A trypanosome species isolated from naturally infected *Haemaphysalis hystricis* ticks in Kagoshima Prefecture, Japan

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SUMMARY

Common arthropod vectors for trypanosomes are flies, fleas and bugs. This study reports on an unknown trypanosome species isolated from naturally infected *Haemaphysalis hystricis* ticks, hereby, referred to as *Trypanosoma* KG1 isolate. The parasite has been successfully cultured *in vitro* with L929 or HEK 293T cell line as feeder cells. This trypanosome cannot survive *in vitro* without feeder cells. Following experimental infections of ticks, the trypomastigote-like and the epimastigote-like forms of this trypanosome could be detected by Giemsa-stained smears of the midgut and salivary glands of *Ornithodoros moubata* ticks which were made to feed on a culturing medium containing *Trypanosoma* KG1 isolate through an artificial membrane. *Trypanosoma* KG1 isolate could also be detected from Giemsa-stained smears of the haemolymph up to 30 days post-inoculation into the *O. moubata* haemocoel. *Trypanosoma* KG1 isolate cannot be propagated in laboratory animals including mice, rats, rabbits and sheep. A phylogenetic tree constructed with the 18S rRNA gene indicates that *Trypanosoma* KG1 is a member of the stercorarian trypanosomes.

Key words: *Trypanosoma* KG1 isolate, *Haemaphysalis hystricis*, *Ornithodoros moubata*.

INTRODUCTION

The genus *Trypanosoma* comprises unicellular flagellates that are parasites of all vertebrate classes. The vectors can be haematophagous arthropods for mammalian, avian, reptilian as well as amphibian trypanosomes, whereas fish, certain amphibian and reptilian trypanosomes are transmitted by leeches (Haag *et al*. 1998). Salivarian trypanosomes are known to be transmitted by biting flies of the genus *Glossina* commonly called tsetse flies (Donelson, 2003; Ravel *et al*. 2003; Stevens and Brisse, 2004) with the exception of *Trypanosoma evansi* and *T. equiperdum* which are transmitted mechanically by the *Stomoxys* and *Tabanid* flies and by copulation, respectively (Brun *et al*. 1998; Gibson, 2003). Furthermore, the stercorarian trypanosomes such as *T. cruzi*, *T. lewisi* and *T. rangeli* are transmitted by bugs and fleas through contaminated faeces (Stevens and Brisse, 2004).

The current study reports on the unknown trypanosome species that has been isolated from naturally infected *Haemaphysalis hystricis* ticks isolated in Kagoshima Prefecture, Japan. The *H. hystricis* tick is a vector of *Ehrlichia* and *Rickettsia* sp. that cause spotted fever group (SFG) ehrlichiosis and rickettsiosis, respectively (Mahara, 1997; Parola *et al*. 2003). We refer to this unknown trypanosome as *Trypanosoma* KG1 isolate. With the aim of identifying the taxonomic status of this unknown trypanosome species, a series of experiments was conducted including the establishment of an *in vitro* culture system for *Trypanosoma* KG1 isolate (KG1), an attempt to determine the laboratory mammalian host, experimental infection of other tick species and the development of PCR and loop-mediated isothermal amplification (LAMP) for specific detection of this trypanosome. Furthermore, in this study we cloned and sequenced the internal transcribed spacer 1 (ITS1), 18S rRNA, large subunit 28S rRNA and kinetoplast DNA (kDNA) genes of *Trypanosoma* KG1 isolate.

MATERIALS AND METHODS

Isolation of parasites from ticks

Three male and 3 female adult *Haemaphysalis hystricis* ticks were collected from vegetation at Uchinoura-cho on the Osumi Peninsula in

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Kagoshima prefecture, situated on the Southernmost part of Kyusyu Island, Japan. The average temperature, relative humidity and rainfall in Kagoshima prefecture are 15–23 °C, 60–75 RH, and 2200–2900 mm, respectively. Vegetation is a typical subtropical forest, in which *Cinnamomum camphora* and *Cycas revoluta* plantations are dominant. Midguts of the ticks were removed aseptically and suspended as pooled samples in the sucrose-potassium-glutamate medium. A suspension of the pooled midgut contents was then inoculated into the monolayer of L929 cells cultivated with Eagle's MEM (Nissui Pharmaceutical Co. Ltd, Tokyo) supplemented with 2% fetal bovine serum (FBS) and incubated at 33 °C (Honda *et al*. 2006). However, in the current study the parasites were propagated at 37 °C with the HEK 293T cell line as feeder cells. The KG1 cultures were maintained in Medium 199 (Sigma® Aldrich, U.K.) supplemented with 2% FBS and Penicillin-Streptomycin (100 U-100 μg/ml) by replacing the culture medium every second day.

Tick infection

A volume of 12 μl of KG1 culture supernatant (containing approximately 10^3 parasites) was experimentally injected into the haemocoel of 12 *Ornithodoros moubata* ticks. Another set of ticks was made to feed on the culture supernatant containing 1×10^6 parasites/ml through an artificial membrane as described previously (Waladde *et al*. 1996). However, in this study we used laboratory film (Parafilm® M, Pechiney Plastic Packaging, Chigago, IL) as an artificial feeding membrane. The presence of the parasites was examined by Giemsa-stained smears made from the haemolymph, salivary glands, and midgut every 5 days for a period of 30 days. All ticks were kept at 25 °C and 50–60% relative humidity in continuous darkness throughout the experiment.

