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Identification and lineage genotyping of South American trypanosomes using fluorescent fragment length barcoding

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ABSTRACT

Trypanosoma cruzi and *Trypanosoma rangeli* are human-infective blood parasites, largely restricted to Central and South America. They also infect a wide range of wild and domestic mammals and are transmitted by a numerous species of triatomine bugs. There are significant overlaps in the host and geographical ranges of both species. The two species consist of a number of distinct phylogenetic lineages. A range of PCR-based techniques have been developed to differentiate between these species and to assign their isolates into lineages. However, the existence of at least six and five lineages within *T. cruzi* and *T. rangeli*, respectively, makes identification of the full range of isolates difficult and time consuming. Here we have applied fluorescent fragment length barcoding (FFLB) to the problem of identifying and genotyping *T. cruzi*, *T. rangeli* and other South American trypanosomes. This technique discriminates species on the basis of length polymorphism of regions of the rDNA locus. FFLB was able to differentiate many trypanosome species known from South American mammals: *T. cruzi cruzi*, *T. cruzi marinkellei*, *T. dionisii*-like, *T. evansi*, *T. lewisi*, *T. rangeli*, *T. theileri* and *T. vivax*. Furthermore, all five *T. rangeli* lineages and many *T. cruzi* lineages could be identified, except the hybrid lineages TcV and TcVI that could not be distinguished from lineages III and II respectively. This method also allowed identification of mixed infections of *T. cruzi* and *T. rangeli* lineages in naturally infected triatomine bugs. The ability of FFLB to genotype multiple lineages of *T. cruzi* and *T. rangeli* together with other trypanosome species, using the same primer sets is an advantage over other currently available techniques. Overall, these results demonstrate that FFLB is a useful method for species diagnosis, genotyping and understanding the epidemiology of American trypanosomes.

1. Introduction

Trypanosoma cruzi and *Trypanosoma rangeli* are the two species of human-infective trypanosomes occurring in overlapping areas of South and Central America. *T. cruzi* causes Chagas disease, a condition that affects at least 8 million people, with 100 million at risk and 14,000 deaths annually (Jannin and Salvatella, 2006). Despite recent advances in disrupting vector transmission in Southern Cone countries, this disease remains a major public health problem in Latin America (Schofield et al., 2006; Miles et al., 2009). In regions endemic for Chagas disease, *T. cruzi* circulates between humans and domestic animals and is transmitted by domiciliated triatomine bugs. However, infection by *T. cruzi* is a widespread zoonosis, ranging from the southern half of the USA to the southernmost countries of South America (Marcili et al., 2009c). *T. rangeli* is not believed to cause disease in humans. A high prevalence of *T. rangeli* in humans has been reported in Central America and northwestern South America, where concomitant infections and serological cross-reactivity with *T. cruzi* make diagnosis of Chagas disease difficult (Vallejo et al., 2009). Both *T. cruzi* and *T. rangeli* have a wide mammalian host range and are transmitted by a large diversity of triatomine bugs, although only species of the genus *Rhodnius* transmits *T. rangeli* (Maia da Silva et al., 2007; Vallejo et al., 2009).

Molecular studies have revealed high genetic diversity in *T. cruzi* and *T. rangeli*, with isolates of both species distributed into several lineages, also called discrete taxonomic units (DTU) within *T. cruzi* (Stevens et al., 1999; Maia da Silva et al., 2007; Miles et al., 2009; Vallejo et al., 2009). At least six lineages of *T. cruzi cruzi* have been described using molecular markers including RAPDs, SSU

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rRNA gene sequences, microsatellites and mitochondrial genes (e.g. Brisse et al., 2001; Machado and Ayala, 2001; Freitas et al., 2005; Westenberger et al., 2005; Miles et al., 2009). These lineages differ in their host range, ecotope and geographical distribution (Miles et al., 2009) and are potentially associated with variable forms of Chagas disease (Anez et al., 2004). *T. cruzi* lineages until recently designed as TcI and TcIIa–e (Brisse et al., 2001; Miles et al., 2009) were recently redesigned as follows: TcI, TcII (former TcIIb), TcIII (TcIIc), TcIV (TcIIa), TcV (TcIId) and TcVI (TcIIe) (Zingales et al., 2009).

Accurate identification of *T. cruzi* and *T. rangeli*, and their respective lineages is important for diagnosis and understanding their epidemiology. In addition, other species of mammalian trypanosome can be found in the vertebrate hosts of these species, and there are potentially species that are yet to be discovered (Stevens et al., 1999; Maia da Silva et al., 2008; Marcili et al., 2009a,c; Cavazzana et al., 2010). Distinguishing between *T. cruzi* and *T. rangeli* lineages is still problematic, especially in regions where man, wild reservoirs and triatomines can be found infected with different combinations of isolates from different lineages of both *T. cruzi* and *T. rangeli* (Yeo et al., 2005, 2007; Vallejo et al., 2009). Morphology is insufficient for species identification, particularly in mixed infections in vectors. In mixed cultures *T. cruzi* prevails over *T. rangeli* and, after successive passages, typically only one lineage of *T. cruzi* is selected (Yeo et al., 2007; Maia da Silva et al., 2008). PCR assays have increased sensitivity and accuracy of diagnosis of *T. cruzi*, allowing identification directly from tissue samples, and triatomine guts and faeces (Hamano et al., 2001; Virreira et al., 2003). Several PCR-based methods are able to differentiate *T. cruzi* from *T. rangeli*, including PCR with species-specific primers developed from several genomic regions: kDNA minicircles (Avila et al., 1991); telomeric repeats (Chiurillo et al., 2003); repetitive DNA (Vargas et al., 2000); randomly amplified polymorphic DNA (RAPD)-derived markers (Maia da Silva et al., 2004a), spliced leader gene (Maia da Silva et al., 2007) and Cathepsin L-like gene (Ortiz et al., 2009). Length differences in a region of the 24S rRNA gene permitted the identification of the three common trypanosomatid species in triatomines: *T. cruzi*, *T. rangeli* and *Blastocrithidia triatoma* (Schijman et al., 2006).

