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Trypanosoma avium: experimental transmission from black flies to canaries

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Abstract Trypanosomes identified as *Trypanosoma avium* were found in the ornithophilic black flies (*Eusimulium latipes*) attacking buzzard nestlings (*Buteo buteo*). Parasites formed plugs and rosettes in the hindgut of the vector and were attached on the cuticular lining of the black fly anterior intestine (ileum) by hemidesmosome-like plaques. Hindgut stages from infected black flies were experimentally transmitted into canaries (*Serinus canaria*) by ingestion of vectors and by contamination of host conjunctiva. This is the first clear evidence of such transmission in avian trypanosomes. Parasites survived in peripheral blood of birds at the least 10 months. In contrast to the direct inoculation of insect stages, parasites from culture failed to produce infection in experimental birds; this has consequences for laboratory studies of host susceptibility and transmission.

Introduction

A wide variety of blood-sucking arthropods (mites, hippoboscids, biting midges and mosquitoes) have been described as vectors for avian trypanosomes (see Bennett 1970; Baker 1976; Molyneux 1977). In black flies, several routes of transmission to birds have been reported: Bennett (1961) infected several bird species with trypanosomes from ornithophilic black flies (genus *Eusimulium*) by intraperitoneal injection and by placing parasites on the scarified skin of hosts. Fallis et al. (1973) reported experimental transmission of *Trypanosoma numidae* by intraperitoneally injecting a suspension of infected *Simulium* sp. into African guinea fowl (*Numida mitrata*) and chicken (*Gallus domesticus*). Desser et al. (1975) experimentally transmitted stages (reported as epimastigotes) of *T. avium* from the hindguts of *Simulium rugglesi* naturally infected by feeding on Peking ducklings (*Anas platyrhynchos*) to uninfected ducklings. The authors demonstrated several ways of transmission: ingestion of black flies, contamination of abraded skin, and intraperitoneal inoculation. The role of black flies in transmission of avian trypanosomes has been supported by using molecular biology methods (Dirie et al. 1990; Votýpka et al. 2002).

More than 100 species of avian trypanosomes have been described thus far. For most of these species, host and vector specificities are unknown, and clear-cut evidence of a host-parasite relationship, as well as evidence of the natural route of transmission, are still lacking. For those vectors in which kinetoplastids (trypanosomes and leishmanias) develop in the fore part of the alimentary tract (e.g. phlebotomine sand flies) or in salivary glands (e.g. tse-tse flies), transmission of parasites is accomplished by the bite of the blood-sucking vectors. Two other routes of infection have been reported for vectors in which trypanosomes develop in their digestive tract (e.g. ticks) or in the hind part of the alimentary tract (e.g. kissing bugs): (1) by ingestion of whole vectors, (2) by contamination of host skin or conjunctiva by vector faeces during blood sucking.

In our previous study, we found that ornithophilic simuliids (*Eusimulium* spp.) are vectors for raptor trypanosomes (Votýpka et al. 2002), and that parasites occurred only in hindguts of dissected black flies. Trypanosome forms from vectors and cultured cells were different in their morphology, but it is known for other kinetoplastids that culture conditions may affect the development of infection forms (Figueiredo et al. 2000).

The aim of this study was to determine the route of transmission, infectivity of culture stages, and host specificity of the black fly-transmitted raptor trypanosomes characterized by molecular biology in our previous study.

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Materials and methods

Collection and dissection of black flies

Blood-sucking insects attacking the nestlings of buzzards were collected using air-sucking miniature CDC traps, installed overnight at the level of the nest, in the forest area next Ovčáry village, 10 km south of Prague, Czech Republic. Black flies were kept in nets in humid and cold conditions. Insect determination, dissection and light microscopy examination of the intestine and salivary glands for the presence of parasites was performed within 2 days of capture. Location in the gut and cell morphology of kinetoplastids of infected black flies was observed by light microscope after dissection.

