How does *Trypanosoma equiperdum* fit into the *Trypanozoon* group? A cluster analysis by RAPD and Multiplex-endonuclease genotyping approach

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SUMMARY

The pathogenic trypanosomes *Trypanosoma equiperdum*, *T. evansi* as well as *T. brucei* are morphologically identical. In horses, these parasites are considered to cause respectively dourine, surra and nagana. Previous molecular attempts to differentiate these species were not successful for *T. evansi* and *T. equiperdum*; only *T. b. brucei* could be differentiated to a certain extent. In this study we analysed 10 putative *T. equiperdum*, 8 *T. evansi* and 4 *T. b. brucei* using Random Amplified Polymorphic DNA (RAPD) and multiplex-endonuclease fingerprinting, a modified AFLP technique. The results obtained confirm the homogeneity of the *T. equiperdum* group tested. The *T. b. brucei* clustered out in a heterogenous group. For *T. equiperdum* the situation is more complex: 8 out of 10 *T. equiperdum* clustered together with the *T. evansi* group, while 2 *T. equiperdum* strains were more related to *T. b. brucei*. Hence, 2 hypotheses can be formulated: (1) only 2 *T. equiperdum* strains are genuine *T. equiperdum* causing dourine; all other *T. equiperdum* strains actually are *T. evansi* causing surra or (2) *T. equiperdum* does not exist at all. In that case, the different clinical outcome of horse infections with *T. evansi* or *T. b. brucei* is primarily related to the host immune response.

Key words: *Trypanosoma equiperdum*, characterization, RAPD, multiplex-endonuclease fingerprinting, AFLP.

INTRODUCTION

Dourine, Surra and Nagana are all lethal diseases in horses caused by *Trypanosoma equiperdum*, *T. evansi* and *T. b. brucei*, respectively (Office International des Epizooties, OIE list B). They are all members of the *Trypanozoon* subgenus and have morphologically identical bloodstream forms. *T. equiperdum* and *T. evansi* are transmitted respectively by sexual contact and by blood-sucking flies explaining their worldwide distribution, while the dependence on tsetse flies as the vector limits *T. b. brucei* to sub-Saharan Africa. (Stephen, 1986).

Most research on the genome of pathogenic Sali- varian trypanosomes is performed on *T. b. brucei*. The genomes of *T. equiperdum* and *T. evansi* have not been thoroughly studied and most investigations focus on the sequence of variable surface glycoproteins (VSGs) (Baltz et al. 1986; Roth et al. 1986; Urakawa et al. 2001), on expression sites (Florent, Raibaud & Eisen, 1991), and on the kinetoplast DNA (kDNA) (Riou & Saucier, 1979; Frasch et al. 1980; Borst, Fase-Fowler & Gibson, 1987; Masiga & Gibson, 1990; Ou, Giroud & Baltz, 1991; Lun, Brun & Gibson, 1992b).

Despite numerous attempts, researchers have not been able to differentiate *T. equiperdum* from *T. evansi* consistently, neither at the serological, nor at the molecular level (Baltz, unpublished observations; Hide et al. 1990; Lun et al. 1992a; Lun et al. 1992; Biteau et al. 2000). Previous studies performed in our laboratory further underline the close relationship between both species (Claes et al. 2002). Only 2 of the 10 putative *T. equiperdum* strains, the BoTat 1.1 (Morocco) and the Ondersteopot Veterinary Institute (OVI) strain (South Africa), seem to differ from the rest of the *T. equiperdum* strains in Variable Antigen Repertoire. All other *T. equiperdum* have the same characteristics as *T. evansi* strains. In the present study, we examined the
characteristics of several *T. equiperdum*, *T. evansi* and *T. b. brucei* populations with 2 molecular techniques, Random Amplified Polymorphic DNA (RAPD) and the multiplex-endonuclease fingerprinting method.

**MATERIALS AND METHODS**

**Trypanosome populations**

A collection of 4 *T. b. brucei*, 8 *T. evansi* and 10 *T. equiperdum* populations, derived from strains isolated all over the world, was used in this study (Table 1). All populations were kept as cryostabilates in liquid nitrogen. For the *T. equiperdum* strains, the history is mostly unknown. Only the OVI strain from South Africa, was well documented.

