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First record of *Trypanosoma theileri*-like flagellates in horseflies from Northwest Russia

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Summary

Trypanosomatid infections of female horseflies were documented in three regions of Northwest Russia (Leningrad Oblast, Novgorod Oblast and the Republic of Karelia). The molecular analysis of four intestinal specimens and one culture isolated from the recta of infected horseflies revealed the presence of flagellates related to *Trypanosoma theileri*. This is the first registered case of such infection in the Northwestern Federal District and the first record of *T. theileri*-like trypanosomes in the vectors on the territory of the Russian Federation.

Key words: *Trypanosoma theileri*, horseflies, morphology, 18S rRNA

Introduction

The flagellates of the family Trypanosomatidae (Euglenozoa, Kinetoplastea) are widely distributed parasites of animals and plants. The group is famous mostly for the members that are able to cause dangerous diseases such as American trypanosomiasis, sleeping sickness and various leishmaniases in humans. *Trypanosoma* Gruby, 1843 is the largest genus of the family. It unites dixenous trypanosomatids, whose life cycles include parasitizing vertebrate hosts and invertebrate (arthropod or leech) vectors (Frolov et al., 2015; Frolov, 2016). *Trypanosoma theileri* Laveran, 1902 is a cosmopolite flagellate inhabiting the blood of domestic and wild ruminants (Hoare, 1972; Wells, 1972). Various species of horseflies (Diptera, Tabanidae) are considered to be its main vectors (Kraneveld, 1931; Page, 1972; Böse et al., 1987; Böse and Heister, 1993). However, *T. theileri* apparently represents rather a species complex than a single species. On the phylogenetic reconstructions including the isolates from different hosts and geographic regions, these trypanosomes always form a clade comprising several groups regarded as either genotypes or closely related species (Rodrigues et al., 2010a, 2010b; Martinkovic et al., 2012; Fisher et al., 2013). This taxonomic uncertainty forces researchers to refer to the flagellates of this group as "*T. theileri*-like trypanosomes". At present, it comprises two additional species of trypanosomes: *T. melophagium* Flu, 1908 and *T. cervi* Kingston et Morton, 1975 parasitizing sheep and deer, respectively (Martinkovic et al., 2012; Fisher et al., 2013). The sheep ked, *Melophagus ovinus* (Diptera: Hippoboscidae) is the vector of the former species,

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whereas the latter appears to be transmitted by the deer keds, *Lipoptena* spp. (Hippoboscidae) (Hoare, 1923; Gibson et al., 2010; Martinkovic et al., 2012). Other hematophagous arthropods are also likely to play this role for *T. theileri*-like trypanosomes (Fisher et al., 2013; Calzolari et al., 2018).

The vertebrate hosts acquire trypanosomes through oral mucosa upon the contact with fresh feces of tabanids either upon accidental ingestion of the vectors or in the process of grooming (Nöller, 1916; Kraneveld, 1931; Böse et al., 1987). The horseflies become infected when sucking the blood of infected animals. In the vector's midgut, the trypomastigotes transform into elongated epimastigotes. The latter invade the hindgut, where they undergo intensive proliferation, accompanied by the consecutive change of the following developmental stages: elongated epimastigotes, pyriform epimastigotes, spheromastigotes and metacyclic amastigotes (Böse and Heister, 1993). Thus, the traditional diagnosis is based on the observation of four morphotypes in the hindgut.

Although these parasites are considered as cosmopolitan, for many geographic regions there are no data about their occurrence. In particular, this concerns the vast territory of the Russian Federation. Taking into account the asymptomatic course of the infection and the low level of parasitemia in cattle and wild ruminants, these trypanosomes usually remain unnoticed when traditional diagnostic methods are applied. The only exceptions are the early records in European bison (*Bison bonasus*) from Belovezhskaya Pushcha (Wrublewski, 1909) and zebu (*Bos indicus*) from the Caucasus (Yakimoff et al., 1933). No data concerning the vectors of *T. theileri*-like trypanosomes on the territory of Russia have been published yet.

Here we present the first record of these flagellates in tabanid flies from the territories of three adjacent regions in Northwest Russia and characterize them using morphological and molecular methods.

Material and methods

Insect collection

The human-attacking female horseflies were collected in 2018 from the end of May to July in Leningrad Oblast (village Tolmachyovo, 58°51'N, 29°54'E), Novgorod Oblast (village Oksochi, 58°39'N, 32°47'E) and the Republic of Karelia (Lakhdenpokhya town, 61°31'N, 30°12'E). The captured insects were kept in the individual vials containing tubes with drinking water.

