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## ***Trypanosoma* spp. in Swedish game animals**

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### **Abstract**

Serum and blood samples from 36 game animals, shot during the hunting seasons 2007-2009, were collected and analyzed for the presence of *Trypanosoma* spp. by three methods; Isolation, PCR and serology. Only fished animals were included, four different ruminants and wild boar. Trypanosomes could be isolated from two of the animals, and eight had detectable parasite DNA. Seven animals had high titers of anti trypanosoma IgG antibodies. The two isolated strains, one from roe deer and one from European elk, were determined to *T. theileri* by partial DNA sequencing of the 18 S ribosomal gene. In the seven boars no *Trypanosoma* were detected, but 4 out of 7 strongly positive serological samples came from this group. This is the first study in Scandinavia on the presence of *Trypanosoma* in game animals. The results indicate that trypanosomiasis is frequently occurring among Swedish game animals.

### **Keywords**

Trypanosomiasis, *Trypanosoma theileri*, game animals, PCR, isolation, serology

## Introduction

Trypanosomiasis is of considerable importance both from health aspects and financial aspects. Examples of human diseases caused by trypanosomes are the African sleeping sickness, *Trypanosoma brucei* (Kennedy 2008) and the Chagas disease, *T. cruzi* (Rassi et al. 2010). Most domestic animals, such as cows, horses and camels, and most wild animals, such as deers, buffalos and elephants, can also host trypanosome parasites. Such infections are particularly frequent in tropical and subtropical areas (Magona et al. 2009). Trypanosomes that are common in animals include *T. vivax*, *T. congolense* and *T. brucei*. There are also some reports suggesting that a considerable part of the European cattle population carries trypanosomes (Verloo et al. 2000).

Trypanosomes are parasites transmitted by ectoparasites such as the tse-tse fly, Tabanidae (Horse flies), and by various ticks (Magona et al. 2009; Burgdorfer et al. 1973).

In Europe, *T. theileri* is found in cattle and *T. equiperdum* (shown to be sexually transmitted and not vector borne!) is found among horses (Antoine-Moussiaux et al. 2009). The clinical signs of animal trypanosomiasis (also called “Nagana”) vary with type of animal and with parasite species. Common signs of primary infection are an intermittent fever, anemia and weight loss. Infection by trypanosomes can sometimes cause acute disease but chronic infections are also common. Morbidity and mortality numbers varies. Many untreated animals eventually die, but some breeds of animals are genetically resistant to trypanosome infection (Magona et al. 2009; Jelinek et al. 2002).

Classification of isolates of *Trypanosoma* has traditionally been done by microscopy of blood smears. There are several morphological criteria of the parasite, which is useful in the identification of the species. More recently developed techniques based on DNA-analysis now also contribute information that is useful for species characterization. (Botero et al. 2010)

There are only a few studies of *Trypanosoma* in Scandinavia. Three strains have previously been isolated, two from reindeer (*Rangifer tarandus*) and one from moose (*Alces alces*). The moose strain were by, isoenzyme pattern analysis, similar to American isolates from *T. theileri*. However, the reindeer isolates were considerably different (Dirie et al. 1990). Our finding of *T. theileri* in the blood of an European elk made it interesting to investigate the frequency and species distribution of *Trypanosoma* infections in game animals. For this investigation, we have used a set of sera from animals available from hunting in the counties of the Stockholm

area. Isolation, microscopy, serology and PCR were used to identify blood samples for trypanosoma exposure. The PCR studies were performed using a sequence of ribosomal DNA. Ribosomal DNA has previously been shown to be useful for trypanosome analysis (Geysen et al. 2003; Desquesnes and Dávila 2002).

## **Material and methods**

### **Sample collection**

Blood samples from 36 newly killed animals, 12 Roe deer (*Capreolus capreolus*); 9 fallow deer (*Dama dama*); 7 European elk (*Alces alces*); 1 red deer (*Cervus elaphus*); and 7 wild boar (*Sus scrofa*) (Table 2) were collected into heparinized or serum BD Vacutainer<sup>R</sup> tubes (Becton Dickinson and Company NJ USA). . The samples were collected on the Vevelsta farm in central Sweden during the hunting seasons of 2007 to 2009.

