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Incrimination of *Eratyrus cuspidatus* (Stal) in the transmission of Chagas' disease by molecular epidemiology analysis of *Trypanosoma cruzi* isolates from a geographically restricted area in the north of Colombia

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ABSTRACT

Following the report of two cases of acute Chagas' disease and the appearance of several triatomine species in human dwellings in an area considered non-endemic for domestic transmission of *Trypanosoma cruzi*; a epidemiological, entomological and *T. cruzi* molecular epidemiology analysis was performed in order to establish the transmission dynamic of the parasite in the studied area. 2 *T. cruzi* isolates from human patients, 5 from *Eratyrus cuspidatus*, 4 from *Rhodnius pallescens*, 4 from *Panstrongylus geniculatus* and 7 reference stocks were analyzed by mini-exon gene, random amplified polymorphic DNA (RAPD) and multilocus enzyme electrophoresis (MLEE).

All isolates from vectors and human resulted *T. cruzi* group I by mini-exon, RAPD and MLEE. While mini-exon and MLEE did not showed any differences between the studied isolates, RAPD analysis identified a common *T. cruzi* genotype for the *E. cuspidatus* isolates and human isolates and distinguished different strains from *R. pallescens* and *P. geniculatus* isolates. The presence of the same *T. cruzi* genotype in isolates from patients and *E. cuspidatus* suggests that this species can be responsible for the transmission of Chagas' disease in the study area. RAPD analysis showed better resolution and discrimination of *T. cruzi* strains than mini-exon and MLEE and can be considered a useful tool for molecular epidemiology studies. Incrimination of sylvatic triatomine species in the transmission of Chagas' disease indicates that more knowledge about the ecology of these vectors is necessary to improve control strategies.

1. Introduction

Chagas' disease represents the first cause of cardiac lesions in young, economically productive adults in the endemic countries of Latin America. It is transmitted in ecological units composed of sylvatic or domestic mammals and sylvatic or domestic Triatomine species, both infected with *Trypanosoma cruzi* within well-defined geographical environment (Moncayo, 2003). The transmission cycles are complex. Transmission cycles of *T. cruzi* are enzootic when no domestic triatomine colonies exist and sporadic cases of Chagas' disease are present (Miles et al., 2004). More than 130 species of triatomine are known (Galvão et al., 2003) but only some species have adapted to human dwellings conforming the domestic transmission cycle. Domestic and sylvatic transmission cycles can be considered as separate or overlapping according to the locality and the dynamics between them.

Phenotypic and genotypic characters have been used to divide the species *T. cruzi* into at least two principal divisions (Brisse et al., 2001; Campbell et al., 2004). These divisions have been named *T. cruzi* I and *T. cruzi* II by international consensus (Anonymous, 1999). In the southern cone countries *T cruzi* II and *T. cruzi* I have been found mainly in the domestic and sylvatic cycles respectively (Miles et al., 1977; Zingales et al., 1998; Zingales et al., 1999); in contrast *T. cruzi* I predominates in both, domestic and sylvatic cycles from the Amazon basin northwards (Miles et al., 1981; Añez et al., 2004; Carrasco et al., 2005). Five lower phylogenetic subdivisions have been identified within *T. cruzi* II lineage, designated *T. cruzi* IIa-e, whereas no clear subdivision has been found within *T. cruzi* I using random amplified polymorphic DNA (RPD) and multilocus enzyme electrophoresis (MLEE) (Barnabé et al., 2000). However, most recently studies found genetic variability and phylogenetic relationships within *T. cruzi* I using sequences of the non-transcribed spacer of mini-exon genes (O'Connor et al., 2007). The mini-exon gene have led to the development of molecular tools to identify phylogenetic haplotypes in *T. cruzi* I isolates (Herrera et al., 2007). Thereby, mini-exon is an attractive marker to establish the phylogeny of lineage I and explore relationships between *T. cruzi* hosts and geographic distribution (Falla et al., 2009). Intraspecific strain characterization

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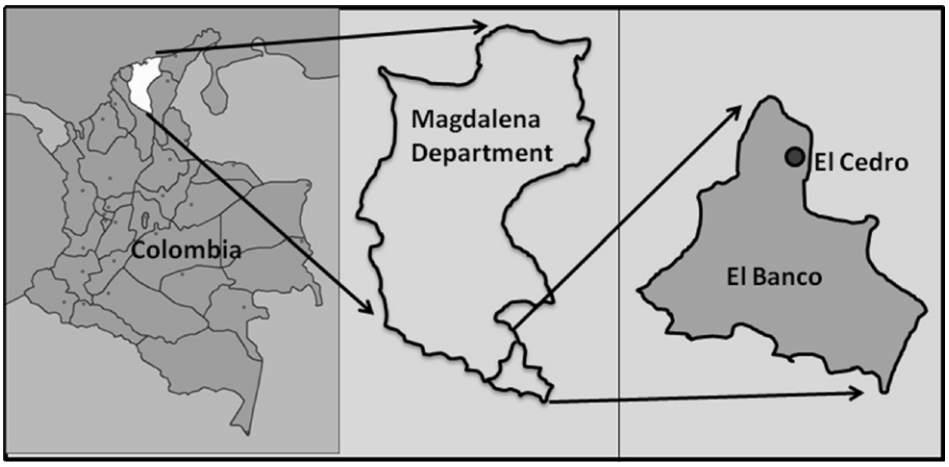

Fig. 1. Geographic location of the study area in the north of Colombia. El Cedro is the village where the patients in the acute-phase of Chagas' disease were detected.

is crucial in molecular epidemiology of Chagas' disease. Considering the association between *T. cruzi* genotypes with natural hosts and vectors, comparative genetics could possibly resolve the extensive diversity of *T. cruzi* and could guide the design of cost effective and improved control strategies.