**Preparation of trypanosome DNA**

Blood-stream form trypanosomes were expanded in mice and rats and were purified from the blood by DEAE chromatography (Lanham & Godfrey, 1970), followed by repeated centrifugation (3 times 20 min, 2000 g) and sediment washes with phosphate-buffered saline glucose (PSG) (38 mM Na₂HPO₄, 2H₂O, 2 mM NaHPO₄, 80 mM glucose). Finally, trypanosome pellets were subsequently stored at −80 °C.

Twenty μl of trypanosome pellets (approximately 2 × 10⁷ cells) were resuspended in 200 μl of phosphate-buffered saline (PBS) and the trypanosome DNA was extracted using the QIAamp DNA mini kit (Westburg, Leusden, The Netherlands), resulting in pure DNA in 200 μl of Milli-Q water. The typical yield of DNA extracted from a 20 μl pellet was 150 ng/μl or 30 μg of total DNA. The extracts obtained were diluted in Milli-Q water to a standard concentration of 50 ng/μl and stored at −20 °C.

**Random Amplified Polymorphic DNA (RAPD)**

Ten μl of extracted DNA (50 ng/μl) were mixed with 40 μl of a PCR-mix containing: 0.5 U Taq DNA recombinant polymerase (Promega, UK), PCR buffer (Promega, UK), 3.0 mM MgCl₂ (Promega, UK), 200 μM of each of the 4 dNTPs (Roche, Mannheim, Germany) and 0.5 μM of the oligonucleotide 10-mer (Gibco BRL, UK). The different oligonucleotides used were (in 5’→3’ direction): RAPD 606 CGG TCG GCC A (Ventura et al. 2001) and RAPD ILO 525 CGG ACG TCG C (Waitumbi & Murphy, 1993).

Amplifications were performed in a Biometra® Trio-block thermocycler. Cycling conditions were as follows: denaturation for 4 min at 94 °C, followed by 40 amplification cycles of 2 min denaturation at 94 °C, 2 min primer-template annealing at 40 °C and 2 min polymerization at 72 °C. A final elongation step was carried out for 5 min at 72 °C.

**Multiplex-endonuclease fingerprinting method**

A fine-scale genotyping approach involving multiple endonucleases in combination with a pair of cognate