Infection of laboratory animals and parasite detection

Five female ICR mice, BALB/C mice, and SD rats aged 6 weeks were inoculated intraperitoneally with 1 ml of culture supernatant containing approximately 1×10^6 *Trypanosoma* KG1 parasites, and corresponding numbers of each animal were kept as uninfected control groups. One Japanese white rabbit was also inoculated with 1×10^6 parasites, and another was kept as control. Furthermore, a splenectomized rat and sheep were also inoculated with 1×10^6 and 2×10^6 *Trypanosoma* KG1, respectively. Corresponding numbers of each animal species were kept as uninfected controls. All the controls of the above-mentioned mammalian species were inoculated with 1 ml of the new culture medium (Medium 199) (Sigma® Aldrich, UK). The inoculated animals were bled every 3 days for a period of 30 days and parasitological examinations (microscopy of wet smears, Giemsa-stained thin blood smears, and buffy coat preparations), PCR and LAMP were performed in order to detect the parasite from blood samples. All animals were euthanized 30 days post-infection. In order to clarify infectivity of the KG1 parasite in the animal species tested, total DNA of spleen, heart, liver, kidneys, and lymph nodes was examined by PCR and LAMP.

DNA extraction

Total DNA of the KG1 isolate, blood, and visceral organs was extracted as previously described (Sambrook and Russell, 2001). Briefly, the extraction buffer (10 mM Tris-HCl, pH 8·0, 10 mM EDTA, 1% sodium dodecyl sulphate) and 100 μg/ml proteinase K were added to the samples and incubated overnight at 55 °C. DNA was extracted with phenol-chloroform isoamyl alcohol (25 : 24 : 1) and precipitated with isopropanol. DNA was dissolved in 250 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8·0) buffer.

PCR

In the initial experiments, several primers of commonly known trypanosomes, including the *Trypanosoma* (*Trypanozoon*) group, *T. evansi*, *T. rangeli*, Kinetoplastida (Desquesnes and Davila, 2002), and the *T. lewisi* primers (Desquesnes *et al*. 2002), were used in our attempts to amplify DNA of *Trypanosoma* KG1 isolate. We also used primers for eukaryotic 18S rRNA (Countway *et al*. 2005) and primers designed from the 18S rRNA of *Trypanosoma pestanai* (Accession no: AJ009159) for amplification of KG1 DNA and 28S rRNA LSU (Accession no: X14553) of *T. brucei* group (Table 1 and Fig. 1). The PCR reaction mixture contained 10 × PCR buffer (100 mM Tris-HCl, pH 8·3, 500 mM KCl, 15 mM $MgCl_2$, 0·01% (w/v) gelatin), 2 mM of each dNTP, 5 pmol of each primer, and 0·5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Japan) in a final volume of 50 μl. The reaction mixture was heated at 94 °C for 10 min and subjected to 35 cycles at 94 °C for 45 sec, 1 min at 55 °C, and 1 min at 72 °C, with a final extension at 72 °C for 7 min.

LAMP

The following LAMP primer set targeting ITS-1 gene of the *Trypanosoma* KG1 isolate was designed using the Primer Explorer V2 software program (Fujitsu, Japan): FIP: 5′-GAT TCC AGC TGC AGG TTC ACC AAT AGT AGG GAA GCA AAG TC-3′, BIP: 5′-GCA TGT ATG TAT GTG TAG TAT GCG TTA GAA GCT GTT GCT TCA TAC C-3′, F3: 5′-ACC GAA AGT TCA CCG

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Table 1. PCR primers used for amplification of *Trypanosoma* KG1 genomic DNA

Primer name	Sequence	Target gene	Specificity	Primer position* 5′	Primer position* 3′
EUK-A and B	A: 5′-AACCTGGTTGATCCTGCCAGT-3′	18S rRNA	Eukaryote cell	ND**	ND
	B: 5′-GATCCTTCTGCAGGTTCACCTAC-3′			ND	ND
TPEF1 and B1	F1: 5′-CCATGCATGCCTCAGAATCACTGC-3′	18S rRNA	*T. pestanai*	ND	ND
	B1: 5′-GGCACTGCCGGCTCTATTTC-3′			345	364
TPEF2 and B2	F2: 5′-GCAGCGAAAAGAAATAGAGCCGG-3′	18S rRNA	*T. pestanai*	335	357
	B2: 5′-GTTCGTCCTGGTGCGGTCTAAG-3′			1,073	1,094
LSU1 and 2	LSU1: 5′-TGGAAATGCGAAACACTTGC-3′	28S rRNA	*T. brucei* group	ND	ND
	LSU2: 5′-ACACCCCAGGTTTTTGCTT-3′			ND	ND
KIN1 and 2	KIN1: 5′-GCGTTCAAAGATTGGGCAAT-3′	kDNA	Kinteplastida	ND	ND
	KIN2: 5′-CGCCCGAAAGTTCACC-3′			ND	ND
TRYP IS and IR	TRYP-IS: 5′-CGTCCCTGCCATTTGTACACA-3′	ITS1-5.8S	*T. lewisi*	ND	ND
	TRYP-IR: 5′-CGATGGATGACTTGGCTTCC-3′			ND	ND

* Primer position on the nucleotide sequence of *Trypanosoma* KG1 18S rRNA (Accession no.: AB281091).
** ND, Not determined.

