

SHORT REPORT

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Natural infection of the sand fly *Phlebotomus kazeruni* by *Trypanosoma* species in Pakistan

Hiroto Kato^{1*}, Hiroshi Uezato², Hiroshi Sato³, Abdul M Bhutto⁴, Farooq R Soomro⁵, Javed H Baloch⁵, Hiroyuki Iwata¹, Yoshihisa Hashiguchi⁶

Abstract

The natural infection of phlebotomine sand flies by *Leishmania* parasites was surveyed in a desert area of Pakistan where cutaneous leishmaniasis is endemic. Out of 220 female sand flies dissected, one sand fly, *Phlebotomus kazeruni*, was positive for flagellates in the hindgut. Analyses of cytochrome *b* (*cyt b*), glycosomal glyceraldehyde phosphate dehydrogenase (*gGAPDH*) and small subunit ribosomal RNA (SSU rRNA) gene sequences identified the parasite as a *Trypanosoma* species of probably a reptile or amphibian. This is the first report of phlebotomine sand flies naturally infected with a *Trypanosoma* species in Pakistan. The possible infection of sand flies with *Trypanosoma* species should be taken into consideration in epidemiological studies of vector species in areas where leishmaniasis is endemic.

Findings

Phlebotomine sand flies are blood-sucking insects belonging to the family Psychodidae in the order Diptera [1]. The identification of sand fly species is epidemiologically very important because less than 10 percent of over 800 species described are responsible for the transmission of human pathogens such as flagellate protozoa of the genus *Leishmania* [1-3]. Some sand fly species are reported to transmit non-pathogenic flagellates of *Endotrypanum* species, originally identified as intraerythrocytic parasites of sloths in the New World [4]. In addition, some *Trypanosoma* species of mammals, lizards, snakes and toads are transmitted by phlebotomine sand flies [5-11]. Since the flagellated forms of these parasites in the insect gut are morphologically similar to those of *Leishmania*, careful differentiation is needed for the epidemiological study of the vectors responsible for circulating *Leishmania* species.

In this study, the natural infection of sand flies by *Leishmania* was surveyed in a desert area of Pakistan where cutaneous leishmaniasis is endemic. Sand flies were captured at Sono Khan (26° 52'N, 68° 03'E), Sindh Province on the 28-29th of June 2004. Shannon traps with a single compartment (1.0 × 1.0 × 1.3 meters in

width, length and height, respectively) were used for the collection. The captured sand flies were dissected and the species identified based on the morphology of their spermathecae. These flies were also examined for *Leishmania* in the gut microscopically at × 400 magnification. The flagellates detected were inoculated into Difco blood agar (USMARU) biphasic medium containing 20% defibrinated rabbit blood, and later co-cultured with *Spodoptera frugiperda* Sf9 insect cells in Grace's medium supplemented with 10% fetal calf serum. The rest of the flagellates in the gut of the sand fly were fixed in absolute ethanol for molecular biological analyses. Genomic DNA was extracted from the ethanol-fixed specimen, and the cytochrome *b* (*cyt b*) gene was amplified from the flagellate with primers prepared for the leishmanial *cyt b* gene (L.cyt-S: GGTGTAGGTTTATAGTYTAGG and L.cyt-R: CTACAATAAACAAATCATAATATRCAATT) [12]. The glycosomal glyceraldehyde phosphate dehydrogenase (*gGAPDH*) and small subunit ribosomal RNA (SSU rRNA) genes were also amplified from the parasite using *gGAPDH* (G3: TTYGCCGYATYGGYCGCATGG and G5: ACMAGRTCCACCACRCGGTG) and SSU rRNA-specific primers (TRY927F: GAAACAAGAAACACGGGAG and TRY927R: CTACTGGGCAGCTTGGA) designed for trypanosomatids [13,14]. The PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI), and the sequences of the inserts of the plasmids were determined by the dideoxy chain