Table 1. Trypanosome populations used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Clone/strain</th>
<th>ITMAS</th>
<th>Origin</th>
<th>Year</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. b. brucei</em></td>
<td>AnTat 2.2</td>
<td>100297B</td>
<td>Nigeria</td>
<td>1970</td>
<td>Tsetse fly</td>
</tr>
<tr>
<td><em>T. b. brucei</em></td>
<td>AnTat 5.2</td>
<td>220197</td>
<td>The Gambia</td>
<td>1975</td>
<td>Cattle</td>
</tr>
<tr>
<td><em>T. b. brucei</em></td>
<td>AnTat 17.1</td>
<td>210596</td>
<td>R. D. Congo</td>
<td>1978</td>
<td>Sheep</td>
</tr>
<tr>
<td><em>T. b. brucei</em></td>
<td>KETRI 2494</td>
<td>270881</td>
<td>Kenya</td>
<td>1980</td>
<td>Tsetse fly</td>
</tr>
<tr>
<td><em>T. evansi</em></td>
<td>AnTat 3.1</td>
<td>070799</td>
<td>South America</td>
<td>1969</td>
<td>Capybara</td>
</tr>
<tr>
<td><em>T. evansi</em></td>
<td>RoTat 1.2</td>
<td>020298</td>
<td>Indonesia</td>
<td>1982</td>
<td>Water buffalo</td>
</tr>
<tr>
<td><em>T. evansi</em></td>
<td>Merzouga 56</td>
<td>120399D</td>
<td>Morocco</td>
<td>1998</td>
<td>Camel</td>
</tr>
<tr>
<td><em>T. evansi</em></td>
<td>Zagora 1.17</td>
<td>040399B</td>
<td>Morocco</td>
<td>1997</td>
<td>Camel</td>
</tr>
<tr>
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<td>KETRI 2480</td>
<td>110297</td>
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<td>1980</td>
<td>Camel</td>
</tr>
<tr>
<td><em>T. evansi</em></td>
<td>CAN 86 K</td>
<td>140799B</td>
<td>Brazil</td>
<td>1986</td>
<td>Dog</td>
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<tr>
<td><em>T. evansi</em></td>
<td>Stock Colombia</td>
<td>150799</td>
<td>Colombia</td>
<td>1973</td>
<td>Horse</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>Stock Vietnam</td>
<td>101298</td>
<td>Vietnam</td>
<td>1998</td>
<td>Water buffalo</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>AnTat 4.1</td>
<td>210983A</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>Afort</td>
<td>241199A</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
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<tr>
<td><em>T. equiperdum</em></td>
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<td>241199B</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
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<tr>
<td><em>T. equiperdum</em></td>
<td>ATCC 30019</td>
<td>020301</td>
<td>France</td>
<td>1903 ?</td>
<td>Horse</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>ATCC 30023</td>
<td>280201</td>
<td>France</td>
<td>1903 ?</td>
<td>Horse</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>STIB 818</td>
<td>010999</td>
<td>P. R. China</td>
<td>1979</td>
<td>Horse</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>American</td>
<td>220101</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
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<tr>
<td><em>T. equiperdum</em></td>
<td>Canadian</td>
<td>290101</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>OVI</td>
<td>241199C</td>
<td>South Africa</td>
<td>1975</td>
<td>Horse</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>BoTat 1.1</td>
<td>240982A</td>
<td>Morocco</td>
<td>1924</td>
<td>Horse</td>
</tr>
</tbody>
</table>
Cluster analysis of Trypanosoma equiperdum

Cluster analysis

The GelCompar II program was used for cluster analysis of RAPD and AFLP profiles by Unweighted Pair Group Method with Arithmetical Mean (UPGMA) based on the Dice coefficient.

With the obtained data matrices, Wagner Parsimony analysis was performed on bootstrapped data using the Seqboot, Mix and Consence programs from the PHYLIP software (Felsenstein, 1989).

RESULTS

RAPD and AFLP reactions were performed with the same set of samples on different days and by different persons. The DNA banding patterns obtained confirmed the repeatability of both techniques in our laboratory (data not shown).

Dendrograms from the RAPD results, analysed by pairwise fragment comparison using the Dice coefficient and by data clustering using UPGMA, are shown in Figs 1 and 2. In RAPD 606, all T. evansi strains cluster out in 1 homogenous group with a 95–100% similarity level. Also, in this cluster 8 out of the 10 tested T. equiperdum strains are found. All T. b. brucei and the 2 remaining T. equiperdum isolates (BoTat 1.1 and OVI) cluster out in a more heterogenous way (with 72–88% similarity coefficient). The similarity level between these two T. equiperdum strains is 75% with, respectively, a 76 and 74% similarity coefficient with the T. evansi/T. equiperdum cluster. T. equiperdum BoTat 1.1 shares the highest similarity with T. b. brucei AnTat 2.2, while the OVI strain is distinct from the rest of the group.

In RAPD ILO 525, the T. evansi strains are grouped in 1 cluster with 90–100% similarity. This cluster harbours the same 8 out of 10 T. equiperdum strains. T. b. brucei forms a more heterogenous groups (Dice coefficients ranging from 74 to 83%) including the T. equiperdum Botat 1.1 and OVI. With this RAPD T. equiperdum BoTat 1.1 relates most to T. equiperdum OVI and T. b. brucei KETRI 2494 and AnTat 2.2; OVI is highly similar to KETRI 2494.

In the UPGMA clustering data obtained from the modified AFLP analysis branches of the homology tree are longer, indicating the higher resolution power of this technique (Fig. 3). All T. evansi are grouped in 1 cluster with a similarity of 85–95%, together with the same 8 T. equiperdum strains. Also with this technique the T. b. brucei group appeared as a heterogenous cluster, including the BoTat 1.1 and OVI strains. Based on the modified AFLP data, the level of similarity of these two latter strains was calculated at 74%. In this analysis, OVI seems closely related to T. b. brucei KETRI 2494, while BoTat 1.1 shares more homology with T. b. brucei AnTat 2.2.

