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Trypanosoma varani and *T. grayi*-like trypanosomes: development in vitro and in insect hosts

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Abstract. The growth in vitro and the development in insect hosts of two reptilian trypanosomes was studied. The first was a *Trypanosoma grayi*-like isolate (Kiboko F_4) from *Glossina pallidipes* in Kenya, East Africa, and the second was *T. varani* V54, isolated from *Varanus exanthematicus* in Senegal. *T. varani* V54 grew well in blood-agar culture media, but for successful long-term cultivation of the F_4 trypanosomes, the presence of feeder cells from a triatomine or *Xenopus* cell line was essential. Experimental infection of tsetse (*G. morsitans morsitans* and *G. palpalis gambiensis*) and phlebotomine sandflies (*Phlebotomus duboscqi*) showed that *T. grayi*-like F_4 trypanosomes, known and shown to develop in tsetse, did not multiply in the sandflies, whereas *T. varani* V54 grew well in the sandflies but not in the tsetse. It is concluded that these two reptilian trypanosome stocks probably represent different species, subject to confirmation from biochemical studies currently in progress. Attempts to isolate *T. grayi* sensu stricto from 13 wild African crocodiles in Zambia and Zaire were unsuccessful.

Since their description, there has been uncertainty about the possible synonomy of the crocodile trypanosome *Trypanosoma grayi* Novy, 1906 and *T. varani* Wenyon, 1908 from the African monitor lizard *Varanus niloticus*.

Detailed studies by Hoare (1929, 1931) revealed the complete life cycle of *T. grayi* in both the Nile crocodile (*Crocodilus niloticus*) and the tsetse vector (*Glossina fuscipes fuscipes*). Hoare, working in Uganda, found that most crocodiles were infected with *T. grayi* but that monitor lizards (*V. niloticus*) did not carry the parasite and could not be infected experimentally. Hoare (1929) therefore concluded that *T. grayi* and *T. varani* were distinct species. Figure 1 shows the bloodstream forms of *T. grayi* in the crocodile and the insect-gut stages of *T. grayi*-like "Kiboko F_4" in experimentally infected *G. morsitans*.

Lloyd et al. (1924) reported that *G. tachinoides*, when fed on naturally infected monitors (*V. exanthematicus*) or toads (*Bufo regularis*), developed mid- and hindgut infections indistinguishable from those caused by *T.*

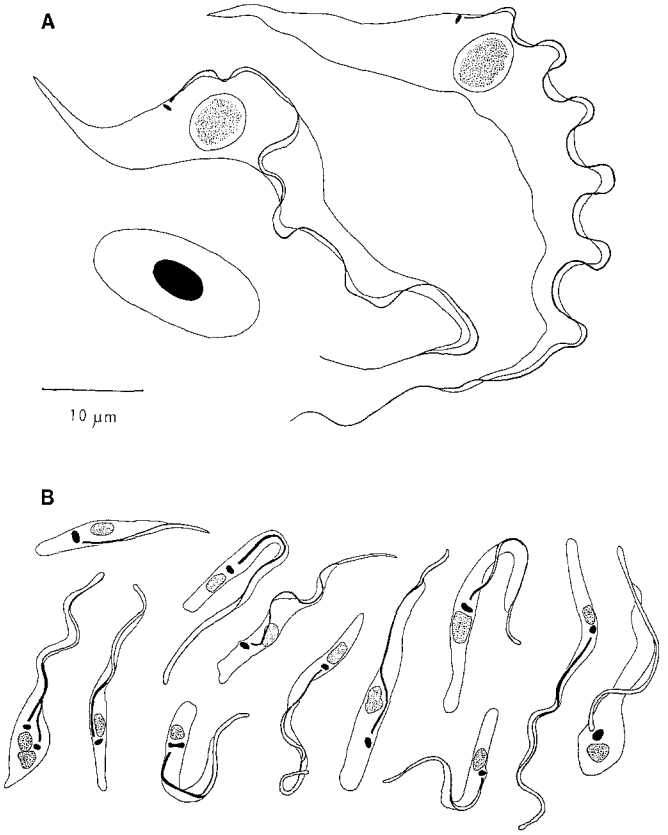

Fig. 1. A Bloodstream forms of *Trypanosoma grayi* Novy 1906 in crocodile blood (*Crocodilus niloticus*); after Hoare 1931. **B** Insect stages of the *T. grayi*-like "Kiboko F_4" trypanosomes in the midgut and hindgut of experimentally infected *Glossina morsitans* (after Minter-Goedbloed et al. 1983)

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grayi. They considered that *T. grayi* infected not only crocodiles but also monitor lizards and, possibly, other reptiles.