Dissection

The horseflies were dissected within 24 hours after capturing. Each insect was euthanized with chloroform and placed into normal saline solution. The whole intestine was isolated, then carefully (without its disruption) placed onto a slide with a drop of normal saline solution, covered with cover glass and examined in light microscope. In the case of detection of trypanosomatids, the cover glass was carefully removed and the infected part of the intestine was isolated for subsequent studies.

Cultivation

The flagellates were seeded onto the blood agar with an overlay consisting of RPMI-1640 medium (Sigma-Aldrich, United Kingdom), 10% Fetal Bovine Serum (Biolot, Saint Petersburg, Russia) and supplemented with 500 μg/ml of streptomycin and 500 Units/ml of benzylpenicillin. In the subsequent passages, the antibiotics were not added. The purification from fungal contamination was performed using the U-shaped glass tube construct described earlier (Podlipaev and Frolov, 1987); however, it was successful only for one culture, KrSl7. Throughout the study, it was maintained at 20° C and passaged monthly. All eight obtained cultures were cryopreserved in the liquid phase of the above medium supplemented with 10% DMSO (Sigma-Aldrich) and stored at -86° C.

Light microscopy

The smears prepared from the intestinal contents were fixed with ethanol, stained with Giemsa stain (Sigma) or 4',6-diamidino-2-phenylindole (DAPI) as described earlier (Yurchenko et al., 2008). Digital images were acquired in DM 2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with UCMOS14000KPA 14-Mpx camera (Toup Tek, Hangzhou, China) at ×1,000 magnification. All measurements and statistical data analyses were accomplished in the software UTHSCSA Image Tool for Windows v. 3.0. The significance of the differences between means was estimated using a paired T-test with Bonferroni correction for multiple comparisons (Lehmann, Romano, 2005).

Molecular identification of insects and parasites

Total genomic DNA was isolated from the intestinal fragments of infected horseflies, their flying muscles as well as the axenically grown trypanosomatid culture using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

The standard 0.65 kb-long barcoding fragment of the insect mitochondrial cytochrome C oxidase subunit 1 (COI) was amplified using the primers LCOI490 and HCO2198 (Folmer et al., 1994) as described previously (Cywinska et al., 2010) and sequenced directly with the same oligonucleotides. The identification of insects was performed with the use of Barcode of Life Data System (http://www.boldsystems.org). In all cases, we were able to identify species of infected horseflies with high fidelity, since they demonstrated over 99% identity with records in the database.

The nearly full-length 18S rRNA gene of trypanosomatids was amplified using the primers S762 and S763 (Maslov et al., 1996) and sequenced directly as described earlier (Kostygov et al., 2012). The obtained trypanosomatid sequences were deposited in the GenBank (Table 1). Their comparison with the previously published sequences of related trypanosomes was performed by Blast searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against Genbank nr database.

Results

Out of total 29 investigated horseflies only 9 (~31 %) were infected (Table 1). The prevalence by region was as follows: 100% (2/2) in Novgorod Oblast, ~23% (3/13) in Leningrad Oblast and ~27 % (4/15) in the Republic of Karelia. The infected horseflies belong to four species: *Hybomitra tarandina, H. muehlfeldi, H. bimaculata* and *Chrysops divaricatus* (Table 1).

Trypanosomatids were detected only in the rectum, where they formed heteromorphic high-density micropopulations represented mainly by semi-motile or immotile parasites (Fig. 1 A–C). Small amastigote-like cells were the prevailing morphotype (Fig. 1 D–F). In four random samples from various hosts and localities, their measurements were similar (Table 2) and the differences between means proved to be statistically non-significant. Apart from the small size, these cells were characterized by the unstable position of the kinetoplast, which was located anteriorly, laterally or posteriorly in relation to the nucleus (Fig. 1 D, E, F, respectively).

The second morphotype was represented by relatively small spherical cells of 4.95–6.23 (5.17 ±0.67 on average) μm in diameter possessing a conspicuous but slowly moving flagellum (Fig.1 G). Such cells were abundant on the smears from the hindgut contents of all infected horseflies.

Pyriform epimastigotes were the third morphotype (Fig. 1 H). Their sizes varied in a wide range with length being 6.8–15.2 (9.3±2.7 on average) μm and width of 3.8–5.7 (11±1.0 on average). The free part of the flagellum in these cells was not present, but its apical part was transformed to an attachment organelle (Fig. 1 H) similar to that in many other trypanosomatids (Frolov and Skarlato, 1995). Most of the pyriform cells were in the process of division.