Cultivation and light and transmission electron microscopy of trypanosomes

Parasites for cultivation were aspirated in an insulin syringe, inoculated on SNB-9 blood agar and cultivated as described in Votýpka et al. (2002). The slides with remaining parasites were then air-dried, fixed for 5 min with methanol, stained with Giemsa's stain (Sigma, St. Louis, Mo.) for 30 min, and examined by light microscopy at 1,000× magnification. The cells were measured with a calibrated micrometer.

For transmission electron microscopy (TEM), parasite-loaded hind parts of the dissected digestive tract (anterior intestines and rectal ampullas) of black flies were washed in 0.1 M phosphate-buffered saline (PBS) solution and fixed in 2.5% glutaraldehyde in the same buffer for 1 h at 4°C. After dehydration in graded series of ethanol, the tissues were embedded in Epon-Araldite, and thin sections were stained with lead citrate and uranyl acetate and examined with a JEOL 1010 microscope. Cells collected from 7-day-old cultures were processed by the same method. The kinetoplast DNA (kDNA) structure of parasites was analysed as described previously (Lukeš and Votýpka 2000). Statistics (one-way ANOVA) were performed using Statistica for Windows, and data are presented as means ± SD.

Experimental infections of birds

Hindguts from infected black flies were homogenized in saline, and applied orally or placed on the conjunctivas of experimental birds: canaries (*Serinus canaria*) and ducklings (*A. platyrhynchos* f. *domestica*) (see Table 1), which were negative for trypanosomes. Each bird was inoculated with three infected black flies (*Eusimulium latipes*). Control birds (three canaries and two ducklings) were kept in the same cages. Presence of trypanosomes was detected by in vitro cultivation on SNB-9 blood agar. Small amounts of blood from the metatarsus vein articulation (*vena metatarsalis plantaris superficialis media*) of experimental birds were taken at 8, 20, 40, 60, 120, and 300 days post infection (DPI). Positive samples were kept for subsequent analysis.

To test if cultured forms of trypanosomes are able to infect bird hosts, parasites were isolated from an infected bird (canary 2), cultivated in RPMI growth medium and used for experimental infection. Canaries were infected with 10^7 trypanosomes from a 7-day-old culture and the method of application was the same as in the previous experiment (see Table 1).

DNA analysis

The identity of trypanosomes from black flies and infected birds and the identification of different isolates from black flies were confirmed by analysis of DNA. Total DNA was isolated from three cultures of trypanosomes originating from naturally infected black flies (SIM-M5, SIM-M6, SIM-M22) and three cultures of experimentally infected birds (canary 2, 5, 6) using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification of the V4 hypervariable domain of the 18S rRNA genes was performed using specific primers S662 and S713 (see Votýpka et al. 2002). Amplicons were purified on 0.75% agarose gels and cloned using a TOPO TA cloning kit version E (Invitrogen, La Jolla, California). Both strands were sequenced on an automated DNA sequencer using a BigDye DNA sequencing kit (Perkin-Elmer, Foster City, California).

RAPD (random amplified polymorphic DNA) reactions were performed in a final volume of 25 µl containing 25 ng parasite DNA (or no DNA as a control), 15 ng of the decamer primer (three

Table 1 Transmission of trypanosomes from the hindguts of black flies (*Eusimulium latipes*) and transmission of trypanosome culture stages (derived from an experimentally infected canary) to canaries and ducklings. *PO* Per oral (by ingestion), *CON* via conjunctiva, *DPI* days post inoculation