To add to the confusion concerning *T. grayi*-like trypanosomes, Lainson (1977) described a new trypanosome (*T. cecili*) of the neotropical cayman (*Caiman crocodilus crocodilus*) from Brazil, which morphologically resembles *T. grayi* but clearly cannot be naturally transmitted by *Glossina* spp.

More recently, a total of six *T. grayi*-like trypanosome stocks were isolated from three species of tsetse: one from *G. pallidipes* in Kenya (Minter-Goedbloed et al. 1983), four from *G. palpalis gambiensis* in Gambia (McNamara and Snow 1991) and one from *G. tachinoides* in Nigeria (Dirie et al. 1991). The Kenyan stock (F_4, Kiboko) and two of the four Gambian stocks were isolated from areas where crocodiles were believed to be absent. All four Gambian stocks were genetically homogeneous (Dirie et al. 1991) and one successfully infected a young crocodile, using trypanosomes defaecated by experimentally infected *G. m. morsitans* (McNamara, unpublished data). Dirie et al. (1991) compared the *T. grayi*-like Kenyan stock with three West African isolates by isoenzyme electrophoresis and found only minor differences between them.

Although in some of the areas referred to above there were apparently no crocodiles, monitor lizards were certainly present. Ranque (1973) in Senegal isolated a trypanosome from *V. exanthematicus* considered to be *T. varani*. Neither crocodiles nor tsetse were apparently present, but phlebotomine sandflies (*Sergentomyia* spp.) were abundant in the area and Ranque suggested that the sandflies might transmit the trypanosomes. Taylor (1929) reported that *V. exanthematicus* were infected with trypanosomes by eating infected sandflies (*S. africana*), and other authors also report an association in nature between reptilian trypanosomes and sandflies (Anderson and Ayala 1968; Ayala 1971; Ayala and McKay 1971; Ashford et al. 1973). Minter and Goedbloed (1971) recorded a 13% infection rate with trypanosomatid parasites in sandflies (*Sergentomyia* spp.) collected from the same area as, and contemporary with, the *G. pallidipes* from which the Kenyan *T. grayi*-like trypanosome was isolated.

The objectives of the present investigation were, firstly, to examine the growth of *T. varani* and *T. grayi*-like organisms in conventional culture media and in a novel co-cultivation system, with either an insect or an amphibian feeder-cell line; and secondly, to compare the development of the same trypanosomes in colony tsetse and sandflies. Our results support the view that *T. varani* is sandfly-associated, whereas the *T. grayi*-like trypanosomes are primarily tsetse-associated. Attempts were also made to re-isolate *T. grayi* sensu stricto from African crocodiles, with a view to define the features that characterise this imperfectly known species and to provide a basis for comparison with other reptilian trypanosome isolates.

Materials and methods

Trypanosome stocks and cultivation

Trypanosoma varani V54 was isolated in 1968 by haemoculture of the microscopically negative blood of a *Varanus exanthematicus* in Senegal (Ranque 1973). Cultures in Tobie's diphasic medium and Diamond's medium were incubated at 22° C and subpassaged every 1–2 weeks. After 47 passages the stock was cryopreserved. For the present studies the stock was resuscitated, grown in NNN, 4N and Evans' modified Tobie's medium at 23° C (Evans 1989) and used to infect sandflies. The V54 stock was also grown in a biphasic blood-agar medium (Oxoid number 2) with horse blood. The slopes had an overlay of Cunningham's MEM (McNamara and Snow 1991), 20% heat-inactivated foetal bovine serum (FBS) and 20 µg gentamycin/ml. These cultures were used to infect tsetse flies.

The *T. grayi*-like F_4 stock was isolated at Kiboko (Kenya) from *Glossina pallidipes* in 1969 by direct cryopreservation of the mid- and hindgut stages (Minter-Goedbloed et al. 1983). Following their recovery from liquid nitrogen storage, the trypanosomes were grown in vitro at 28° C in a cell-culture system.

Two cell lines were used: the triatomine cell line (BTC-32) of Pudney and Lanar (1977), which originated from *Triatoma infestans*, and the *Xenopus* cell line (XTC-2) of Pudney et al. (1973), from the South African clawed toad (*Xenopus laevis*). The two cell-line culture media (Pudney et al. 1973; Pudney and Lanar 1977) were: (a) L15 plus 10% tryptosephosphate broth (TPB) and 20% heat-inactivated FBS and (b) RPMI 1640 supplemented with 10% TPB with HEPES buffer (pH 7.0), 20% heat-inactivated FBS, streptomycin (100 µg/ml) and penicillin (1000 units/ml).