The fourth morphotype was represented by elongated epimastigotes (Fig. 1 I). They were 10.9 –14.2 (12.1±1.0) μm in length and 1.9–2.2 (2.0 ±0.4) μm in width. Their free flagella were roughly as long as the cell body. Only few cells of such kind were detected on the smears from the majority (8/9) of infected horseflies. In the rectum of one specimen (isolate TolSl1 from *H. bimaculata*), these epimastigotes were present in a considerable proportion (Fig. 1 A).

All cultures obtained after seeding the hindgut contents on the biphasic blood-agar medium contained the same two morphotypes: pyriform and elongated epimastigotes (Table 1; Fig. 1 J–L). The former ones prevailed in the early phase (2–10 days) and proliferated in rosettes (Fig. 1 J), while the latter were more abundant in the late phase (11–20 days) and divided individually by binary fission (Fig. 1 K, L).

Trypanosome SSU rRNA gene sequences were obtained from the rectal contents of four infected horseflies and the axenic culture KrSl7 (Table 1). Out of four remaining specimens, one failed and other three demonstrated mixed signal in sequence chromatograms suggesting infections by more than one parasite strain/species. As judged by the high level of sequence identity to the representatives of *T. theileri*-like trypanosomes (> 99 %), our isolates belonged to this particular species complex (Table 3). The 18S rRNA gene sequence of KrSl1 was most similar (99.81–100 % identity) to those of the isolates TREU_124, D30 and TrPhp1 obtained from cow, fallow deer (*Dama dama*) and sandly (*Phlebotomus perfiliewi*), respectively (Table 3). Along with the isolate TmHR1 from sheep ked they are part of the clade TthII (Rodrigues et al., 2010; Martinkovic et

Table 1. Isolates of *T. theileri*-like trypanosomes studied in the current work.

Vector	Isolate	Culture	Trypanosome 18S rRNA GenBank accession number
Novgorod Oblast			
Hybomitra tarandina	NovSl 1	xenic	mixed infection
	NovSl 2	xenic	MK156794
Republic of Karelia			
Hybomitra tarandina	KrSl1	xenic	MK156791
Hybomitra muehlfeldi	KrSl4	xenic	MK156792
Chrysops divaricatus	KrSl 7	axenic	MK156793
	KrSl 9	–	–
Leningrad Oblast			
Hybomitra bimaculata	TolSl2	xenic	mixed infection
	TolSl3	xenic	MK156795
	TolSl13	xenic	mixed infection

al., 2012; Fisher et al., 2013; Calzolari et al., 2018). The sequences of four other isolates (NovSl2, KrSl4, KrSl7, and TolSl3) had only 99.52–99.60 % identity with that of KrSl1 (Table 3). At the same time, they showed greater similarity (99.67–100 %) to the sequences of the TthI clade members PJH-WTD_A1 and ELK_142 isolated from white-tailed deer *Odocoileus virginianus* and wapiti (elk) *Cervus canadensis*, respectively (Table 3).

Discussion

The presence of four morphotypes (elongated and pyriform epimastigotes as well as spheromastigotes and amastigotes) in the hindgut of horseflies points to the infection with *T. theileri*-like trypanosomes. We observed such heteromorphic micropopulations of flagellates in the recta of 9 out of 29 female horseflies. The morphological data were corroborated by the analysis of the trypanosome 18S rRNA gene sequences obtained from the infected intestinal fragments. This is the first registered case of such infection in the Northwestern Federal District and the first record of *Trypanosoma theileri*-like flagellates in the vectors on the territory of Russian Federation.

The taxonomy of *T. theileri*-like trypanosomes is still very uncertain. The existing morphological characters, because of their scarcity, do not allow identifying species of these trypanosomes in most cases (Calzolari et al., 2018). The data on host and vector specificity are not of much help as well. The only exception is *T. melophagium* parasitizing sheep and transmitted by wingless flies *Melophagus ovinus*. So far, this species has never been reported from other hosts and vectors (Martinkovic et al., 2012). Molecular phylogenetic analyses of *T. theileri*-like trypanosomes also do not guarantee unambiguous taxonomic interpretation (Votypka et al., 2015). In most studies, two main lineages (TthI and TthII) each comprising several genotypes were revealed in this group of trypanosomes (Rodrigues et al., 2010; Martinkovic et al., 2012; Fisher et al., 2013). Importantly, only one genotype (IID), being part of the TthII clade, has been assigned to the nominal species *T. melophagium* so far (Martinkovic et al., 2012). Other genotypes are not associated with any particular species of the *T. theileri* group. Our isolates belonged to both clades of *T. theileri*-like

Table 2. Morphometry of amastigotes from four randomly selected isolates (n=25).

