicates transmission cycles between these species evidencing sympatry of caiman species or exposure to the same vector. The T. grayi in Africa is transmitted by tse-tse flies [35]. In Brazil, different species of tabanids can bite reptiles like C. crocodilus and Eunectes murinus [36]. The low host specificity of tabanids and host switch of caiman trypanosomes suggest an important role of insects in the transmission cycle of these parasites in Brazil.

The addition of new isolates of caiman trypanosomes of different vertebrate hosts is necessary for better understanding of diversity and phylogenetic relationships of these parasites and demonstrates the low host specificity of cayman trypanosomes in Brazil.

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