Initially, the fly stages were grown with BTC or XTC cells in plastic-sealed 96-well microtitre plates (Sterilin, UK). Each well held 0.2 ml of culture medium. Plates were sealed with plastic film. Later, for bulk culture, plastic 25-ml tissue-culture flasks (Becton Dickinson, UK) were used. Prior to trypanosome inoculation, a monolayer of either BTC or XTC cells was first grown in the flasks (or wells) for 2–5 days and then inoculated with trypanosomes. The inoculum for both culture flasks and wells was approximately 10^5 trypanosomes. Cultures were examined daily with an inverted microscope and passaged weekly into another well containing a fresh cell-line monolayer; at the same time, the medium of the previous well was partially replaced with fresh medium. After 16 culture passages in the cell-line systems, the F_4 was inoculated into NNN, Evans' modified Tobie's medium, EBLB and "Sloppy Evans" (Evans 1989).

Infection of phlebotomine sandflies

Phlebotomus duboscqi (Senegal strain) were obtained from colonies at Imperial College Field Station, Ascot (UK). Unfed female flies aged 5–7 days were infected via a hamster cheek-pouch membrane with a blood meal and a suspension of cultures of either *T. varani* V54 or *T. grayi*-like F_4 trypanosomes.

T. varani V54 cultures growing in Evans' modified Tobie's medium were washed in phosphate-buffered saline (PBS) by centrifugation (2500 rpm). The sediment was resuspended in defibrinated rabbit blood, previously heat-inactivated. The individual infecting dose per fly was 10^{3-4} organisms. Gentamycin (50 µg/ml) was added to the blood meal. The infected blood was fed to the flies at 37° C via the hamster cheek-pouch membrane feeding apparatus (Bastien 1990). The fed sandflies were dissected at intervals for up to 12 days post-infection. Initially, only live sandflies were examined, but because trypanosomes usually survive for some time in dead flies, the flies that had died within the previous 24 h were also examined.

The *T. grayi*-like F_4 trypanosomes were grown in L15 medium together with XTC as described above. The blood meal was pre-

pared as described for the *T. varani* V54 stock, with the infecting dose being about 10^{3-4} organisms per fly. Flies were dissected daily during the first 7 days after infection and, as before, flies that had died within the previous 24 h were included in the results.

Infection of tsetse flies

G. m. morsitans (colony FX9) were obtained from the colonies of the Tsetse Research Laboratory, Bristol. Teneral males were infected with cultured *T. varani* V54 suspended in washed horse blood via a silicone membrane at 37° C.

In two preliminary experiments, each fly was infected with approximately 10^6 organisms and dissected after 9 days (22 flies) and 21–28 days (150 flies). In a third experiment, flies were infected with about 10^4 organisms each and groups of 10 were dissected daily from day 1 until day 7 post-infection. In further experiments conducted at the Institute of Tropical Medicine, Antwerp (ITMA), *G. m. morsitans, G. p. gambiensis* and *G. tachinoides* from the ITMA colonies were similarly infected with cultured *T. grayi*-like F_4 and *T. varani* V54 and examined for trypanosomes 15 and 23 days later.

Haemocultures from crocodiles

During 1989 and 1990, blood was collected from 13 adult African crocodiles (*C. niloticus*) and examined for *T. grayi*. In all, 12 of the crocodiles were from Zambian crocodile farms (Newrere and the Luangwa Valley) and 1 was from Zaire (Mbandale, Mobeka, Haut Zaire). *G. pallidipes* and *G. morsitans* are putative local vectors of *T. grayi* in Zambia, as are *G. m. morsitans* and *G. m. centralis* in Zaire.

Immediately after the crocodiles had been shot, blood samples were taken either from the bullet wounds or from an incision made in the neck/head region. From each crocodile, 2–6 ml blood was inoculated into 2–5 vials containing Noguchi-Wenyon base with Locke's overlay, brain-heart infusion and antibiotics (penicillin, gentamycin and 5-fluorocytosine). Cultures were incubated at 20° C and/or 25° C and examined for trypanosomes for up to 8 weeks. In some cases, blind culture passages were also carried out.

Results and discussion

Growth in vitro: Trypanosoma varani V54 and the T. grayi-like F_4 compared

The *T. varani* V54 showed abundant growth in blood-agar-based media, with development of epimastigotes and trypomastigotes as described by Ranque (1973).