### **Trypanosome culturing**

The samples were prepared by mixing 1 ml heparinized blood with 1 ml DMEM containing 50 µg ampicillin, and placed as an overlay on 7 ml tryptic soy agar (TSA), supplemented with 5% sheep blood, in 15 ml tubes. The tubes were incubated at 28° C for 15 days. On day 5, 10 and 15, a wet smear from the upper phase was examined by direct microscopy for presence of motile parasites. Alternatively, the smear was Giemsa-stained and inspected by light microscopy. *Trypanosoma*, when present, were sub-cultured by transferring samples to new TSA-tubes with DMEM and 1 ml sheep blood as above.

### **Cloning, sequencing and phylogenetic analysis**

A part of the ribosomal RNA-gene was amplified by PCR from the two isolates of *Trypanosoma*, using the primers 18stnF2 and tryp-theIR. The PCR condition was an initial denaturation for 94 °C 4 minutes, followed by 40 cycles at 94 °C for 30 seconds, 55 °C 30 seconds, and 72 °C 2 minutes, and a final step of 72 °C for 10 minutes. The 940 bp product of was cloned with the “Topo T/A cloning for sequencing kit” (Invitrogen, Carlsbad, CA, USA). Colonies were subcultured and the cloned fragment was sequenced from both directions using internal vector primers. The sequencing reaction was performed with the “Primer sequencing kit” (GE Healthcare UK Ltd) using an ALF express DNA sequencer (Pharmacia Biotech, Uppsala Sweden). Phylogenetic analysis of DNA sequences was performed using CLUSTAL W multiple sequence alignment program.

### **PCR screening of blood samples**

DNA was isolated from blood samples using the QIAGEN DNeasy® Blood & Tissue kit (QIAGEN Nordic, Solna, Sweden). A *Trypanosoma* specific semi-nested PCR assay was performed using the purified DNA as template. A semi-nested assay was chosen in order to increase sensitivity and specificity of the assay. The PCR-amplification were performed with Illustra™PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare UK Ltd.). The primers were adopted with minor modifications from Geysen et al (2003). All primers were purchased from InVitrogen. To obtain longer fragment we also designed a *T. theileri* specific primer, Tryp-theIR. All primer sequences are shown in Table 1.

The amplification was performed with 18stnF2 and 18stnR3 as the first set of primers and 18stnF2 and 18stnR2 as the second set of primers. PCR condition was an initial denaturation for 94 °C 4 minutes, followed by 40 cycles at 94 °C for 30 seconds, 55 °C 30 seconds, 72 °C 1,5 minutes, and a final step of 72 °C for 10 minutes. The PCR products were analyzed by standard agarose gel electrophoresis, using TBE buffer, ethidium bromide and a 1,5 % agarose gel. 1kB Size markers from Fermentas were used. Gels were run at 100 V constant voltage for one hour.

### **Serological analysis**

To investigate the presence of antibodies reactive to *Trypanosoma*, in the blood of the animals, microscopic slides with fixed *Trypanosoma* were prepared: Cultured *Trypanosoma* were sedimented by centrifugation and resuspended in 10% neutralized buffered formalin. After addition of egg yolk sac solution to a final concentration of 10%, spots of samples were dried on microscopic slides and fixed with dry acetone. Serum samples from the investigated animals were diluted 1/20 and 1/200 and added to the microscopic slides. After 1 hr of incubation, the slides were washed for 3 x 10 minutes with phosphate buffered saline (PBS). FITC-labeled protein A (Invitrogen, Carlsbad, CA, USA), in dilution 1/100 was used to detect the presence of IgG-antibodies in the primary serum after one hour incubation and 3x10 minutes wash in PBS. The slides were examined using a UV-microscope. Fluorescence of at least 2+, at a dilution of 1/200 was considered strongly positive.

## Results

*Trypanosoma* were isolated from one roe deer and one European elk. Both animals were calves and both exhibited signs of weakness that may be associated with *Trypanosoma* infection, e.g. reduced weight and fatigue (Table 2).. The isolated parasites exhibited typical morphological characteristics of *Trypanosoma* (Figure 1 and Figure 2).

DNA samples from the isolated strains were amplified by PCR and the 18S RNA gene was partly sequenced (GeneBank accession number EF490992 for the isolate from European elk and HM138533.1 for the one from roe-deer). BLAST alignment showed that the sequences of both isolates correlated most strongly to the sequence of *T. theileri* (GeneBank accession number AJ009164). When comparing the 18S DNA sequence of the European elk with the sequence of *T. theileri* in GenBank, 800 of 806 nucleotides matched. A similar comparison between the roe deer *Trypanosoma* 18S DNA and *T. theileri* 18S DNA gave 806 matching nucleotides. However, there were 3 unpaired nucleotides in the roe deer *Trypanosoma* DNA.

