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Research paper

Rediscovery of *Trypanosoma* (*Pycnomonas*) *suis*, a tsetse-transmitted trypanosome closely related to *T. brucei*☆

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ABSTRACT

The African tsetse-transmitted trypanosomes are considered to be a well-known group of parasitic protozoa, but in 2008 a novel and distinctive trypanosome related to *Trypanosoma brucei* was discovered among tsetse isolates from Msubugwe in Tanzania. The host range, distribution and potential pathogenicity of this new trypanosome remain to be elucidated; such studies would be facilitated by a sensitive and specific identification method. Here, we identified two highly repetitive elements in the genome of the new trypanosome: a 177 bp repeat, which was located predominantly on the highly abundant minichromosomes, and a 138 bp repeat, which was widely dispersed in the genome. A PCR test based on each repeat was specific for the new trypanosome and sensitive to <0.1 trypanosome equivalent. These PCR tests were used to identify trypanosomes in archival pig blood smears from the 1950's, confirming the identity of the Msubugwe trypanosome as *Trypanosoma* (*Pycnomonas*) *suis*. We also present data on the molecular karyotype and spliced leader (SL, miniexon) repeat of the new trypanosome, both of which distinguish *T. suis* from other, better-known African tsetse-transmitted trypanosomes. The rediscovery of *T. suis* opens new lines of research into the evolution and biology of the African trypanosomes.

1. Introduction

The African trypanosomes are parasitic protozoa transmitted by the blood feeding tsetse fly (*Glossina spp.*) and cause sleeping sickness in humans and the livestock disease Nagana. These diseases continue to have devastating social and economic impacts in many countries in sub-saharan Africa. Since the 1980's molecular methods have proved invaluable for the detection and identification of trypanosomes in epidemiological studies [reviewed by (Enyaru et al., 2010)]. In particular the sensitivity and specificity of species-specific PCR tests have greatly increased our knowledge of the trypanosomes carried by tsetse, e.g. (Adams et al., 2006; Lefrancois et al., 1999; Lehane et al., 2000; Malele et al., 2003). Whereas the classical dissection and microscopy method failed to detect the majority of mixed infections (Lloyd and Johnson, 1924), we now appreciate that many flies carry mixed infections of two or more trypanosome species. Similarly, microscopy fails to discriminate morphologically similar species, and the application of PCR identification has led to the description of several new trypanosome species or subgroups in addition to the well-known species *T. brucei*, *T. congolense*, *T. simiae* and *T. vivax* (Adams et al., 2010).

There are currently at least 10 different African trypanosomes that can be identified by species-specific PCR tests. While these PCR tests provide excellent discriminatory power, it is cumbersome and expensive to perform multiple PCR's on each field sample. Identification by a single PCR followed by gel electrophoresis has proved more efficient and economical, e.g. species discrimination based on size differences in the internal transcribed spacer (ITS-1) of the ribosomal RNA (rRNA) repeat unit (Adams et al., 2006; Desquesnes et al., 2001). This test too has limitations, in that trypanosomes with the same size ITS-1 fragment cannot be resolved, and this led to the development of the fluorescent fragment length barcoding (FFLB) method, which uses fine-scale size differences in four PCR products from the rRNA locus (Hamilton et al., 2008).

A notable achievement of FFLB was the discovery of a new tsetse-transmitted African trypanosome related to *T. brucei*; this trypanosome had the same size ITS-1 fragment as *T. simiae* and *T. simiae* Tsavo (380 bp) and was therefore missed previously (Adams et al., 2008; Hamilton et al., 2008). This trypanosome was identified in *Glossina pallidipes* from the coastal region of Tanzania, specifically the Msubugwe National Park (Adams et al., 2008). The Msubugwe trypanosome was found in both tsetse midguts and proboscides, but the vertebrate host remains unknown (Adams et al., 2008, 2010). Phylogenetic analysis of the 18S rRNA and gGAPDH genes showed that the new trypanosome is most closely related to *T. brucei*, but is outside the clade containing subgenus *Trypanozoon* (Adams et al., 2010; Hamilton et al., 2008). This phylogenetic position, between subgenera *Trypanozoon* and *Nannomonas*, is that