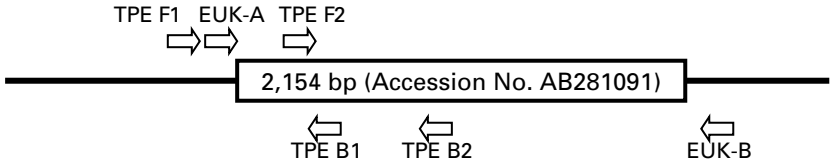

Fig. 1. Schematic diagram of primer positions for 18S rRNA. The entire sequence of 18S rRNA of *Trypanosoma* KG1 is indicated by a solid line. The sequence used for phylogenetic tree construction is shown by an open box with its length and Accession number. Each primer location is indicated by open arrow and primer name.

TAT T-3′, and B3: 5′-TTG TGT GCG AAG AGA ACA-3′. The reaction was carried out as previously reported (Notomi *et al.* 2000), briefly, in a final volume of 25 μl reaction mixture containing 12·5 μl of ×2 LAMP reaction buffer (40 mM Tris-HCl, pH 8·8, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0·2% Tween 20, 1·6 M Betaine, 2·8 mM of each dNTPs), 2 μl template DNA and 1 μl (8 units) *Bst* DNA polymerase (New England BioLabs Inc., USA), 0·9 μl of primer mix (FIP and BIP at 40 pmol each, and F3 and B3 at 5 pmol each), and 8·6 μl of distilled water. The reaction mixture was incubated at 63 °C for 1 h followed by 80 °C for 2 min for termination of the reaction. The F3 and B3 LAMP primers were also used for PCR with the same PCR conditions as mentioned above except for annealing temperature that was modified to 57 °C. Both the PCR and LAMP products were electrophoresed in a 1·5% Tris-acetic acid-EDTA (TAE) agarose gel and stained with ethidium bromide for visualization.

Gene cloning, sequencing, and analysis

The PCR products were purified using the QIAquick gel extraction Kit (Qiagen, USA) and cloned to pT7Blue-T vector (Novagen Inc., Germany) with DNA ligation kit Ver 2.1 (Takara Bio Inc., Japan). The plasmid was cut with *Bam* HI and *Xba* I restriction enzymes, and the nucleic acid sequence was determined with the BigDye terminator cycle sequencing kit (Applied Biosystems, Japan). In order to analyse similarities between the nucleic acid sequences from KG1 and the known sequences of other trypanosomes, the sequences of KG1 were subjected to NCBI BLASTn search (www.ncbi.nlm.nih.gov/BLAST). Furthermore the KG1 18S rRNA sequence was aligned with other selected stercorarian trypanosome genes using ClustalW program, and a phylogenetic tree (see Supplementary data, Figs S1 and S2 in Online version) was constructed by the bootstrapped maximum likelihood method using the PHYLIP 3.6 package program downloaded from the University of Washington website (http://evolution.gs.washington.edu/phylip.html). The Seqboot and Consense programs were used to statistically assess the strength of the tree using bootstrap resampling. The model used for the maximum likelihood classification is one that allows for inequalities of equilibrium base composition and for inequalities of the rate of transitions and transversions. It is related to the model reported by Felsenstein (1981) but generalizes it to allow for unequal rates of transitions and transversions (Felsenstein and Churchill, 1996).

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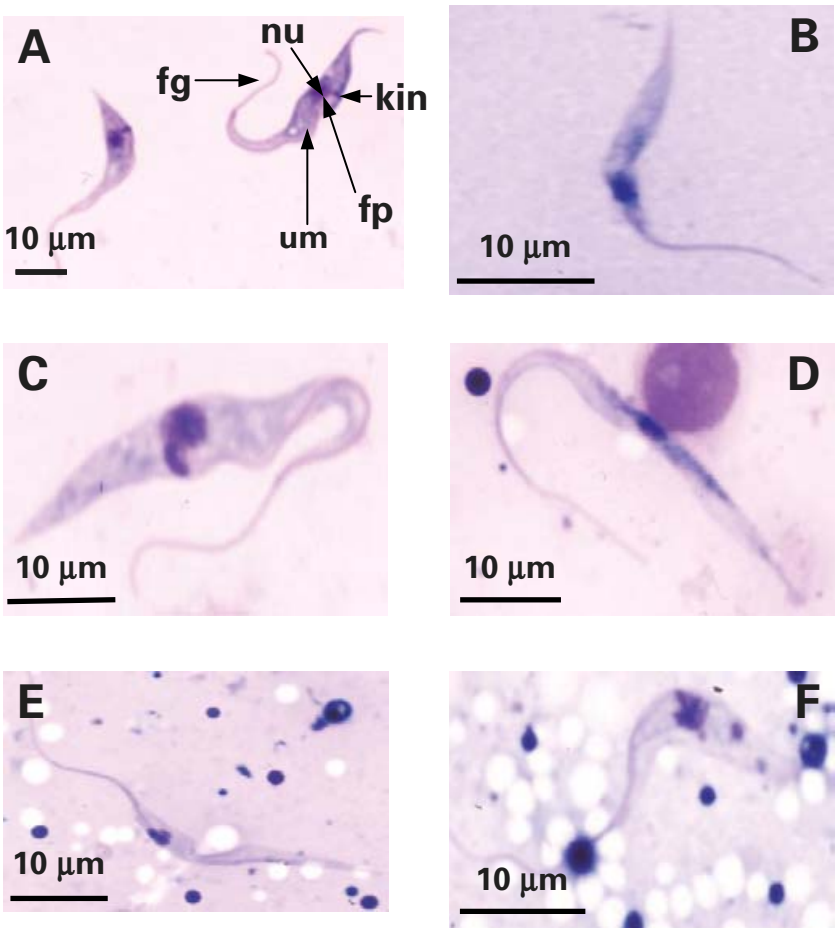

Fig. 2. Giemsa-stained slides of different forms of *Trypanosoma* KG1 from *in vitro* culture and within different parts of an *Ornithodorus moubata* tick. (A) Trypomastigote-like form in the *in vitro* culture with nucleus (nu), kinetoplast (kin), flagellar pocket (fp), undulating membrane (um), and the flagellum (fg). (B) Trypomastigote-like form in the haemocoel. (C) Trypomastigote-like form in the midgut. (D) Epimastigote-like form in the midgut. (E) Trypomastigote-like form in the salivary glands. (F) Epimastigote-like form in the salivary glands.