* Correspondence: kato@yamaguchi-u.ac.jp

¹Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

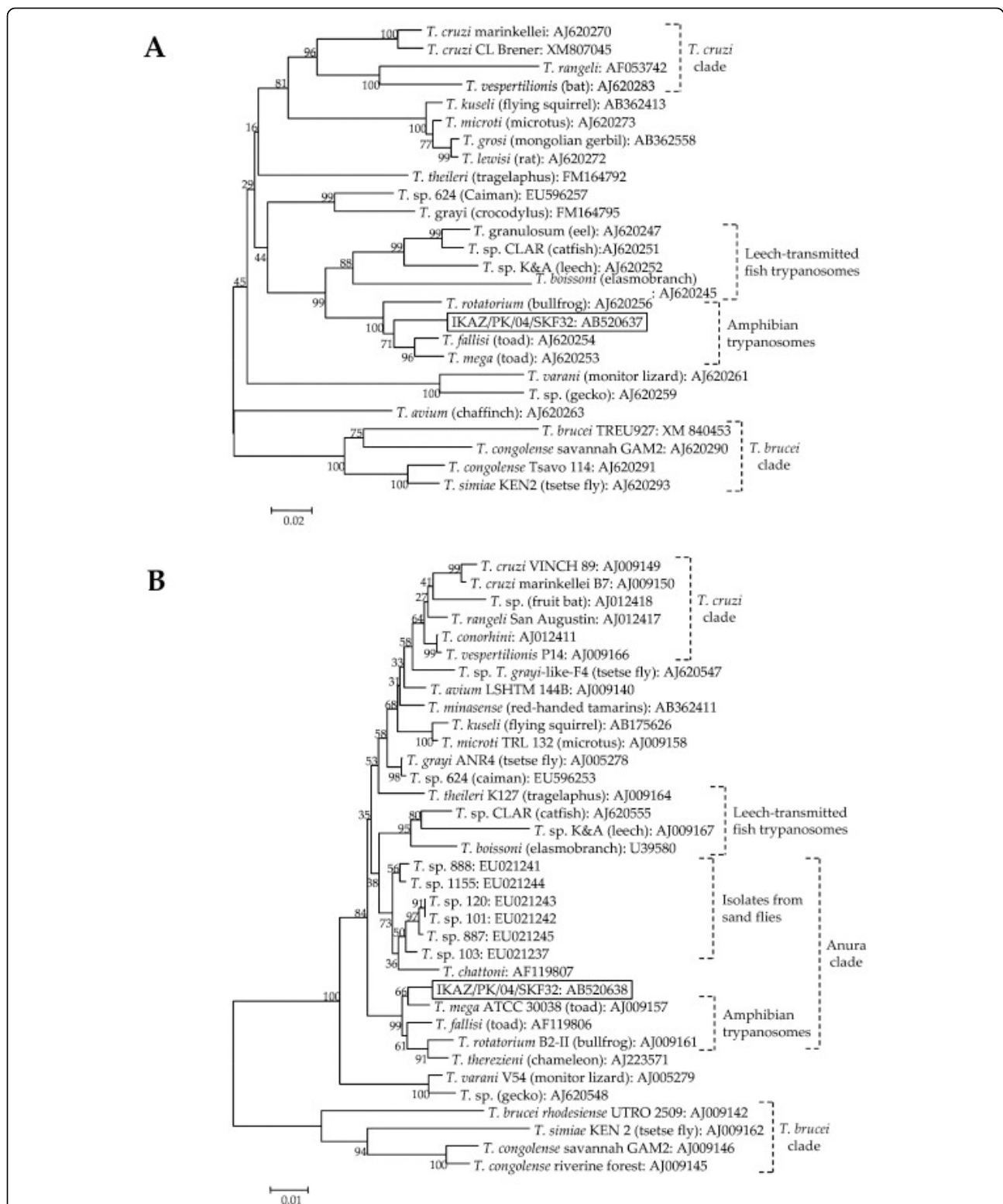


Figure 1 Phylogenetic tree of *gGAPDH* (A) and *SSU rRNA* (B) gene sequences among species. The *gGAPDH* and *SSU rRNA* genes were amplified from isolates of flagellates from *P. kazeruni* (IKAZ/PK/04/SKF32), and the sequences were determined. Phylogenetic analyses of *gGAPDH* and *SSU rRNA* gene sequences were performed by the neighbor-joining method together with those from 25 and 34 *Trypanosoma* species, respectively. The sequences from the database are represented by "the name of the species and isolate (host of the isolate): GenBank accession number". The scale bar represents 0.02 and 0.01% divergence, respectively. Bootstrap values are shown above or below branches.

termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The *gGAPDH* and SSU rRNA gene sequences were aligned with CLUSTAL W software [15] and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 [16]. Phylogenetic analyses were performed by the neighbor-joining (NJ) and maximum parsimony (MP) methods with the distance algorithms available in the MEGA package [16].