The most widely used methods for differentiating between *T. cruzi* lineages are based on polymorphism of 24S alpha rDNA and spliced leader DNA, although these methods are unable to distinguish all lineages (Souto et al., 1996; Fernandes et al., 2001). Indeed, some studies have shown that use of a single molecular marker can lead to misclassification of *T. cruzi* isolates (Brisse et al., 2001; Burgos et al., 2007; Marcili et al., 2009a,b,c). The five lineages of *T. rangeli* can be distinguished through length and sequence polymorphisms of the internal transcribed spacer (ITS) rDNA regions, cathepsin L-like and spliced leader (SL) genes (Maia da Silva et al., 2004b, 2007, 2009; Ortiz et al., 2009).

Fluorescent fragment length barcoding (FFLB) is a method that discriminates species by size polymorphisms in specific regions of the 18S and 28S ribosomal RNA genes (Hamilton et al., 2008). It has been applied to the identification of African trypanosomes, both in tsetse flies and in vertebrates, and its use has led to the discovery of new strains and species (Adams et al., 2008, 2009, 2010; Adams and Hamilton, 2008; Hamilton et al., 2009). It has proved to be quick, accurate, and able to detect mixed infections of up to three different strains (Hamilton et al., 2008; Adams et al., 2009). The high diversity and complexity of *T. cruzi* and *T. rangeli* suggest that many genotypes remain to be described, especially from the generally poorly investigated sylvatic vertebrate and invertebrate hosts of unexplored geographical regions and ecotopes. Here we apply this technique to the issue of species and lineage identification of the American trypanosomes, *T. cruzi* and *T. rangeli*. Our results provide evidence that FFLB is a useful tool for elucidating the genetic diversity present within these species and for better understanding of the epidemiology of American trypanosomes.

2. Materials and methods

2.1. T. cruzi and T. rangeli isolates

The isolates of *T. cruzi* and *T. rangeli* were selected for this study to represent the broad genetic diversity found in a range of vertebrate and vector species from their full geographical range (Table 2). They represented the six recognised lineages of *T. cruzi cruzi*, one new genotype of this species associated with bats (TCbat) and *T. c. marinkellei*, the subspecies most closely related to *T. cruzi cruzi* (Stevens et al., 1999) and thought to be restricted to bats from Central and South America (Marcili et al., 2009a; Cavazzana et al., 2010). Isolates of the five currently recognised lineages of *T. rangeli* (Maia da Silva et al., 2004b, 2007, 2009; Ortiz et al., 2009) were also selected for this study. Identity of species/isolates was as confirmed in previous studies (Maia da Silva et al., 2008; Marcili et al., 2009a,b,c).

2.2. Fluorescent fragment length barcoding

FFLB analysis was carried out using primers and the PCR programme described previously (Hamilton et al., 2008), except REDTaq® DNA Polymerase (Sigma) was used. All DNA samples were isolated from cultured trypanosomes. A total of four primer sets were used (two sets within the 18S rRNA gene and two within the 28S α rRNA gene) to create a barcode for each sample, consisting of the lengths of the four amplified regions. These were then compared to barcodes from other trypanosomes obtained in previous studies (Hamilton et al., 2008, 2009; Adams et al., 2009).

3. Results and discussion

3.1. Identification of American trypanosomes using fluorescent fragment length barcoding

In this study, we evaluated the use of fluorescent fragment length barcoding (FFLB) for identification and diagnosis of species of American trypanosomes and genotyping of lineages of *T. cruzi* and *T. rangeli*. We compared isolates from all recognised major lineages of *T. cruzi* and *T. rangeli*. All isolates examined gave peaks with the four primer sets. Figs. 1 and 2 show example FFLB profiles from a range of species. The method was able to differentiate *T. cruzi* from *T. rangeli* independent of lineages of these two species, as the size ranges of 18S1, 18S3 and 28S1 did not overlap between the two species (Tables 1 and 2). The FFLB patterns of *T. cruzi* and *T. rangeli* also differed from those of several other species that are known from South American mammals: *T. evansi*, *T. dionisii*-like, *T. lewisi*, *T. theileri* and *T. vivax* (Table 1). Additionally, all *T. rangeli* lineages and the several of *T. cruzi* lineages could be distinguished, demonstrating that FFLB could be useful for epidemiological studies. Indeed, two loci 18S1 and 28S, used together, were sufficient to discriminate all lineages except the two *T. cruzi* hybrid lineages, while the other loci often provided additional confidence in the results.

3.2. Identification of T. cruzi lineages

Sixty-four *T. cruzi cruzi* isolates belonging to the six established *T. cruzi* lineages (TcI–TcVI), together with two genotypes that are apparently restricted to bats: TCbat and *T. c. marinkellei*, were characterised using the FFLB method (Tables 1 and 2 and Fig. 1).