RESULTS

Identification of the taxonomic status of the Trypanosoma *KG1 isolate*

Giemsa-stained smears of KG1 from *in vitro* cultures clearly showed common morphological features of trypanosomes such as nucleus, kinetoplast, undulating membrane, flagella pocket and the flagellum (Fig. 2A). The trypomastigote-like form of KG1 is 15–30 μm in length and has a width of 2–5 μm. The free flagellum length varied amongst cells, with a minimum of 5 μm and a maximum of 20 μm. Out of the several primers of commonly known trypanosomes that were used in an attempt to amplify the unknown trypanosome DNA, only the *T. lewisi* primers targeting the ITS1-5.8S, and the universal primers for the 18S rRNA of eukarytic cells, 28S rRNA and the kDNA genes, with product size of approximately 600 bp, 2000 bp, 200 bp and 500 bp, respectively, amplified *Trypanosoma* KG1 isolate DNA (Fig. 3). These PCR products were successfully cloned and sequenced. However, for the 18S rRNA genes we only obtained a nucleotide sequence which was about 1000 bp in length due to the quality

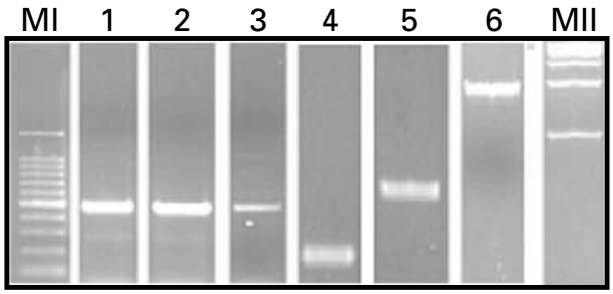

Fig. 3. Amplification of *Trypanosoma* KG1 genomic DNA by PCR with different primers targeting different genes. Lane MI, 100 bp Marker; Lane 1, kDNA; Lane 2, TPEF1B1 for 18S rRNA; Lane 3, TPEF2B2 for 18S rRNA; Lane 4, LSU 28S rRNA; Lane 5, ITS1-5.8S; Lane 6, EUK-AB for 18S rRNA; and Lane MII, 1 kb Marker.

of the sequencing reaction. Hence, in order to obtain additional sequences for more reliable phylogenetic analysis based on the 18S rRNA gene sequence which is at least 2000 bp, we further used two sets of primers designed from the 18S rRNA of *T. pestanai* (Table 1 and Fig. 1) with PCR products of 513 bp

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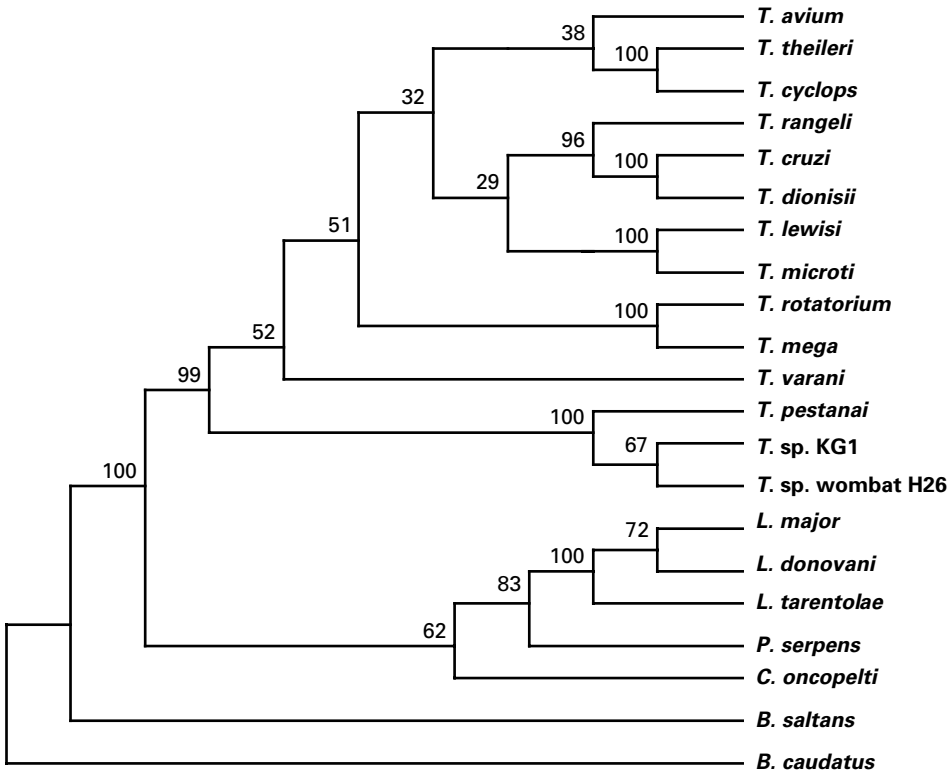