Neither with the RAPD nor the modified AFLP, the position of the strains amongst the clusters seemed to be related to their geographical origin, original host species or the year of isolation. In RAPD 606, T. evansi and T. equiperdum from different regions and hosts (RoTat 1.2, AnTat 3.1, CAN 86K, stock Vietnam, STIB 818, American stablishate, ATCC 30019 and ATCC 30023) gave a 100% similarity coefficient (Fig. 1). On the other hand, with RAPD ILO 525 (Fig. 2), T. evansi stocks from different origins (RoTat 1.2, stock Colombia, Zagora I.17) showed exactly the same pattern.
When mixed parsimony analysis was performed on bootstrapped data from both RAPDs and the modified AFLP, the homogenous T. evansi/T. equiperdum cluster differed from the more heterogenous group with an 80% and 100% probability coefficient, respectively for the modified AFLP and both RAPD’s (data not shown).

DISCUSSION

Comparison of the RAPD 606 results in the present study with those from Ventura et al. (2001), reveals a similar close genetic relationship between T. evansi populations from different origins, and approximately the same distance between T. equiperdum BoTat 1.1 and the T. evansi cluster (76% similarity versus 60%, respectively).

With RAPD ILO 525, Waitumbi & Murphy (1993) were able to divide the Trypanozoon sub-genus into 3 groups: (1) T. b. brucei and T. b. rhodesiense, (2) T. b. gambiense and (3) T. evansi. No T. equiperdum was included in their analysis.

Other previous characterization studies mainly focused on the T. brucei subspecies or on T. evansi and only few T. equiperdum were included. Hide et al. (1990), analysed 42 T. brucei by repetitive DNA probes, together with only 1 T. equiperdum and 1 T. evansi. A separate T. b. gambiense type I cluster was found while T. b. brucei and T. b. rhodesiense were more heterogenous. The T. equiperdum and T. evansi appeared to have a dissimilarity level of 56% with the T. brucei group and a dissimilarity level of 45% between each other. Unfortunately from their paper, it is not clear which T. equiperdum and T. evansi strains were used. By both kDNA and isoenzyme analysis, Lun et al. (1992a, b) could not find differences which would distinguish 12 stocks of T. evansi from 1 T. equiperdum (STIB 818). Agbo et al. (2002) included 2 T. evansi (AnTat 3.1 and RoTat 1.2) and 2 T. equiperdum (AnTat 4.1 and STIB 818) in their AFLP analysis of Trypanosoma spp., again without conclusive results on the differentiation between T. evansi and T. equiperdum.

Using microsatellite markers on 3 T. equiperdum (BoTat 1.1, STIB 818 and a South African strain), Biteau et al. (2000) observed heterogenous patterns amongst them and concluded that ‘previous interpretation of the close relationship of T. evansi and T. equiperdum by isoenzyme and RFLP analysis might have been simplistic’. Only Zhang & Baltz (1994) found some differences between T. equiperdum and T. evansi stocks using repetitive DNA probes. BoTat 1.1 and a South African strain were separated from the T. evansi group. They were more similar to T. b. brucei than to the T. evansi cluster which contained a third T. equiperdum (STIB 818). Zhang & Baltz concluded that this outlier T. equiperdum STIB 818 could reflect the limit of sensitivity of the RFLP technique used or could be due to the misclassification of this
strain. To our knowledge, the South African strain is the Onderstepoort Veterinary Institute (OVI) strain (T. De Waal, personal communication).

Taken together, the above-mentioned results correspond well with the present results based on a larger collection of \textit{T. evansi} and \textit{T. equiperdum} strains. Irrespective of the DNA amplification method, 2 major groups can be formed: 1 homogenous group including all \textit{T. evansi} and most of the \textit{T. equiperdum} strains and 1 heterogenous group including all \textit{T. b. brucei} and 2 \textit{T. equiperdum}, the BoTat 1.1 clone and the OVI strain.