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expected for *T.* (*Pycnomonas*) *suis*, a little known trypanosome first described in 1905 by Ochmann as a trypanosome causing acute trypanosomiasis in domestic pigs in Tanzania (Hoare, 1972). Decades later, Peel and Chardome re-described *T. suis* in their detailed work on the biology of pig-infective trypanosomes isolated from tsetse (*G. brevipalpis*) in Burundi (Peel and Chardome, 1954a). Study of their evidence of the morphology and developmental cycle of this trypanosome in tsetse, involving the midgut, proboscis and salivary glands, convinced C. A. Hoare that *T. suis* was indeed a real species rather than a synonym of *T. simiae*, and could be an important link in understanding the evolution of trypanosome developmental cycles in tsetse. He placed *T. suis* in a separate subgenus, *Pycnomonas*, to reflect its unique biology (Hoare, 1972). Since then, there have been no substantiated reports of *T. suis* (Gibson et al., 2001; Janssen and Wijers, 1974) and to our knowledge, the archival giemsa-stained slides of Peel and Chardome prepared in the 1950's and also examined by C. A. Hoare are the only type specimens available for *T. suis*.

Here, we set out to develop a sensitive and specific identification test for the Msubugwe trypanosome as a tool to explore its biology and epidemiology and to determine whether it represents the little known *T. suis*. The isolate of the Msubugwe trypanosome cultured from the midguts of tsetse flies collected in Tanzania (Adams, 2008) formed the starting point for this work.

2. Materials and methods

2.1. Trypanosome culture and DNA purification

The G1-62 and F2-J isolates of the Msubugwe trypanosome came from individual *G. pallidipes* midguts collected from the Msubugwe forest reserve in Tanzania in 2006 by Emily Adams in collaboration with Imna Malele of the Tsetse and Trypanosomiasis Research Institute (Adams, 2008; Adams et al., 2008). Briefly, pieces of infected tsetse midgut were placed in sterile tubes containing approximately 0.5 ml of either Cunningham's medium (Cunningham, 1977) with 10% *v*/*v* heat-inactivated foetal calf serum and 5 μg/ml hemin, or modified Sloppy Evans medium (Noyes et al., 1999), both with 5× anti-contamination cocktail [1×: 60 μg ml^{-1} penicillin G, 100 μg ml^{-1} kanamycin sulphate, 10 μg ml^{-1} chloroamphenicol, 50 μg ml^{-1} flucytosine; (Maser et al., 2002)]; this method proved suitable for primary isolation of a range of trypanosomes from the tsetse midgut. G1-62 trypanosomes then required a period of co-culture with insect cells (*Anopheles stephensi*) before an axenic culture could be established in Cunningham's medium at 27 °C with 10% *v*/*v* heat-inactivated foetal calf serum, 5 μg ml^{-1} hemin and 10 μg ml^{-1} gentamycin.

Total DNA from G1-62 was purified by standard methods of phenol-chloroform extraction and alcohol precipitation (Van der Ploeg et al., 1982). Samples for pulsed field gel electrophoresis (PFG) were made by lysing and deproteinizing trypanosomes in agarose blocks as described (Van der Ploeg et al., 1984). DNA was purified from archival giemsa-stained slides essentially as described (Tasker et al., 2010) using a genomic DNA extraction kit (Thermo Scientific). Techniques were initially trialled on *T. brucei* rodent blood slides prepared in 1980 using the standard *T. brucei* PCR primers (Masiga et al., 1992; Moser et al., 1989), before application to the archival porcine blood smears originally prepared by E. Peel in 1952/3. Digital images were previously made of these slides (Gibson et al., 2001). Potential contamination with environmental trypanosome DNA was avoided by carrying out all preparative work on archival material in a separate laboratory where trypanosome material was not handled.

Other DNAs used in the study were from the following isolates: *T. b. brucei* TSW 65 (MSUS/CI/82/TSW 65), J10 (MCRO/ZM/73/J10); *T. b. gambiense* DAL 972 (MHOM/CI/86/DAL 972); *T. congolense* savannah 1/148 (MBOI/NG/60/1–148), GAM 2 (MBOT/GM/77/GAM 2), WG 81 (MCAP/KE/81/WG 81); *T. congolense* forest TSW 103 (MSUS/LR/77/TSW 103-A), TV031 (GPAG/GM/85/TV031); *T. congolense* kilifi WG 84 (MCAP/KE/81/WG 84), WG 5 (MOVS/KE/80/WG 5); *T. godfreyi* KEN 7 (GMOS/GM/88/KEN 7), KEN 10 (GMOS/GM/88/KEN 10); *T. simiae* BAN 7 (GMOS/GM/88/BAN 7), TV008 (GMOS/GM/85/TV008); *T. simiae* Tsavo KETRI 3436 (MSUS/KE/70/KETRI 1864); *T. vivax* Y58 (MBOT/NG/63/Y58), Y486 (MBOT/NG/63/Y486); *T. grayi* BAN1 (GPAG/GM/88/BAN1).