Fig. 4. Phylogenetic tree based on bootstrapped maximum likelihood analysis of the 18S rRNA gene sequences performed using PHYLIP 3.6 program. The tree includes 21 taxa, values at the nodes are bootstrap values (%: 100 replicates), and the length of the 18S rRNA gene sequence of KG1 for alignment is 2154 bp. The Accession no. of *T.* sp. wombat H26 sequence is AJ009169. The sequences of other trypanosomes, *Leishmania* parasites, bodonids, *Phytomonas serpens* and *Crithidia oncopelti* were obtained from the GenBank database according to Accession numbers published by Hughes and Piontkivska (2003*a*).

and 681 bp. The locations of EUK-A and B, TPE F1 and B1, and TPE F2 and B2 are schematically indicated in Fig. 1. As a result, we were able to construct a 2154 bp sequence of 18S rRNA from *Trypanosoma* KG1. The partial gene sequences were submitted to the GenBank as ITS1 (AB259643); 18S rRNA (AB281091); kDNA (AB259645) and 28S rRNA (AB259646). Accordingly, the nucleic acid sequences of the above-mentioned genes mainly showed close homology to a group of non-pathogenic trypanosomes as well as some *Bodo*, *Leishmania* and the *Crithidia* species by BLASTn search (data not shown). Similarly, the phylogenetic tree constructed with the partial 18S rRNA gene of *Trypanosoma* KG1 which is a conserved region with a length of 2154 bp, *Trypanosoma* KG1 is clustered together with *Trypanosoma* sp. wombat H26 and *T. pestanai* in the bootstrap majority-rule consensus unrooted tree obtained from 100 maximum likelihood replicates (Fig. 4). *Bodo caudatus* was used as an outgroup parasite.

In vitro *and* in vivo *propagation of* Trypanosoma *KG1 isolate*

Ticks were collected in the field with the aim of determining *Ehrlichia* infections hence, initially tick extracts were cultured with L929. However, actively motile trypanosome-like flagellates were observed in the cultures. In the current study, *Trypanosoma* KG1 isolate actively propagated in the presence of HEK 293T cell as a feeder cell layer. We could not find HEK 293T cells infected with the parasite throughout the culture period. Giemsa-stained smears of the haemolymph showed the presence of the *Trypanosoma KG1* isolate in the *O. moubata* ticks for up to 30 days post-injection (time at which the experiment was terminated) into the haemocoel (Fig. 2B). In another set of *O. moubata* ticks that were made to feed on a medium containing KG1 parasites through an artificial membrane, the trypanosomes were detected in the tick midgut and salivary glands for up to 30 days post-infection. Two different life-cycle forms (trypomastigote-like and epimastigote-like) of this trypanosome were observed in both midgut and salivary glands of the tick, whereas in the haemolymph only the epimastigote-like form was visible (Fig. 2C–F). No parasites were observed from haemolymph of ticks fed through an artificial membrane. Following attempts to infect mice, rats, rabbits and sheep with the *Trypanosoma* KG1 isolate, the parasite could not be detected by microscopical examination as well as by specific gene amplification tests (data not shown).

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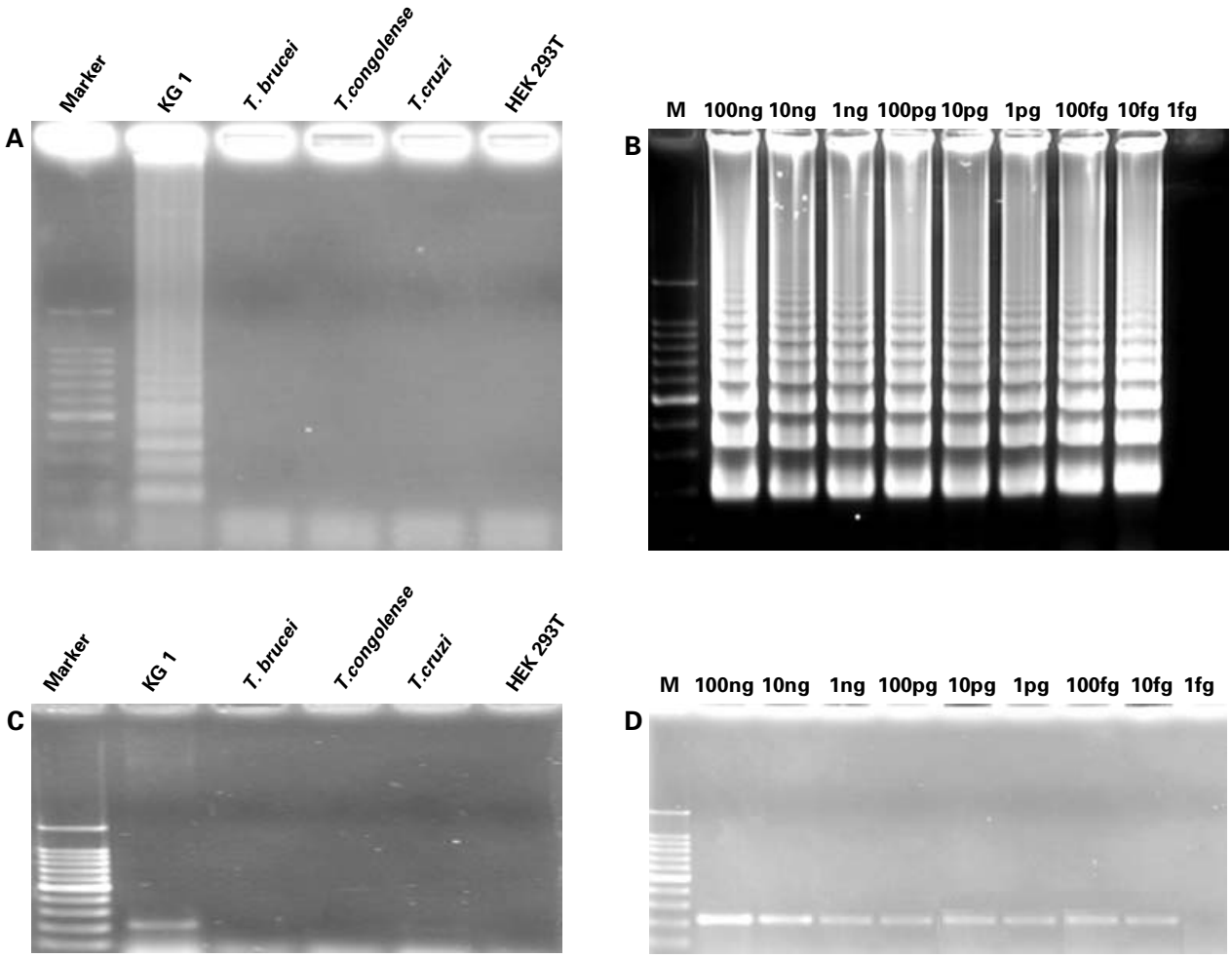