Following the report of two cases of acute Chagas' disease and the appearance of several triatomine species in human dwellings from a geographically restricted area in the north of Colombia considered non-endemic for domestic transmission of *T. cruzi*; epidemiological and entomological surveys and *T. cruzi* molecular analysis by mini-exon gene, RAPD and MLEE were performed in order to establish the transmission dynamic of the parasite in the studied area.

2. Materials and methods

2.1. Study area

The field work was carried out in Magdalena Department, Municipality of El Banco, settlement of El Cedro (09°00′17″N; 73°58′41″W) (Fig. 1) with a population of 116 inhabitants distributed in 32 human dwellings dispersed in patches of primary and secondary forest alternated with crops field. The ecological life zone is tropical dry forest, with an altitude of 35 m above sea level, average temperature of 24–34 °C, an average relative humidity of 65%, and a mean rainfall of 1300 mm/year. This area was considered non-endemic for domestic transmission of *T. cruzi* by the National Chagas Control Surveillance Program.

2.2. Serological survey

A serum sample was obtained from the 116 individuals after written consent. Two serological methods were carried out with the same serum from each person. Indirect immunofluoresence antibody test (IFAT) and an enzyme-linked immonosorbent assay (ELISA) were performed following standard procedures (Camargo, 1966; Voller et al., 1975). Titers equal or greater than 1:64 for the IFAT and an optical absorbance equal or greater than 0.2 for the ELISA were considered positive for the infection with *T. cruzi*. Patients were considered positive when they showed reactivity in the IFAT and the ELISA. Although this method do not discard the presence of *Trypanosoma rangeli* in the studied area, all stocks were confirmed as *T. cruzi* by MLEE and molecular analysis.

2.3. Entomological survey

The totality of human dwellings from El Cedro (32) was examined for the presence of triatomine vectors using the same methods (manual capture during 60 min by a trained health care worker and passive capture by dwelling inhabitants during 1 week). The passive capture is described as the capture of insects during normal activities by dwelling inhabitants. All captured bugs were placed in plastic bottles containing filter paper and transported to the laboratory for morphological identification according to Lent and Wigodzinsky (1979).

Faeces were obtained by gently squeezing live insects after feeding. The faeces were mixed with phosphate buffered saline and examined for the presence of flagellates.

2.4. Isolation of T. cruzi from vectors and human

13 stocks of *T. cruzi* were isolated, 5 from *Eratyrus cuspidatus*, 4 from *Panstrongylus geniculatus*, 4 from *Rhodnius pallescens* and 2 from the two patients with acute Chagas' disease. Stocks from patients could not be obtained by hemoculture and were collected by xenodiagnosis using 30 fourth instars nymphs of *R. prolixus*. Bugs captured inside houses as well as nymphs used for xenodiagnosis were examined by fresh faeces smears. The positive samples were inoculated in BALB/c mice and later blood of these mice was submitted to hemoculture in liver infusion tryptose (LIT) medium. The isolated parasites were maintained by passages in LIT medium at 28 °C. Six strains previously characterized by MLEE (Tibayrenc et al., 1986) were used as reference stocks to represent the main phylogenetic subdivisions of *T. cruzi* species: X10cl1 (*T. cruzi* I), CANIIIcl1 (*T. cruzi* IIa), TU18cl2 (*T. cruzi* IIb), M6241cl6 (*T. cruzi* IIc), Mncl2 (*T. cruzi* IId) and CL-Brener (*T. cruzi* IIe).

2.5. Characterization of T. cruzi strains

Stocks were harvested by centrifugation (2800 × *g*, 20 min, at 4 °C) and washed in PBS (Na_2HPO_4 10 mM, NaH_2PO_4 10 mM, NaCl 150 mM, pH 7.2). Cells were lysed in an equal volume of hypotonic enzyme stabilizer (EDTA 2 mM, dithiotreitol 2 mM, ε-aminocaproic acid 2 mM), on ice for 20 min. The lysates were again centrifuged (13,000 × *g*, 10 min, 4 °C). The soluble fraction was stored at −70 °C until used in MLLE analysis, whereas the pellet of lysed cells was used for DNA extraction, according to the following protocol. Pellets were resuspended in 400 μl Tris–HCl 100 mM (pH 8.0), NaCl 400 mM EDTA-Na_2 10 mM. SDS was then added to a final concen-