The *T. grayi*-like F_4 trypanosomes grew well in the cell-line systems; clumps of developing organisms were seen after 1–2 days and in about 2 weeks grew into a dense mass of aggregated and free-moving organisms, with densities of up to 10^9 organisms/ml being observed. The *T. grayi*-like F_4 could be subpassaged virtually indefinitely. After 16 passages, the stock was cryopreserved with 10% dimethylsulphoxide (DMSO) or 10% glycerol as the cryoprotectant. Both the recovery after cryopreservation and further growth were excellent. During the weekly partial medium replacements, high trypanosome density was maintained for up to 6 weeks; afterwards growth decreased, with an increase in cellular and trypanosome debris and further deterioration of the cell monolayer being noted. The stages and sizes of the developing trypanosomes were as reported by Minter-Goedbloed et al. (1983); epimastigotes were apparently the dominant stage. Long-term cultivation of the *T. grayi*-like F_4 trypanosomes in conventional culture media was less successful than that in the cell-line systems. The F_4 stock showed good initial growth in the blood-agar-based media (Evans 1989), but there was a gradual decline and, usually after 4–5 passages, the culture was lost.

The growth difference between *T. varani* V54 and *T. grayi*-like F_4 trypanosomes in blood-agar-based media appears to be a stock- rather than species-specific feature. Other *T. grayi*-like isolates are reported to grow well in these media (Dirie et al. 1991). Hoare (1929) also reported good growth of *T. grayi* in NNN, Noguchi-Wenyon and Nöller's media. The tissue-culture system in microtitre plates had the important practical advantage that repeated direct observations could be made without disturbing the culture system in any way.

Table 1. Infection of *Phlebotomus duboscqi* with *Trypanosoma varani* V54

Days after infection	Number infected/ number dissected	% Infected
1	4/ 4	100
2	1/ 2	50
3	5/ 5	100
5	14/20	70
6	1/ 1	100
7	7/ 9	78
9	12/16	75
12	2/13	15

Infection of phlebotomine sandflies and tsetse flies with T. varani V54 and T. grayi-like F_4 trypanosomes

Development of T. varani V54 in phlebotomine sandflies (P. dubosqi). Table 1 summarises the results of two experiments. In the first, 21 survivors from an original 30 *P. duboscqi* were dissected on days 5 and 12 post-infection (8 and 13 flies, respectively). Three of the 5-day-old flies and two of the 12-day-old flies were heavily infected with trypanosomes. In the second experiment, 49 flies were dissected. A total of 41 flies were infected with trypanosomes, including 31 that died during the observation period of 9 days. Blood-meal residues were mostly visible in the midgut up to day 7; in two flies dissected on day 9, small blood-meal residues were detected. Dense midgut trypanosome infections, with nests and rosettes, were recorded from day 3 onwards, indicating trypanosome multiplication in the flies. Heavy hindgut infections were also seen in nine flies from day 5 onwards. One infected fly dissected on day 12 had infections in the midgut and cardia with densely packed, long, slender trypanosomes. Most flies, both live and dead, were also infected with bacteria. A possible interaction between trypanosomes and bacteria was not studied.

Table 2. Infection of *P. duboscqi* with *T. grayi*-like F_4 trypanosomes

Days after infection	Number infected/ number dissected	% Infected
1	1/ 1	100
2	4/ 4	100
3	5/ 6	88
4	2/ 9	22
5	2/10	20
6	2/13	23
7	0/14	0

Table 3. Infection of *Glossina morsitans morsitans* with *T. varani* V54

Days after infection	Number infected/ number dissected	% Infected
1	10/ 10	100
2	9/ 10	90
3	10/ 10	100
4	3/ 10	30
5	1/ 10	10
6	0/ 10	0
7	0/ 10	0
9	0/ 22	0
21–28	0/150	0

Table 4. Infection of *G. m. morsitans, G. palpalis gambiensis* and *G. tachinoides* with *T. varani* V54 and *T. grayi*-like F_4 trypanosomes

Trypanosome stock	*Glossina* spp. (ITMA)	Number infected/ number dissected	% infected
T. varani V54	*G. m. morsitans*	0/ 8	0
	G. p. gambiensis	0/ 8	0
	G. tachinoides	0/ 3	0
T. grayi-like F_4	*G. m. morsitans*	2/ 9	22[a]
	G. p. gambiensis	2/ 6	33[b]
	G. tachinoides	0/12	0

[a] Days 15 and 23
[b] Day 15

Development of T. grayi-like F_4 trypanosomes in phlebotomine sandflies (P. duboscqi). In all, 2 groups of 32 flies were fed; in the second group, feeding was carried out in two stages and the same blood meal was re-used following 48 h storage at 4° C after the presence of active trypanosomes had been microscopically confirmed. A total of 57 flies were dissected, 8 of which had died during the previous 24 h; 16 of the 57 flies, all with a visible blood-meal residue, had scanty trypanosome infections in the midgut (Table 2). There was no indication of trypanosome multiplication, as observed with *T. varani* V54.