In this survey, 220 female sand flies were dissected for identification at the species level, and four species of the genus *Phlebotomus* were recognized. Among them, the two most prevalent species were identified as *Phlebotomus (P.) sergenti* (39 flies) and *P. papatasi* (27 flies). Other *Phlebotomus* species were *P. nuri* (10 flies) and *P. kazeruni* (1 fly), and the rest (143 flies) were identified as *Sergentomyia* species. The natural infection of sand flies by flagellates was detected in the hindgut of *P. kazeruni*. The *cyt b* gene sequence of the parasite was determined and analyzed with the BLASTn program. Unexpectedly, the sequence showed only 80.6-82.8% homology with those of *Leishmania* species and 83.6% homology with that of *Trypanosoma (T.) brucei brucei*, suggesting the flagellate belonged to the genus *Trypanosoma*. For further characterization, the *gGAPDH* and SSU rRNA genes were amplified from the flagellate and the sequences were analyzed since these genes have been well studied in trypanosomatids [13,14]. The *gGAPDH* and SSU rRNA genes from the parasite (DDBJ accession numbers: AB520637 and AB520638, respectively) had more than 90% sequence similarity with those of amphibian trypanosomes such as *T. fallisi*, *T. mega* and *T. rotatorium* isolated from toad and frog. Phylogenetic analyses of the *gGAPDH* and SSU rRNA genes showed that the flagellate within *P. kazeruni* belongs to a clade of amphibian trypanosomes that includes a chameleon trypanosome, *T. therezieni*, but the sequences did not completely match those from any reported species (Fig. 1A and 1B). In addition, the parasite from *P. kazeruni* located in a separate clade from *Trypanosoma* species isolated from Amazonian sand flies, which have closer relationships with anuran trypanosomes captured in Amazonia [10] (Fig. 1B). These results strongly suggested that the parasite is a novel or genetically uncharacterized *Trypanosoma* species of an amphibian or possibly reptile. The parasite was successfully isolated as a culture (code number: IKAZ/PK/04/SKF32), which was confirmed to correspond to the original by the SSU rRNA gene analysis. The parasites markedly grew in Grace's medium in a co-culture with *Sf9* insect cells. On glass-slide smears prepared from the liquid culture, rosettes of parasites with an oval shape and epimastigotes varying in shape and length were observed (Fig. 2). The parasites showed the features of

Trypanosoma species, demonstrating characteristics such as a flagellum, a kinetoplast and an undulating membrane (Fig. 2). The research area was a desert community, and no amphibian was observed during the field trip. However, numbers of geckoes were found on the walls of houses, including sites where the Shannon traps were set. Thus, two Japanese reptile species (*Takydromus tachydromoides* and *Gekko japonicus*) and one Japanese amphibian (*Hyla japonica*) were intraperitoneally injected with 5×10^6 parasites as a preliminary search for the vertebrate host of the parasite; however, no parasites were observed in peripheral blood 3 weeks after the inoculation. The possibility of mammalian hosts was also addressed since the frog trypanosome, *T. rotatorium*, has been reported to infect mice under experimental conditions [17]. The flagellate was inoculated intracutaneously into BALB/c mice and Mongolian gerbils (*Meriones unguiculatus*), but its DNA was not detected in peripheral blood and lymphoid tissues by PCR after 6 weeks. Based on the findings obtained by phylogenetic analyses and experimental infection, the parasite is presumably infectious to certain species of reptiles or amphibians in Pakistan. Further study is needed to identify the vertebrate host of the parasite species. The procedures were performed according to the recommendations of the ethics committee for animal experimentation, Faculty of Agriculture, Yamaguchi University.

