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Research paper

Ribosomal DNA analysis of tsetse and non-tsetse transmitted Ethiopian *Trypanosoma vivax* strains in view of improved molecular diagnosis

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ABSTRACT

Animal trypanosomosis caused by *Trypanosoma vivax* (*T. vivax*) is a devastating disease causing serious economic losses. Most molecular diagnostics for *T. vivax* infection target the ribosomal DNA locus (rDNA) but are challenged by the heterogeneity among *T. vivax* strains. In this study, we investigated the rDNA heterogeneity of Ethiopian *T. vivax* strains in relation to their presence in tsetse-infested and tsetse-free areas and its effect on molecular diagnosis.

We sequenced the rDNA loci of six Ethiopian (three from tsetse-infested and three from tsetse-free areas) and one Nigerian *T. vivax* strain. We analysed the obtained sequences *in silico* for primer-mismatches of some commonly used diagnostic PCR assays and for GC content. With these data, we selected some rDNA diagnostic PCR assays for evaluation of their diagnostic accuracy. Furthermore we constructed two phylogenetic networks based on sequences within the smaller subunit (SSU) of 18S and within the 5.8S and internal transcribed spacer 2 (ITS2) to assess the relatedness of Ethiopian *T. vivax* strains to strains from other African countries and from South America.

In silico analysis of the rDNA sequence showed important mismatches of some published diagnostic PCR primers and high GC content of *T. vivax* rDNA. The evaluation of selected diagnostic PCR assays with specimens from cattle under natural *T. vivax* challenge showed that this high GC content interferes with the diagnostic accuracy of PCR, especially in cases of mixed infections with *T. congolense*. Adding betain to the PCR reaction mixture can enhance the amplification of *T. vivax* rDNA but decreases the sensitivity for *T. congolense* and *Trypanozoon*. The networks illustrated that Ethiopian *T. vivax* strains are considerably heterogeneous and two strains (one from tsetse-infested and one from tsetse-free area) are more related to the West African and South American strains than to the East African strains.

The rDNA locus sequence of six Ethiopian *T. vivax* strains showed important differences and higher GC content compared to other animal trypanosomes but could not be related to their origin from tsetse-infested or tsetse-free area. The high GC content of *T. vivax* DNA renders accurate diagnosis of all pathogenic animal trypanosomes with one single PCR problematic.

1. Introduction

In almost half of the African countries, 30% of the population is primarily dependent on livestock for their survival. Among the devastating animal diseases, African animal trypanosomosis (AAT) holds the lion share in reducing the benefit to be obtained from livestock agriculture (Perry and Grace, 2009). The problem of trypanosomosis, affecting both human and animal health, is significant and eradication of the disease figures among the millennium development goals of African countries (Programme Against African Trypanosomiasis, 2008; Shaw, 2009).

Abbreviations: AAT, animal African trypanosomosis; Indels, inserstion-deletion polymorphisms; ITS1, internal transcribed spacer 1; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; rDNA, ribosomal deoxyribonucleic acid; SSU, small subunit; TvPRAC, *Trypanosoma vivax* proline racemase.

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AAT is a parasitic disease caused by different pathogenic species of Salivarian trypanosomes of which several affect livestock by inducing anaemia, loss of condition and emaciation and many untreated cases are fatal (Batista et al., 2011). Among the Salivarian trypanosomes, *Trypanosoma vivax* (*T. vivax*) is transmitted both cyclically and mechanically and the latter transmission route has enabled it to spread beyond the tsetse-infested areas of Africa and even into South America (Birhanu et al., 2015; Fikru et al., 2012; Osório et al., 2008; Sinshaw et al., 2006). Several reports describe that West African *T. vivax* strains are more pathogenic than East African strains and that they are genetically more closely related to South American strains (Cortez et al., 2006; Gardiner and Mahmoud, 1992; Ventura et al., 2001). A recent study based on the cathepsin L-like gene confirms the relatedness of West African and South American isolates (Nakayima et al., 2013), but in another study, phylogenetic analysis on smaller subunit (SSU) variable (V7-V8 region) region of 18S sequences showed that *T. vivax* strains from Tanzania and Mozambique differ from the East African and West African strains, whilst they still cluster within the *T. vivax* clade (Rodrigues et al., 2008). Similarly, among *T. vivax* strains from wild animals in the Serengeti and Luangwa valley ecosystems, three variants were observed based on ITS1, 5.8S and ITS2 sequences and even a new genotype of *T. vivax* was detected in tsetse flies captured in Tanzania (Adams et al., 2010a,b; Auty et al., 2012).

The degree of genetic heterogeneity was reported to be higher in East African *T. vivax* as compared to West African and South American *T. vivax* (Adams et al., 2010b). In South America, *T. vivax* is transmitted only mechanically, favoring clonal expansion, whereas in Africa both mechanical and cyclical transmission occur, the latter allowing genetic exchange between strains. Clonal expansion resulting from tsetse independent transmission is well documented in *T. evansi* and *T. equiperdum* and has been suggested to occur as well in *T. vivax* populations in The Gambia (Duffy et al., 2009; Tait et al., 2011). However, to confirm that genetic diversity of African *T. vivax* strains is related to cyclical transmission by tsetse flies thus explaining the high homogeneity of South American isolates, analysis of more isolates from tsetse-infested and tsetse-free areas is needed (Rodrigues et al., 2008). In studies on AAT, the genetic heterogeneity of *T. vivax* hampers unequivocal identification of the infecting species because most *T. vivax*-specific diagnostic PCR tests are based on West African *T. vivax* DNA sequences and some fail to recognise many *T. vivax* infections in livestock and tsetse in East Africa (Adams et al., 2010b; Fikru et al., 2014; Gonzatti et al., 2014).