The animals were screened by PCR for *Trypanosoma* DNA in the blood. 35 animals were tested by a *Trypanosoma* specific semi-nested PCR. Seven of the animals were positive, as determined by obtaining an amplicon of correct size. Of these 7 PCR positive samples one was also positive by isolation, and 4 show weak serological response. However, two of the samples could not be tested by serology (Table 2).

Seven of the animals were strongly positive in the serological analysis. None of these were positive by isolation or by PCR. Weak reactions were observed in samples of another 10 of the animals. 9 samples were negative and 11 samples could not be assayed. It can be noted that the rate of serological positive animals was higher for wild boars than for deer's and European elks.

## Discussion

In the present study, trypanosomes were isolated from 2 out of 35 animals. This is a lower fraction than that reported in the few studies on the distribution of *Trypanosoma* in game animals in the northern temperate zone that we have found. In a study of hunter killed elks in Wyoming, 22 of 37 animals were culture positive for *Trypanosoma* sp. (Morton and Kingston 1976). In a later study, 4 of 7 free ranging moose were found to be culture positive (Kingston 1981). These studies also showed that trypanosomes frequently were found in samples from other game animals such as American bison, pronghorn antelope and various deer. These studies thus show a much higher rate of culture positive animals than we observed. This difference could have a number of different explanations:

It is quite plausible that there are true differences in prevalence between different geographical regions.

In a previous Scandinavian study, 2 out of 50 samples from reindeer were found positive and one moose sample was also positive. The authors studied surface carbohydrates and isoenzymes and found the reindeer isolates was so considerably different that the authors suspected that these isolates may represent a new species (Dirie et al. 1990). We can in this study show that roe deer and European elk isolates have different 18S rDNA sequences.

Previous studies have shown that the concentration of parasites in the blood of infected animals may fluctuate substantially, from more than  $1 \times 10^6$  parasites per mL blood to less than 1 parasite per l blood (Antoine-Moussiaux et al. 2009). Hence, there is a risk of false negative results in a culture test using small samples. Furthermore, growth of *Trypanosoma* may require very particular culture conditions. We used a procedure similar to that of the Morton and Kingston group (1976) but other groups have used other conditions. In a study of *T. theileri* in cattle, one procedure for culturing gave 4 out of 10 positive animals and another procedure gave positive positive results in 10 out of 10 cases. (Verloo et al. 2000)

A particular problem with samples from game animals is the risk for contamination of the blood by bacteria as animals are shot and often eviscerated before the blood is collected. We tried to minimize this problem by addition of ampicillin to the culture medium. However, sometimes ampicillin resistant bacteria were observed to grow in the culture.

An interesting observation was that culturing of trypanosomes was possible with bovine blood or sheep blood in the culture medium, but not with blood from one of the authors.

The results of the PCR screening analysis show 7 positive animals out of 35. As reviewed, comparative studies between PCR and parasitological methods, show that in a given collection of animal samples many more are positive for *Trypanosoma* in PCR than in culture assay (Desquesnes and Dávila 2002)

As mentioned previously, the parasitemia can differ a million times. Our DNA samples were extracted from 200  $\mu$ l lysed blood and thus, there need to be at least 5 parasites per ml for an average content of at least one parasite per sample. Furthermore, in the PCR assay we use only 2,5% of the extracted DNA-sample. Thus, there need to be on an average at least 200 parasites per ml to get a positive PCR. It is possible that some *Trypanosoma* carrier animals have lower concentrations of parasites in their blood and there is thus a considerable risk that they would be false negative in PCR.

The serological tests showed that 30% of the tested animals had developed antibodies in high titer ( $>1/200$ ) and that another 40% had any detectable serum antibodies, indicating that Swedish game animals are often exposed to *Trypanosoma*. It is possible that the immunological response rapidly removes most or all of the parasites. The levels of antibodies to trypanosomes, on the other hand, may remain high for a considerable time. This could explain why there are many more seropositive than PCR positive samples.

It can be noted that boars represent a large fraction of the high titer animals (4 out of 7), although none of these were positive either by PCR-analysis or by culturing assay. A possible explanation for this is that the boars often are exposed to insect vector borne parasites but that the trypanosomes do not propagate in the boars. It has been reported that *T. theileri* infects ruminants but that it is not frequent in suidae (pig) animals (Desquesnes and Dávila 2002).

Were there any clinical manifestations that can be associated with trypanosome infection in the animals of our study? This is a difficult question to answer, as the animals were only observed on the hunting occasion.