In the present study, a *Trypanosoma* species was isolated from *P. kazeruni*, which is a xerophilic species distributing broadly throughout North Africa and West Asia with little-known behavior [18-20], and the parasite was genetically characterized. To date, trypanosomes in

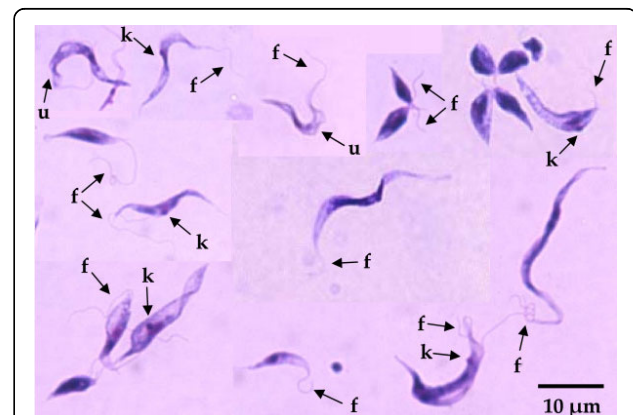


Figure 2 *Trypanosoma* species isolated from a phlebotomine sand fly *P. kazeruni*. Glass-slide smears were prepared from the culture in Grace's medium co-cultivated with *Sf9* insect cells and stained with Giemsa staining. Rosettes of parasites with an oval shape and epimastigotes varying in morphology were observed. k, kinetoplast; f, flagellum; u, undulating membrane.

sand flies have been reported on the African and American continents [5-11], but this is the first record of phlebotomine sand flies naturally infected by a *Trypanosoma* species in Asia. Pakistan is a tropical and subtropical country located in the northwest of South Asia and has areas where leishmaniasis is highly endemic [21,22]. Although information on the endemicity and spread of cutaneous leishmaniasis is accumulating [23-26], the sand fly species responsible for the transmission is poorly understood. To date, no natural infections of sand flies by flagellates have been reported in Pakistan, and further efforts are ongoing on this matter. Thus, careful identification of the parasites is required for the epidemiological study of sand fly species responsible for the transmission of *Leishmania* protozoa.

Acknowledgements

We are indebted to Dr. Juma K. Kakarsulemankhel (Sandflies, Leishmaniasis & Mosquitoes Laboratory, Department of Zoology, Faculty of Science, University of Balochistan, Quetta, Balochistan, Pakistan), Hamed A. Chandio and Nuzhat Seema Bhatti (The Leprosy Unit, Chandka Medical College Hospital and the Executive District Office-Health, Larkana, Sindh, Pakistan) for their support during the field phase of the present study. This study was financially supported by the Ministry of Education, Culture and Sports, Science and Technology (MEXT) of Japan (Grant Nos. 14256004, 18256004 and 18780230)

Author details

¹Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan. ²Department of Dermatology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan. ³Department of Veterinary Parasitology, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan. ⁴Department of Dermatology, Chandka Medical College, Doctors Colony, Bunglow No 14, VIP Road, Larkana, Sindh, Pakistan. ⁵Incharge Leprosy Centre, Larkana, Sindh, Pakistan. ⁶Department of Parasitology, Kochi Medical School, Kochi University, Oko-cho Kohasu, Nankoku-shi, Kochi 783-8505, Japan.

Authors' contributions

HK was fully involved in all phases of the study, including field research, sample collection, laboratory work, and writing the manuscript. HU and HS were involved in the molecular works. AMB, FRS and JHB organized and conducted field research. HU and YH conducted field research and sample collection. HK, HI and YH drafted the manuscript. YH supervised the overall conduct of the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 8 January 2010 Accepted: 25 February 2010

Published: 25 February 2010

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doi:10.1186/1756-3305-3-10

Cite this article as: Kato *et al.*: Natural infection of the sand fly *Phlebotomus kazeruni* by *Trypanosoma* species in Pakistan. *Parasites & Vectors* 2010 **3**:10.

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