Bird number	Source	Application	8 DPI	20 DPI	40 DPI	60 DPI	120 DPI	300 DPI
Canary 1	Black flies	PO	+	+	+	+	+	+
Canary 2	Black flies	PO	+	+	+	+	+	NI[a]
Canary 3	Black flies	PO	+	+	+	+	NI	NI
Canary 4	Black flies	PO	+	+	+	+	+	+
Canary 5	Black flies	CON	+	+	+	+	NI	NI
Canary 6	Black flies	CON	+	+	+	+	+	+
Canary 7	Black flies	CON	+	+	+	+	NI	NI
Canary 8	Black flies	CON	+	+	+	+	+	+
Duckling 1	Black flies	PO + CON	–	–	NI	NI	NI	NI
Duckling 2	Black flies	PO + CON	–	–	NI	NI	NI	NI
Canary 9	Culture	PO	–	–	–	–	NI	NI
Canary 10	Culture	PO	–	–	–	–	NI	NI
Canary 11	Culture	PO	–	–	–	–	NI	NI
Canary 12	Culture	PO	–	–	–	–	NI	NI
Canary 13	Culture	PO	–	–	–	–	NI	NI
Canary 14	Culture	PO	–	–	–	–	NI	NI
Canary 15	Culture	CON	–	–	–	–	NI	NI
Canary 16	Culture	CON	–	–	–	–	NI	NI
Canary 17	Culture	CON	–	–	–	–	NI	NI
Canary 18	Culture	CON	–	–	–	–	NI	NI
Canary 19	Culture	CON	–	–	–	–	NI	NI
Canary 20	Culture	CON	–	–	–	–	NI	NI

[a]Not investigated

selected primers OPA-08, OPA-09, OPA-11; RAPD 10mer kits, Operon Technologies, Alameda, California), 200 μM dNTP, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (MBI Fermentas, St. Leon Rot, Germany). The PCR reaction consisted of an initial denaturation of 5 min at 94°C and 40 cycles of 1 min at 94°C, 1 min at 40°C and 1.5 min at 72°C, with a final extension step at 72°C for 10 min. All reactions were performed in the same thermocycler and RAPD fragments were separated electrophoretically on 1.5% agarose gels stained with ethidium bromide, and visualised under UV light.

Results

Prevalence and observation of trypanosomes in black flies

Two species of ornithophilic black flies were identified from the buzzard nest studied: *Eusimulium latipes* (77%, $n = 219$) and *E. securiforme* (23%, $n = 66$). Investigation of their alimentary tracts showed that 67% of *E. latipes* and 4% of *E. securiforme* were positive for trypanosomes. Trypanosomes were exclusively located in the hindgut: in the anterior intestine (ileum) and the rectal ampulla. Parasites formed plugs and rosettes in both parts of the alimentary tract and about half of the observed trypanosome cells were free. In several cases, the high number of trypanosomes in the anterior part of the alimentary tract resulted in non-physiological enlargement of the rectal ampullas of infected black flies. Parasites were never seen in the salivary glands.

Light microscopy and TEM

Trypanosomes from black fly hindguts and from in vitro cultures differed significantly in their morphology and body length. Insect forms were small trypomastigotes (body length: 9.6 ± 1.9 μm, range 4.6–14.4 μm, $n = 81$, Fig. 1A) while epimastigotes developed in cultures (body length: 20.7 ± 5.8 μm, range 6.8–29.0 μm, $n = 20$, Fig. 1B). Only two trypomastigotes were found on slides from canary blood (Fig. 1C). Culture forms derived from blood and insect trypomastigotes are similar.

The parasites observed by TEM were in close contact with chitin in the cuticular lining of the anterior intestine, towards which they are oriented with their flagellas. The adhesion of the parasite to this cuticle-lined region occurred by the formation of zonal hemidesmosome-like plaques at the extremities of the expanded flagella of epimastigotes (Fig. 1D).

Experimental infections

Trypanosomes were found in the blood of all eight canaries inoculated with hindgut forms of parasites from black flies (*E. latipes*), irrespective of the method of application (orally or via conjunctivas). Parasites were detected from day 8 up to day 300 after infection. No parasites were found in either the blood of experimentally infected ducklings and control birds, or in canaries inoculated with culture stages of trypanosomes (isolated from an infected canary) (Table 1).