Fig. 5. LAMP and PCR for detection of ITS1 gene of *Trypanosoma* KG1 species. (A) Specificity of LAMP. (B) Sensitivity of LAMP. (C) Specificity of PCR. (D) Sensitivity of PCR. M and Marker indicate 100 bp ladder DNA size marker up to 1000 bp and additional band of 1500 bp.

Development of LAMP and PCR for specific detection of Trypanosoma *KG1 isolate*

LAMP detection method targeting ITS1 gene of *Trypanosoma* KG1 isolate has proved to be specific (Fig. 5A) and sensitive, whereby this method detected the genomic DNA up to 10 fg (Fig. 5B). The LAMP primers F3 and B3 have been used for PCR and were also specific (Fig. 5C), with the same sensitivity as LAMP (Fig. 5D).

DISCUSSION

In this study, we report on the unknown trypanosome species that has been isolated in naturally infected *H. hystricis* ticks. The clear visibility of common morphological features such as the kinetoplast, nucleus, the flagellar pocket, undulating membrane and the flagellum in the Giemsa-stained smears strongly suggests that the organism belongs to the genus *Trypanosoma*. The nucleic acid sequences of the cloned PCR products of the ITS1, 18S rRNA, 28S rRNA and kDNA genes of this unknown trypanosome isolate indicated close homology to groups of non-pathogenic trypanosomes. According to the phylogenetic tree constructed in this study from nucleic acid sequences of the 18S rRNA, *Trypanosoma* KG1 is clustered together with *Trypanosoma* (*Megatrypanum*) *pestanai* which has been reported to infect badgers (*Meles meles*). However, the vector is unknown (Hoare, 1972) and *T.* sp. wombat H26 isolated from wombat (*Vombatus ursinus*), whereby the wombat flea (*Lycopsylla nova*) has been suggested as a possible vector (Noyes *et al.* 1999), as are ticks, based on a report in which trypanosomes were found in tick nymphs (*Ixodes holocyclus*) in Australia (Mackerras, 1959).

In the phylogenetic analysis of the 18S rRNA and gGAPDH (Hughes and Piontkviska, 2003*a*, *b*; Hamilton *et al.* 2004) *T. pestanai* clustered with *T.* (*Megatrypanum*) *theileri* and *T.* (*Megatrypanum*) *cyclops*. Whilst, in the phylogenetic analysis of the SSU rRNA *T. pestanai* clustered only with *T.* sp. wombat H26 (Hamilton *et al.* 2004). However, the clustering in the phylogenetic analysis of the current study is different probably because of the differences in the bootstrap support whereby they are generally low or absent for the above-mentioned analyses.

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Another possible reason could be due to different lengths of sequences used in alignments which are longer or shorter than those used in the current study or the fact that the model of evolution in the maximum likelihood analysis of the current study is different to evolution models used in the above-mentioned analyses.

In particular, *T. theileri* has also been reported to be naturally infecting the *Hyalomma anatolicum anatolicum* ticks (Latif *et al*. 2004). However, *Trypanosoma* KG1 is morphologically different from *T. theileri*. Accordingly, the trypomastigote-like form of KG1 has a curved pointed shape 15–30 μm in length (Honda *et al*. 2006). In contrast, *T. theileri* is one of the largest mammalian trypanosomes with a mean length of 60–70 μm (Stevens and Brisse, 2004). There is great variability within trypanosome species, and a lot of incomplete knowledge about these parasites. New trypanosome species as well as new localities of known trypanosomes are constantly being described (Karbowiak and Wita, 2004). We, therefore, refer to this unknown trypanosome as *Trypanosoma* KG1 isolate, with reference to the locality of isolation, Kagoshima prefecture of Southern Japan.