Parameter	KrSl1	TolSl3	NovSl2	KrSl7
Cell length	5.78±0.31 (6.48—5.22)	5.61±0.38 (6.44—5.1)	5.24±0.47 (6.13—4.53)	4.91±0.45 (5.74—4.15)
Cell width	2.00±0.14 (2.33—1.79)	1.88±0.17 (2.26—1.54)	1.63±0.19 (1.99—1.33)	1.65±0.13 (1.85—1.42)
Distance from anterior end to nucleus	3.24±0.28 (3.84—2.67)	2.87±0.36 (3.39—2.3)	3.29±0.35 (4.07—2.93)	3.04±0.59 (4.07—2.04)
Distance from anterior end to kinetoplast	4.26±0.20 (4.63—3.65	3.53±0.34 (4.36—2.74)	4.13±0.53 (4.57—2.61)	3.99±0.43 (4.49—2.62)

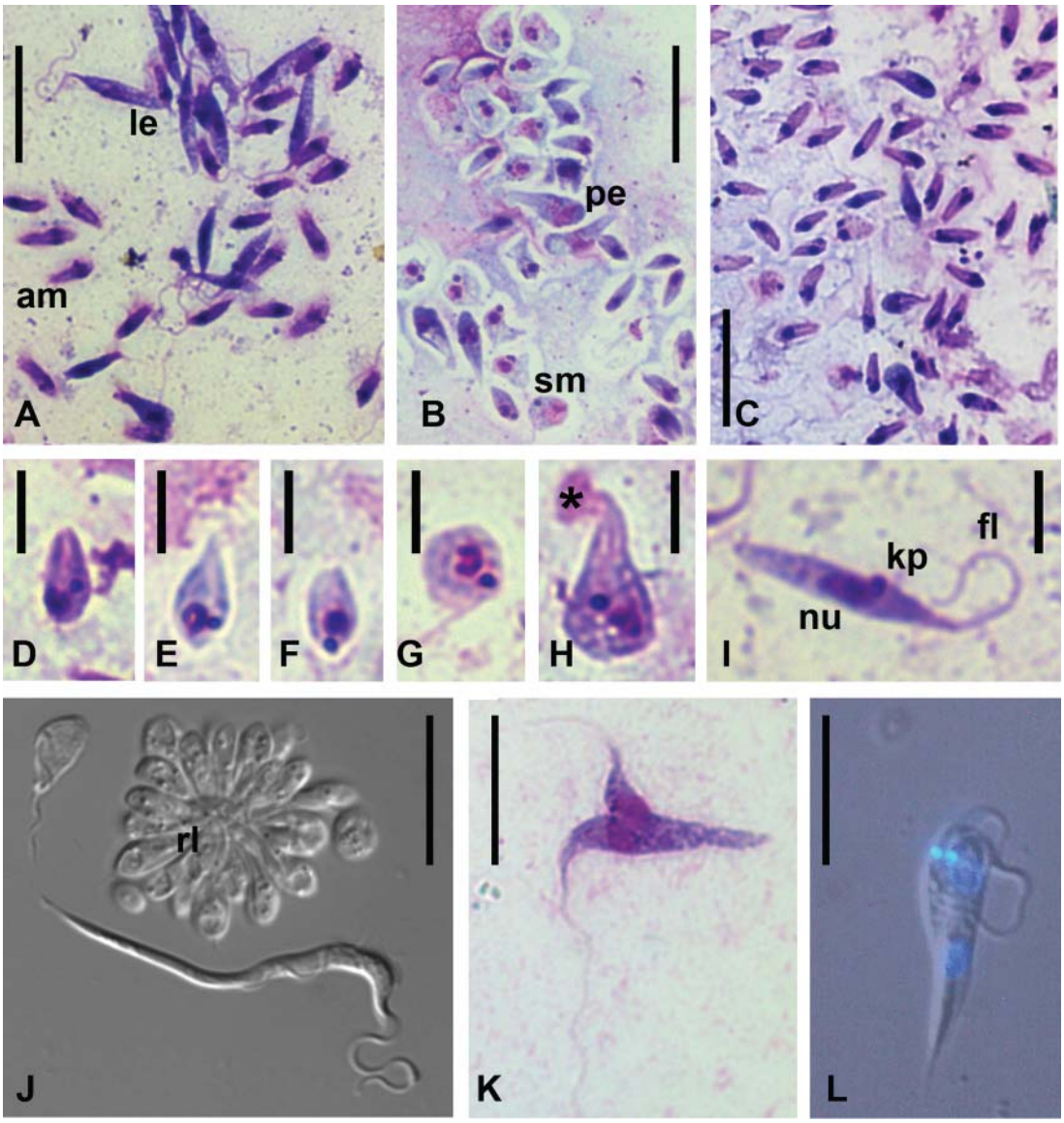

Fig. 1. Morphology of *T. theileri*-like trypanosomes from the horseflies' hindgut (A–I) and in axenic culture (J–L). A – Trypanosomes from *Hybomitra bimaculata*; B – trypanosomes from *H. tarandina* (Novgorod Oblast) ; C – trypanosomes from *Chrysops divaricatus*; D–F amastigotes; G – spheromastigote; H – pyriform epimastigote; I – long epimastigote; J – rosette of pyriform epimastigotes in the culture KrSl7; K, L – binary fission of long epimastigotes in the culture KrSl7. A–I, K – Giemsa staining; J – DIC; L – combination of DIC and DAPI. *Abbreviations:* am – amastigotes, fl – flagellum, kp – kinetoplast, le – long epimastigotes, nu – nucleus, pe – pyriform epimastigotes, rl – rosette of pyriform epimastigotes, sm – spheromastigotes, *asterisk* marks the modified part of the flagellum. Scale bars: A–C, J–L – 10 μm; D–I – 5 μm.

trypanosomes: NovSl2, KrSl4, KrSl7, and TolSl3 proved to be a part of TthI, whereas KrSl1 affiliated with TthII. Interestingly, no correlation could be observed between association to a particular clade and vector species or locality. NovSl2 and KrSl1 were obtained from the same horsefly species (*H. tarandina*) but two different regions (Novgorod Oblast and the Republic of Karelia, respectively). According to our data, they belong to two separate clades of *T. theileri*-like trypanosomes. At the same time, the lineage TthI included the isolates NovSl2, KrSl4, KrSl7 and TolSl3 isolated from four different species of vectors and three different regions (Table 1). Unquestionably, these data are preliminary and