Since the bacterial infection of sandflies occurred in both the V54- and the F_4-fed flies as well as in unfed control sandflies, a comparison of the trypanosome infection rates was feasible. The heavy infections noted in the V54-fed sandflies showed that trypanosome multiplication certainly took place. Neither the location nor the various developmental stages of the trypanosomes nor their infectivity to reptiles was studied in detail.

Anderson and Ayala (1968) and Ayala (1971) have found posterior-station infection of sandflies with toad trypanosomes, but Ayala and McKay (1971) report anterior-station infection with lizard trypanosomes in California. Ashford et al. (1973) describe posterior-station infections in *Sergentomyia bedfordi* in Ethiopia with *T. boueti* of the African three-lined skink (*Mabuya striata*). We found mostly posterior-station (mid- and hindgut) development in *P. duboscqi*, but one fly showed an anterior-station infection with *T. varani.*

Transmission of the reptile trypanosomes is generally thought to take place by ingestion of flies (Anderson and Ayala 1968; Ayala 1971; Ayala and McKay 1971; Ashford et al. 1973).

Development of T. varani V54 and T. grayi-like F_4 trypanosomes in Glossina spp. None of the 172 Bristol colony flies dissected after 9 and 21–28 days was infected. In the subsequent experiment, scanty trypanosome infections were observed during the first 5 days post-infection but not later. Usually, trypanosomes were seen only in the midgut; sometimes a few were seen in the hindgut, but possible contamination from the midgut during dissection cannot be discounted. There was no evidence that infections ever established in the hindgut or elsewhere in the fly. Table 3 gives details of the persistence of *T. varani* V54 in Bristol *G. m. morsitans* during the 1st week after infection.

None of the Antwerp colony tsetse experimentally infected with *T. varani* V54 developed infections, but 4 of the 27 *Glossina* spp. infected with the *T. grayi*-like F_4 organisms developed infections; 2/9 (22%) *G. m. morsitans* examined at 15 and 23 days post-infection had midgut infections, and 2/6 (33%) *G. p. gambiensis* examined at 15 days had midgut and hindgut infections. None of the 12 *G. tachinoides* developed infections. Table 4 shows these results.

The ITMA results are in agreement with the Bristol series and show that *T. varani* V54 did not multiply in *Glossina,* whereas the *T. grayi*-like F_4 developed in two *Glossina* spp. This observation confirms the findings of Minter-Goedbloed et al. (1983) and McNamara and Snow (1991) that *T. grayi*-like trypanosomes from East and West Africa can produce mature stercorarian infections in tsetse. In contrast, in phlebotomine sandflies (*P. duboscqi*), *T. varani* V54 yielded posterior- and, in one case, anterior-station infections, but they died out in *Glossina.*

In summary, the experiments show that a marked difference was found between *T. varani* V54 and the *T. grayi*-like F_4 trypanosomes in their respective development in experimentally infected colony tsetse (*G. m. morsitans* and *G. p. gambiensis*) and phlebotomine sandflies (*P. duboscqi*). The *T. grayi*-like F_4 trypanosomes multiplied into mature hindgut infections in the tsetse but not in the sandflies; *T. varani* V54 produced posterior- and anterior-station infections in the sandflies but

died out in the tsetse. These findings support the conclusion that the *T. grayi*-like F_4 trypanosome and *T. varani* V54 are different species and are also supported by the interim results of isoenzyme and DNA characterisation studies (McNamara et al., unpublished data).

Crocodile haemocultures

The cultures from 4 of the 13 crocodiles (3 from Zambia and 1 from Zaire) were contaminated with bacteria on receipt and were discarded; the cultures of the remaining 9 crocodiles were negative throughout, suggesting that these animals were not infected with *T. grayi*. Although Hoare (1929) found that parasitaemias in crocodiles infected with *T. grayi* were extremely low (approx 200 organisms/ml), the quantity of blood inoculated into culture was considered adequate.

As a check on the suitability of the isolation media, *T. varani* V54 and fish trypanosomes were inoculated into the same culture media used for the crocodile haemocultures; in all cases growth was excellent. Trypanosome hindgut-infection rates in the putative local *Glossina* vectors of *T. grayi* were not investigated. Similarly, in an area of Gambia with *T. grayi*-like infections in the tsetse, two *Varanus niloticus* were found to be negative by microscopy and haemoculture (McNamara, unpublished data).

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