Hard ticks of the genera *Hyalomma* and *Rhipicephalus* have been reported to harbour trypanosome (el Kady, 1998; Latif *et al*. 2004) and *Leishmania* (Coutinho *et al*. 2005) parasites, respectively. *Trypanosoma* KG1 isolate was also isolated in hard tick *H. hystricis*. Additionally we have been able to propagate the trypanosome *in vivo* by infecting the soft tick *O. moubata* and the parasite could be isolated in the midgut and the salivary glands. This observation suggests that this trypanosome has the ability to infect both the hard and soft tick species. Some of the Stercorarian trypanosomes to which *Trypanosoma* KG1 isolate is closely related, have a narrow host range for their vertebrate hosts where they live extracellularly, primarily in the bloodstream (Sato *et al*. 2003). This could explain our unsuccessful attempts to propagate *Trypanosoma* KG1 isolate in the experimental animals (mice, rats, rabbits and sheep).

According to Yamakuti *et al*. (1971), *H. hystricis* distribution is restricted to a subtropical and temperate belt of Eastern Asia, and the adult ticks have a fairly wide host range including humans, domestic dogs, buffalo, pigs, wild boars, tigers, hog-badgers, goat-like deer (*Muntiacus reevesi*), short-eared rabbit (*Lepus sinensis*) and the Ryuku black rabbit (Yamaguti *et al*. 1971; Mahara, 1997; Cao *et al*. 2000; Parola *et al*. 2003). This wide host range of *H. hystricis* suggests that one or more of the above-mentioned mammals could be harbouring *Trypanosoma* KG1 isolate, particularly the wild pig (*Sus scrofa*), as it has been reported to be a host of *H. hystricis* in Kagoshima (Yamaguti *et al*. 1971), badgers also (which are hosts for *T. pestanai* which is clustered together with KG1) are possible hosts. In Sudan, Morzaria *et al*. (1986) demonstrated the first biological transmission of *T. theileri* to cattle by the tick *H. a. anatolicum*. It is therefore highly possible that *Trypanosoma* KG1 might be transmitted by the ticks during feeding since we detected the parasite in the salivary glands of experimentally infected *O. moubata* tick.

LAMP reaction amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions using only simple incubators (Notomi *et al*. 2000), and has recently been developed for diagnosis of African trypanosomes (Kuboki *et al*. 2003; Thekisoe *et al*. 2005), whilst PCR is already an established and widely used diagnostic technique for trypanosomes (Zarlenga and Higgins, 2001; Desquesnes and Davila, 2002; Cox *et al*. 2005). Thus, this study has also developed sensitive and specific LAMP and PCR methods that could later be used in attempts to identify vertebrate host(s) and epidemiological studies of the *Trypanosoma* KG1 isolate.

We are now faced with challenges of identifying the possible vertebrate host(s), which may lead to a better understanding of its transmission and parasite host-relationship. There is also a need for further studies of other trypanosome species and ticks as this might give information on non-survival of other trypanosomes in ticks, eventually identifying responsible molecules that could be used as trypanosome vaccine candidates.

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REFERENCES

Brun, R., Hecker, H. and Lun, Z-R. (1998). *Trypanosoma evansi* and *T. equiperdum*: distribution, biology, treatment and phylogenetic relationship (a review). *Veterinary Parasitology* **79**, 95–107.

Cao, W-C., Gao, Y-M., Zhang, P-H., Zhang, X-T., Dai, Q-H., Dumler, J. S., Fang, L-Q. and Yang, H. (2000). Identification of *Ehrlichia chaffensis* by nested PCR in ticks from Southern China. *Journal of Clinical Microbiology* **38**, 2778–2780.

Countway, P. D., Cast, R. J., Savai, P. and Caron, D. A. (2005). Protistan diversity estimates based on 18S rDNA from seawater incubations in the Western North Atlantic. *The Journal of Eukaryotic Microbiology* **52**, 95–106.

Coutinho, M. T. Z., Bueno, L. L., Sterzik, A., Fujiwara, R. T., Bolteho, J. R., De Maria, M., Genaro, O. and Linardi, P. M. (2005). Participation of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the epidemiology of canine visceral leishmaniasis. *Veterinary Parasitology* **128**, 149–155.

Cox, A., Tilley, A., McOdimba, F., Fyfe, J., Eisler, M., Hide, G. and Welburn, S. (2005). A PCR based assay

for detection and differentiation of African trypanosome species in blood. *Experimental Parasitology* **111**, 24–29.

Desquesness, M., Ravel, S. and Guny, G. (2002). PCR identification of *Trypanosoma lewisi*, a common parasite of laboratory rats. *Kinetoplastid Biology and Disease* **1**, 1–6.

Desquesnes, M. and Davila, A. M. R. (2002). Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Veterinary Parasitology* **109**, 213–231.

Donelson, J. E. (2003). Antigenic variation and the African trypanosome genome. *Acta Tropica* **85**, 391–404.

el Kady, G. M. (1998). Protozoal parasites in the tick species infesting camels in Sinai Peninsula. *Journal of the Egyptian Society of Parasitology* **28**, 765–766.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**, 368–376.

Felsenstein, J. and Churchill, G. A. (1996). A Hidden Markov model approach to variation among sites in rate of evolution. *Molecular and Biological Evolution* **13**, 93–104.

Gibson, W. (2003). Species concepts for trypanosomes: from morphological to molecular definitions? *Kinetoplastid Biology and Disease* **2**, 1–6.

Haag, J., O'Huigin, C. and Overath, P. (1998). The molecular phylogeny of trypanosomes: evidence for an early divergence of the salivaria. *Molecular and Biochemical Parasitology* **91**, 37–49.