Identity of strains

DNA comparison of trypanosome strains from black flies and canaries revealed the following findings: (1) 100% sequence identity of the highly polymorphic V4 domain of the 18S rRNA gene among all studied isolates was observed, and absolute consistency with previously published sequences from raptors (AF416559, AY099318, AY099319 and AY099320) and black fly *E. securiforme* (AF416563) from the Czech and Slovak Republics. (2) The same patterns of DNA fragments produced using the RAPD technique (six studied

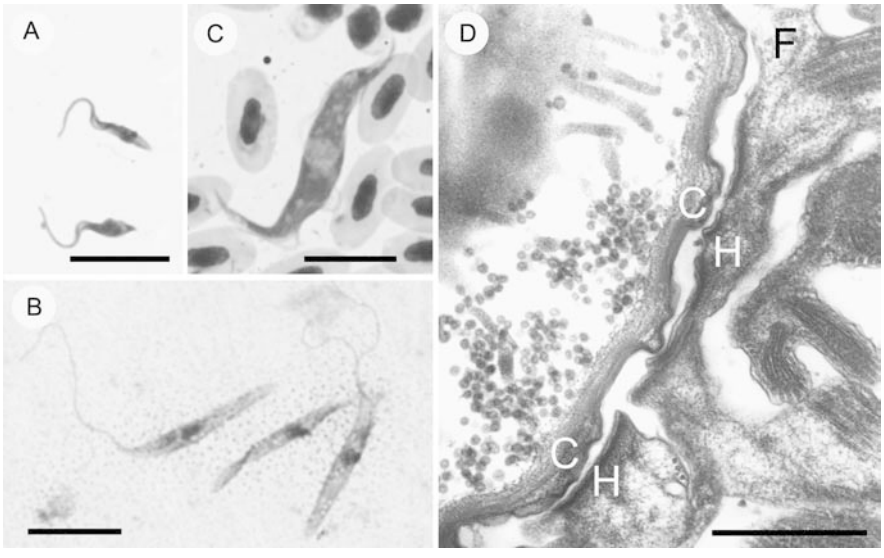

Fig. 1 Micrographs of Giemsa-stained trypanosomes from **A** hindgut of black fly, **B** 7-day-old culture, **C** blood of experimentally infected canary. **D** Electron micrograph of trypanosome adhesion to cuticle-lined regions (*C*) in black fly anterior intestine (ileum) by zonal hemidesmosome-like plaques (*H*) on parasite expanded flagella (*F*). *Bars* **A**–**C** 10 μm, **D** 1 μm

isolates, three random primers). Trypanosome control DNA obtained from blackbird showed a distinct pattern (data not shown).

Electron microscopy revealed no significant differences in morphology (cylindrical shape, number of electron-dense stripes) and thickness of kinetoplast discs ($F_{(2,\ 115)} = 1.08$; $P = 0.34$) in trypanosomes localised in black fly hindguts (0.608 μm ± 0.054 μm, $n = 25$) and culture forms of trypanosomes derived from *E. latipes* (0.601 μm ± 0.049 μm, $n = 49$) and infected canaries (0.617 μm ± 0.052 μm, $n = 42$).