The ribosomal DNA (rDNA) locus is a preferential target to develop species-specific molecular diagnostics because of its multicopy nature (Desquesnes et al., 2001; Geysen et al., 2003; Njiru et al., 2005). The 18S and 28S rRNA genes are ideal targets to design simple generic diagnostic PCR tests because they are composed of alternating conserved and variable domains, allowing the variable domains to be amplified using primers in the flanking conserved regions. Therefore, sequence analysis of the rDNA locus from several representative *T. vivax* strains may help to solve problems associated with accurate identification of all *T. vivax* strains regardless of their geographic origin and/or means of transmission. In this study we sequenced the rDNA locus of seven *T. vivax* strains to assess whether sequence heterogeneity can be related to their geographical origin and whether this has an effect on the accuracy of molecular diagnostics, in particular for detecting multiple-species infections.

2. Methods

T. vivax DNA was extracted from the Nigerian strain Y486 (ILRAD 700) grown in mice, from 4 Ethiopian strains (4337, 4338, Di, Fc) grown in calves and from the blood of two Ethiopian bovines (306, 310) that were naturally mono-infected with *T. vivax* (Fikru et al., 2012) (Table 1). All strains were confirmed as *T. vivax* by *Tv*PRAC PCR and ITS1-PCR (Fikru et al., 2014). All strains are available on request.

The full sequence of the rDNA locus of the Nigerian strain *T. vivax* Y486 has been published in GenBank (U22316). The nucleotide basic local alignment search tool (BLASTn) revealed a 99% sequence identity with another full sequence of the rDNA locus on chromosome 3 of the same *T. vivax* strain (HE573019). Based on these two sequences, we designed with Primer3 software two diagnostic primer pairs (A-B, E-F) to amplify 2 overlapping fragments of about 2350 bp in order to get the full sequence of the rDNA locus (Fig. 1) (Koressaar and Remm, 2007). These primers were designed in compliance with the In-Fusion cloning protocol (Clontech) with addition of 15 nucleotides that are complementary to the cloning site on the plasmid. Two more primer pairs (C–D, G–H) were designed to sequence the inner part of each fragment to get sufficient overlap for sequence assembly. The primer sequences, their binding sites and expected amplicon sizes based on the *T. vivax* rDNA GenBank sequence U22316 are shown in Table 2.

The two overlapping fragments (fragment AB and EF) were PCR amplified with proofreading Phusion® Hot Start Flex DNA polymerase (New England Biolabs). The reaction was carried out in a PCR cocktail containing in 1× Phusion GC buffer: 1 mM dNTPs, 0.5 μM forward and 0.5 μM reverse primer, 5% DMSO and 1 unit Phusion Hot Start Flex DNA polymerase. A template DNA concentration of about 50 ng was used per reaction. The cycling conditions for the amplification of fragment AB were: initial denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, primer annealing at 61 °C for 30 s and polymerisation reaction at 72 °C for 90 s, and a final extension at 72 °C for 7 min. For fragment EF, the cycling conditions were similar but with a primer annealing temperature of 64 °C. The presence of an amplification product was confirmed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV transilluminescence.

The amplicons were cloned into *Bam*HI linearised pUC19 vector (In-Fusion® HD Cloning kit; Clontech) and transformed into chemically competent Stellar *E. coli* cells. To construct the insert, 5 μL of PCR product was mixed with 2 μL of cloning enhancer and incubated for 15 min at 37 °C followed by 15 min at 80 °C. Two μL of this mixture were added to 5× In-Fusion HD enzyme premix, 1 μL of linearised pUC19 vector and 5 μL of distilled water and incubated for 15 min at 50 °C. Transformation was done by mixing 2.5 μL of the plasmid construct with 100 μL of chemically competent Stellar *E. coli* cells, incubating on ice for 30 min followed by a heat shock at 42 °C for 45 s. The cells were recuperated in SOC medium (Clontech) after incubating at 37 °C under shaking at 300 rpm for 1 h. Hundred μL of the cells were plated on selective agar plates, Lysogeny Broth-carbenicilin, containing 40 mg/mL X-gal to allow blue/white selection.

Colony-PCR was carried out on the white bacterial colonies to ascertain the presence of the required fragments. The following primers that anneal on the plasmid and on the insert were used: fragment 1 forward (18SColF, 5′-CTCTTCGCTATTACGCCAGC-3′) and reverse (18SColR, 5′-GTGATTGCGGCAGATTACGT-3′) and fragment 2 forward (5.8SColBF, 5′-CTCTTCGCTATTACGCCAGC-3′) and reverse (5.8SColBR, 5′-TGTTCGACACTGAGACTGCG-3′). Plasmids containing the insert were purified using Qiagen Miniprep plasmid extraction kit. Two clones were sequenced for every stain except for fragment 2 of Y486, 4338 and Di, where only one clone was sequenced. Sequencing was carried out at the VIB gene sequencing facility of the University of Antwerp. The obtained sequences were assembled using DNAMAN and aligned to each other using CLC Sequence Viewer 6.8.2.