Hamilton, P. B., Steven, J. R., Gaunt, M. W., Gidley, J. and Gibson, W. C. (2004). Trypanosomes are monophyletic: evidence from genes for glyceraldehydes phosphate dehydrogenase and small subunit ribosomal RNA. *International Journal for Parasitology* **34**, 1393–1404.

Hoare, C. A. (1972). *The Trypanosomes of Mammals*, 1st Edn. Blackwell Scientific Publications, Oxford and Edingburgh.

Honda, T., Fujita, H., Kuramoto, T., Watanabe, Y. and Takada, N. (2006). A record on Trypanosomatidae protozoa isolated from a *Haemaphysalis hystricis* tick in Kagoshima Prefecture, Japan. *Annual Report of Ohara General Hospital* **46**, 11–13.

Hughes, A. and Piontkivska, H. (2003*a*). Phylogeny of Trypanosomatidae and Bodonidae (Kinetoplastida) based on 18S rRNA: Evidence for paraphyly of *Trypanosoma* and six other genera. *Molecular Biology and Evolution* **20**, 644–652.

Hughes, A. L. and Piontkivska, H. (2003*b*). Molecular phylogenetics of Trypanosomatidae: contrasting results from 18S rRNA and protein phylogenies. *Kinetoplastid Biology and Disease* **2**, 15.

Karbowiak, G. and Wita, I. (2004). *Trypanosoma* (*Herpetosoma*) *grosi kosewiense* subsp. n., the parasite of the yellow-necked mouse *Apodemus flavicollis* (Melchior, 1834). *Acta Protozoologica* **43**, 173–178.

Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F., Grab, D. J., Suzuki, H., Sugimoto, C. and Igarashi, I. (2003). Loop-mediated isothermal amplification (LAMP) for detection of African trypanosomes. *Journal of Clinical Microbiology* **38**, 2778–2780.

Latif, A. A., Bakheit, M. A., Mohamed, A. E. and Zweygarth, E. (2004). High infection rates of the tick *Hyalomma anatolicum anatolicum* with *Trypanosoma theileri*. *The Onderstepoort Journal of Veterinary Research* **71**, 251–256.

Mackerras, M. J. (1959). The haematozoa of Australian mammals. *Australian Journal of Zoology* **7**, 105–135.

Mahara, F. (1997). Japanese Spotted Fever: Report of 31 cases and review of the literature. *Emerging Infectious Diseases* **3**, 105–111.

Morzaria, S. P., Latif, A. A., Jongejan, J. and Walker, A. R. (1986). Transmission of *Trypanosoma* sp. to cattle by the tick *Hyalomma anatolicum anatolicum*. *Veterinary Parasitology* **19**, 13–21.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* **28**, I–VII.

Noyes, H. A., Stevens, J. R., Teixeira, M., Phelan, J. and Holtz, P. (1999). A nested PCR for the ssrRNA gene detects *Trypanosoma binneyi* in the platypus and *Trypanosoma* sp. in wombats and kangaroos in Australia. *International Journal for Parasitology* **29**, 331–339.

Parola, P., Cornet, J-P., Sanogo, Y. O., Miller, R. S., Van Thien, H., Gonzalez, J-P., Raoult, D., Telford, III, S. R. and Wongsrichanalai, C. (2003). Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp. and other Eubacteria in ticks from the Thai-Myanmar border and Vietnam. *Journal of Clinical Microbiology* **41**, 1600–1608.

Ravel, S., Grebaut, P., Cuisance, D. and Guny, G. (2003). Monitoring the development status of *Trypanosoma brucei gambiense* in the tsetse fly by means of PCR analysis of anal and saliva drops. *Acta Tropica* **88**, 161–165.

Sambrook, J. and Russell, D. W. (2001). Preparation and analysis of eukaryotic genomic DNA. In *Molecular Cloning: A Laboratory Manual*, 3rd Edn. (ed. Sambrook, J. & Russell, D. W.), pp. 6.1–6.30. Cold Spring Harbor Laboratory Press, New York.

Sato, H., Ishita, K., Matsuo, K., Inaba, T., Kamiya, H. and Ito, M. (2003). Persistent infection of Mongolian jirds with a non-pathogenic trypanosome, *Trypanosoma* (*Herpetosoma*) *grosi*. *Parasitology* **127**, 357–363.

Stevens, J. R. and Brisse, S. (2004). Systematics of trypanosomes of medical and veterinary importance. In *The Trypanosomiasis* (ed. Maudlin, I. Holmes, P. H. and Miles, M. A.), pp. 1–23. CABI Publishing, UK.

Thekisoe, O. M. M., Inoue, N., Kuboki, N., Tuntasuvan, D., Bunnoy, W., Borisutsuwan, S., Igarashi, I. and Sugimoto, C. (2005). Evaluation of loop-mediated isothermal amplification (LAMP), PCR and parasitological tests for detection of *Trypanosoma evansi* in experimentally infected pigs. *Veterinary Parasitology* **130**, 327–330.

Waladde, S. M., Young, A. S. and Morzaria, S. P. (1996). Artificial feeding of ixodid ticks. *Parasitology Today* **12**, 272–278.

Yamaguti, N., Tipton, V. J., Keegan, H. L. and Toshioka, S. (1971). Ticks of Japan, Korea, and the Ryuku Islands. *Brigham Young University Science Bulletin* **15**, 77–83.

Zarlenga, D. S. and Higgins, J. (2001). PCR as a diagnostic and quantitative technique in veterinary parasitology. *Veterinary Parasitology* **101**, 215–230.