Discussion

Experimental transmission of avian trypanosomes by intraperitoneal inoculation has been reported by several authors (Bennett 1961; Fallis et al. 1973; Desser et al. 1975). Natural transmission was demonstrated only by ingestion of black flies or by contamination of abraded host skin (Bennett 1961; Desser et al. 1975). On the other hand, Bennett (1961) failed to produce infections in birds by feeding them whole black flies or by placing an emulsion containing trypanosomes on their eyes. Our experiments confirm transmission of avian trypanosomes via ingestion of black flies and show successful transmission via conjunctiva. To our knowledge, this is the first clear evidence of such transmission of avian trypanosomes. Our finding of trypanosomes in rectal ampulla of black flies is in agreement with the observations of Bennett (1961). Localisation of parasites in the hindgut of *E. latipes* suggests that the natural routes of infection are by contamination and/or by ingestion. Blood-sucking Nematocera excrete urine during feeding on the host (Briegel and Rezzonico 1985; Sádlová et al. 1998) and infective stages of trypanosomatids are discarded via this fluid (Sádlová and Volf 1999). Therefore, we may speculate that this process, called prediuresis (Briegel and Rezzonico 1985), could occur also in black flies. Haematophagous Nematocera (black flies, biting midges and mosquitoes) very often feed on the parts around bird's eyes (J. Votýpka and M. Svobodová, unpublished observation) and prediuresis could play a role in transmission of trypanosomes by contamination of the conjunctiva. Transmission by ingestion of vectors is more probable in smaller bird species, which exhibit more anti-ectoparasite behaviour (Edman et al. 1974). Thus, we may expect a higher probability of black fly catching by these smaller birds.

In terms of the host specificity, we demonstrated that canaries are suitable hosts for trypanosomes transmitted by black flies; these parasites are identical to raptor isolates. Despite the small number of experimental ducklings, we conclude that ducks (order Anseriformes) are not an appropriate host for this species of avian trypanosomes. All eight canaries inoculated with trypanosomes from black flies were infected, and parasites occurred in their peripheral blood at least 10 months after infection. On the other hand, birds inoculated by culture stages (re-isolated from an infected canary) were not infected. Our experiments show that only hindgut stages from black flies are infectious, whereas culture stages fail to infect birds. This finding is very important for interpretations of experimental transmissions in which only culture stages of trypanosomes were used. It is known that culture conditions could influence infectivity of trypanosomatids for their hosts. For example in *Trypanosoma cruzi*, a human pathogen transmitted by kissing bugs, development of infective forms (metacyclogenesis) is triggered by nutritional stress, which leads to substrate adhesion and transformation of epimastigotes (Figueiredo et al. 2000). In black flies, trypanosomes adhere to the hindgut cuticular lining (Fig. 1D), and this might be a prerequisite for the development of infective forms.

The ultrastructure of the vector stages of *T. avium* in *E. latipes* observed by us corresponds with the findings of other authors. Similar hemidesmosome-like adhesion was reported by Desser (1977) for trypanosomes in the posterior part of the alimentary tract of *S. rugglesi*, by Mungomba et al. (1989) in *Ornithomyia avicularia*, and there is a strong parallel with the adhesion of *Leishmania* on the stomodeal valve of sand flies (Schlein et al. 1992).

Based on data obtained by molecular methods (sequencing and RAPD), and electron microscopy observations, we confirmed that trypanosomes from *E. latipes* are identical with parasites from canaries infected with homogenates of infected black flies. According to their 18S rRNA, all strains studied belong to the same trypanosome species—*T. avium*. Demonstration of kinetoplast identity of trypanosomes localised in the alimentary tract of black flies and trypanosomes from the cell culture suggest that kDNA morphology is constant within introduction into culture. This supports previous findings that the unique structure of the kinetoplast disk (shape and size) of bird trypanosomes could be used as a suitable marker for distinguishing between avian trypanosome species (Lukeš and Votýpka 2000).

Previously, we showed the identity of trypanosomes isolated from black flies (genus *Eusimulium*) and birds of the order Falconiformes (Votýpka et al. 2002). In this study, we demonstrate successful transmission of *T. avium* from ornithophilic black flies (*E. latipes*) caught on raptor nests into experimental bird hosts via ingestion and via conjunctiva. Canaries (order Passeriformes) may serve as model hosts for raptor trypanosomes, because parasites survive in their peripheral blood for long periods. Our finding that *T. avium* fails to produce infective stages after introduction into culture is important for interpretations of experimental transmission of bird trypanosomes and possibly for other kinetoplastids.

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