Table 3. Pairwise identity (%) of SSU rRNA gene sequences obtained in this study and those of *T. theileri*-like trypanosomes from the GenBank. The new sequences obtained in the current study are shown in bold.

N	Name, isolate, accession number	1	2	3	4	5	6	7	8	9	10	Clades
1	*T. theileri* D30 AJ009165.1											
2	*T. theileri* TREU_124 AJ009163.1	99.86										
3	*T. theileri* TrPhp1 KY681802.1	100.0	99.76									TthII
4	*T. melophagium* TmHR1 HQ664912.1	99.63	99.67	99.39								
5	**KrSl1 MK156791**	99.86	99.81	100.0	99.62							
6	**KrSl4 MK156792**	99.53	99.48	99.15	99.34	99.57						
7	**KrSl7 MK156793**	99.57	99.52	99.15	99.38	99.52	100.0					
8	**NovSl2 MK156794**	99.48	99.43	99.15	99.29	99.53	100.0	99.90				
9	**TolSl3 MK156795**	99.74	99.60	99.29	99.34	99.60	100.0	100.0	99.87			TthI
10	T. cf. cervi PJH-WTD_A1 JX178192.1	99.53	99.48	99.02	99.34	99.52	99.86	99.86	99.76	99.74		
11	PJH-2013b Elk_142_Cl_10 JX178178.1	99.21	99.21	99.02	99.02	99.20	99.67	99.73	99.67	100.0	99.61	

require further justification, but they suggest that the specificity to vectors in *T. theileri*-like trypanosomes is low or absent at all.

Of particular interest is the high frequency of mixed infections (33%) we observed in the studied isolates. Monoxenous trypanosomatids were not detected in our specimens and trypanosomes other than those from *T. theileri* species complex have been never recorded in the studied regions. Although occasional *T. evansi* infections were registered in Europe and even on the territory of Russia, they were never documented north of the 53rd parallel (Desquesnes et al., 2013). In addition, we analyzed the material from the hindgut, where *T. evansi* does not appear, because it is transmitted through contaminated vector's mouthparts or via regurgitation of recently ingested blood (Luckins, 1988). Thus, the observed mixed infections suggest that some horseflies may be simultaneously infected with different genotypes or species of *T. theileri*-like trypanosomes.

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