2.2. Electrophoresis, blotting and hybridisation

Genomic DNA from G1-62 was digested with various restriction enzymes (*Sau*3A, *Taq*I, *Rsa*I, *Dde*I, *Sau*96, *Mlu*I, *Alu*I and *Hpy*CH4V) and size-fractionated through 1.2–1.6% agarose gels. PFG was carried out using a Biorad CHEF-DR III with a 3 phase programme (Block 1: switch time 1800 s, 106° angle, voltage 2 V/cm, 15 h; Block 2: switch time 300 s to 900 s, 106° angle, voltage 3 V/cm, 40 h; Block 3: switch time 60 s to 180 s, 120° angle, voltage 4 V/cm, 10 h) using 0.5× Tris-borate-EDTA buffer and 0.9% agarose gels at 14 °C. Chromosomal DNA from *Hansenula wingei* was used as a size marker. Gels were stained overnight by submersion in electrophoresis buffer containing ethidium bromide (1 mg/L). Gels were blotted onto positively charged nylon membrane (Roche) and DNA fixed by baking the membrane at 80 °C for 2 h. Probes were labelled with dioxygenin-11-dUTP (DIG) using the PCR DIG Probe Synthesis Kit (Roche), as per manufacturer's instructions. Blots were hybridised overnight in DIG Easy Hyb hybridisation buffer (Roche) at 42 °C, followed by stringency washes at 68 °C and chemiluminescent detection according to manufacturer's recommendations.

2.3. PCR conditions

PCR's were performed using Reddimix or DreamTaq Green PCR Master Mix (Thermo Scientific) with a standard programme of 95 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 40 s, followed by 72 °C for 5 min. Oligonucleotide primers specific to the 138 bp, 177 bp and 332 bp repeats identified in this study were designed using DNAMAN software package and OligoPerfect™ Designer (Invitrogen). 138 bp 138F 5′-CCCTAAGTTGGGAGTGGTCA-3′, 138R 5′-ACAGTAAGAGTGGCGGTCTGA-3′; 177 bp 177F 5′-TGCACACA CCGTTATTAGCG-3′, 177R 5′-GCGTTTCCGCACATTTGT-3′; 332 bp 332F 5′-GCCGGTATATTGTGGAGGTA-3′, 332R 5′-CCGAAGCACTGAAGGTTT AC-3′. The following primers were used to amplify the spliced leader RNA: MED1 5′-GGGAAGCTTCTGTACTATATTGGTA-3′ and MED2 5′-GGGAATTCAATATAGTACAGAAACTG-3′ (Murthy et al., 1992); MEX1 5′-TCATAAGACCTCTCATCCC-3′.

Quantitative PCR (qPCR) was carried out using a SYBR Green/ROX qPCR Master Mix (Thermo-Fisher) according to manufacturer's instructions with 300 nM primer concentrations. Replicate qPCR reactions were performed; positive controls (dilutions of G1-62 DNA) and a negative control (water) were included in each set of reactions. Resulting data were analysed using MX Pro software (Agilent Technologies).

2.4. Cloning and sequencing

Following restriction enzyme digestion, bands of interest were excised from gel and purified using a GeneJET® Gel Extraction Kit (Thermo Scientific) as per manufacturer's instructions. Purified restriction fragments were ligated into a plasmid vector and cloned in *Escherichia coli* using standard methods. DNA was prepared from transformed colonies using GeneJET® Plasmid Miniprep Kit (Thermo Scientific) as per manufacturer's instructions, and inserts were sequenced using flanking primers in the plasmid vector. The SL repeat was sequenced directly from the PCR product using primers MED1, MED2 and MEX1. Results were analysed using DNAMAN software (Lynnon Biosoft) and compared to sequences of known trypanosome species using NCBI BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and GeneDB BLAST (http://www.genedb.org/Homepage).