However, the hunters made specific remarks regarding the two animals that were later shown to be culture positive. Both were noted to be of small size and in poor general condition. No such observations were made for the other animals. At present, however, we are not prepared to draw any conclusion from these observations. The issue needs to be further investigated.

The strong sequence-similarity between the isolated *Trypanosoma* and *T.theileri* is perhaps not unexpected. *T. theileri* is a trypanosome that has been observed to be primarily transferred by Tabanidae-vectors (Desquesnes and Dávila 2002). Since horse flies (Tabanidae) are present in the Swedish forests, this result is not surprising.

In conclusion, we have found evidence of frequent occurrence of *Trypanosoma* sp. in the game species studied. The two strains isolated were classified as being *T. theileri*. The extent to which these infections have significant clinical implications for the animals remains to be investigated.

**Ethical standards.**

The authors declare that the experiments comply with the current laws of the country.

**Conflict of interests.**

The authors declare that they have no conflict of interests.

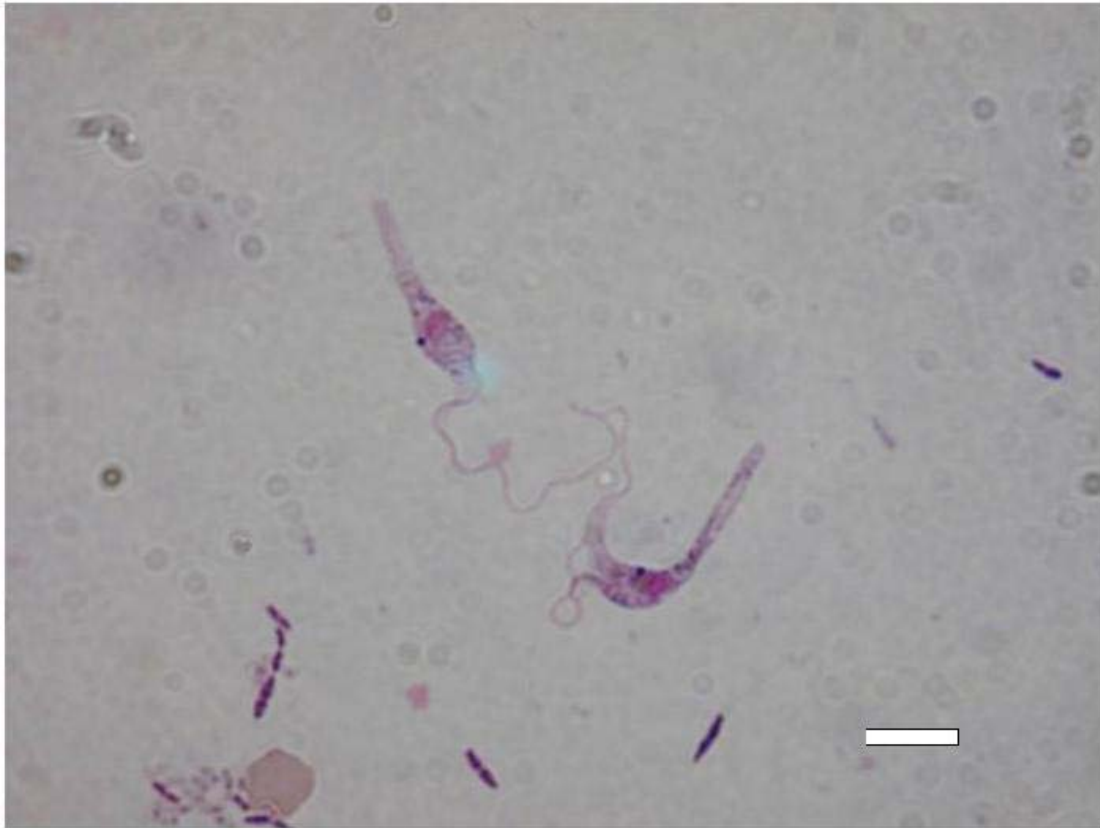
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## Figures and legends