Previous serological and molecular studies on the same collection yielded similar results: all hitherto tested \textit{T. evansi} share the presence and expression of the RoTat 1.2 VSG gene, while for the screened populations of \textit{T. equiperdum} only BoTat 1.1 and OVI, as well as all tested \textit{T. b. brucei}, do not express nor contain this VSG gene (Claes et al. unpublished observations).

Combining these data, 2 hypotheses can be formulated. Firstly, BoTat 1.1 and the OVI strain are the only genuine \textit{T. equiperdum} while all other \textit{T. equiperdum} actually are misclassified \textit{T. evansi}, thus extending the view of Zhang & Baltz (1994). Indeed, in experimental infections with the OVI strain by Barrowman (1976), clinical signs of dourine were observed in the infected horses, while in experimental infections with the American and Canadian stabilates, which in our study are both found in the \textit{T. evansi} cluster, only general signs of trypanosomiasis were observed (Hagebock et al. 1993).

Unfortunately, for most \textit{T. equiperdum} strains, including BoTat 1.1, similar clinical experiments have not been performed.

The question whether BoTat 1.1 and OVI are ‘real’ \textit{T. equiperdum} strains could be solved by following the clinical outcome of horses experimentally infected with both strains and by comparing the result with infections with \textit{T. evansi}-like \textit{T. equiperdum} strains. However, one should keep in mind that strains that have undergone multiple passages in laboratory animals might have lost or changed their pathogenicity and virulence. Alternatively, specific serological or molecular markers could be identified which can differentiate \textit{T. equiperdum} from \textit{T. b. brucei}. In the absence of a conclusive \textit{T. b. brucei} marker this remains a challenging issue.

An alternative hypothesis is that the species \textit{T. equiperdum} actually does not exist but that dourine is caused by particular strains of \textit{T. evansi} and/or \textit{T. b. brucei}. Then the clinical outcome of the infection would merely depend on the virulence or the tissue tropism of the infective strains or the immunological response of the individual host to the trypanosome infection.

Indeed, in horses both acute, subacute and chronic forms of nagana (\textit{T. b. brucei}) have been described, sometimes with clinical signs such as oedema of prepuce and legs, and sporadically the appearance of urticarial plaques. Also for surra (\textit{T. evansi}) in horses, both acute and chronic infections have been mentioned. Here also, oedema is evident as plaques on the ventral surface of the body or as
swelling of the sheath or prepuce (Stephen, 1986). Altogether, some clinical signs of nagana and surra are shared with dourine, i.e. urticarial plaques and genital swellings. Therefore, it might be that differential diagnosis based only on clinical signs is not conclusive for the infecting trypanosome species and certain chronic cases of nagana or surra might have been considered as dourine or vice versa. This enigma would be solved if one considers dourine as the chronic form of both diseases.

Both hypotheses should be checked against the other biological characteristics of the 3 trypanosome species. Until now, *T. b. brucei* is considered to be only cyclically transmitted by tsetse flies, while *T. evansi* and *T. equiperdum* are transmitted respectively mechanically and sexually. However, we have no evidence to exclude sexual or mechanical transmission of *T. b. brucei*. The transmission route could even be linked to host specificity and virulence or tissue tropism. Also for *T. evansi*, sexual transmission might occur. 

Hagebock et al. (1993) indeed were able to infect horses by urogenital inoculation with the American and Canadian *T. equiperdum* strains, which in our study cluster together with *T. evansi*. Thus, if these strains are considered to be *T. evansi*, a first proof of sexual transmission of *T. evansi* in horses is obtained. Nevertheless, to prove the possibility of sexual transmission of *T. b. brucei* and *T. evansi*, more experimental infections with both species should be conducted.

Based on the available clinical, serological and molecular data, we propose that there is not sufficient evidence for the existence of *T. equiperdum* as a separate species.

To further clarify the confusion about *T. equiperdum*, we propose to isolate new trypanosome strains from well defined dourine, surra and nagana cases in horses, to analyse them with the most performant serological and molecular techniques and to study their pathogenicity and transmission routes in horses.

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