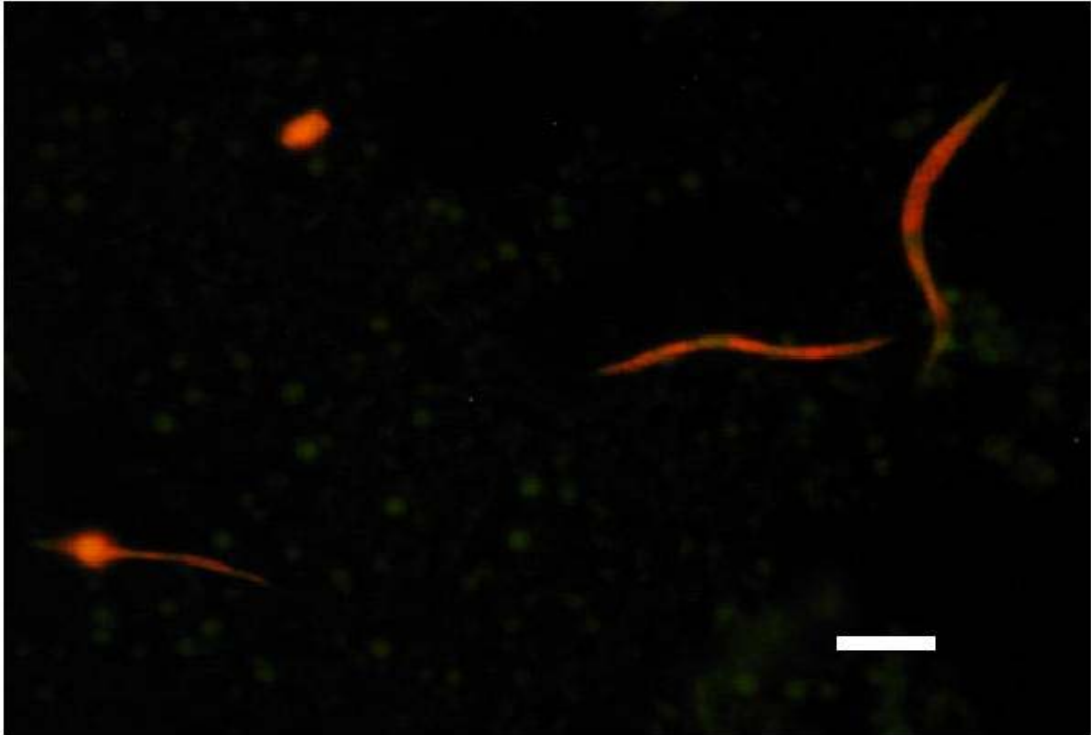
**Figure 1**



Isolated trypanosomes

Light microscopy of Giemsa-stained *Trypanosoma*, primary isolated from blood of a roe deer host (Bar 10  $\mu\text{m}$ ). Two parasites are shown in the centre of the picture. *Bacillus* bacteria can also be seen.

**Figure 2.**



*Trypanosoma* stained with acridine orange and observed by fluorescence microscopy.  
(Bar 10  $\mu\text{m}$ )

**Table 1.**

Annealing positions and sequences for the primers used for PCR of the 18s rDNA gene in *Trypanosoma*.

<b>Primer</b>	<b>Position</b>	<b>Sequence</b>	<b>Length</b>
18stnF2	1272-1297	AAACGATGACACCCATGAATTGGGGA	26
18stnR2	1868-1892	GTGTCTTGTTCTCACTGACATTGTAGTG	28
18stnR3	1969-1991	TGCGCGACCAATAATTGCAATAC	23
Tryp-theIR	2196-2212	TAATCTCATCGGAAAATGATCCAG	24

**Table 2.** Test results for the individual animals. 9 Fallow Deer, 1 Red Deer, 12 Roe Deer, 7 Boars, 7 European Elks. ND = Not done. Serology: 2+ = F.A. titre 1/200, 1+= Any detectable fluorescence.

Sample nr	Animal	Isolation	Serology	PCR results
9, 28, 31	Fallow deer	-	ND	-
15,23, 26	Fallow deer	-	-	-
24	Fallow deer	-	1+	+
30	Fallow deer	-	2+	-
37	Fallow deer	-	1+	-
1	Roe Deer	+	ND	ND
7	Roe Deer	-	ND	+
8, 10	Roe Deer	-	ND	-
13	Roe Deer	-	ND	+
16	Roe Deer	-	-	-
17	Roe Deer	-	1+	+
18, 29	Roe Deer	-	1+	-
19	Roe Deer	-	2+	-
38, 39	Roe Deer	-	ND	-
11	Boar	-	ND	-
20, 21,35, 36	Boar	-	2+	-
22	Boar	-	-	-
32	Red Deer	-	1+	+
2, 6	Elk	-	-	-
3	Elk	-	-	+
4	Elk	+	1+	+
5	Elk	-	2+	-
27, 33	Elk	-	1+	-