まれていないことから，血清型に依存しない広範囲のレプトスピラ感染に有効なワクチンの開発が今後の重要な課題である．

2. イヌのレプトスピラ症の全国調査に向けた初動調査

ヒトにおいてレプトスピラ症は見過ごされた疾患となっていることから，ヒトのレプトスピラ症の実態を明らかにするために，まずは調査対象地域でのイヌのレプトスピラ症の発生状況を明らかにし，ヒトへの感染リスクの存在を示すことによって，医師のレプトスピラ症への関心を向上させることが重要である．そこで本年度は，全国でのイヌのレプトスピラ症の発生状況を明らかにするための初動調査として，千葉，三重，福岡，佐賀，熊本，沖縄各県の衛生研究所の協力の下，宮崎県で行った強化サーベイランス法を参考に各県で検査定点サーベイランスを行った．各県における本年度の検査頭数は以下のとおりであった：千葉県 15 例，三重県 12 例(うち 2 例は奈良県の患畜)，福岡県 2 例，佐賀県 4 例，熊本県 2 例，沖縄県 3 例．その結果，沖縄県を除く各県でレプトスピラ陽性イヌが検出された(表 2)．また鹿児島県からの依頼検査により，同県でのイヌのレプトスピラ症の発生も確認された(表 2)．サーベイランスを始めた時期が各県で異なるため発生状況を単純に比較することはできないが，国内の多くの県でイヌのレプトスピラ症が発生していることが明らかとなった．一方で，国内のレプトスピラ症患者の約 50%が沖縄県から報告されているが，イヌのレプトスピラ感染疑い頭数は同じ期間調査を行った千葉県や三重県に比べて非常に少なかった．沖縄県の患者の多くは河川で感染していることから，ヒトに比べて川遊びを行う機会が少ないイヌはレプトスピラ感染のリスクが低いことを示唆しているのかもしれない．今後とも各県での調査を継続していくことで，国内のイヌのレプトスピラ症発生が明らかとなっていくことが期待される．

3. 港湾区域のネズミからのレプトスピラ分離培養および *flaB* 遺伝子の解析

全国の検疫所の協力により，33 か所の港湾区域で捕獲されたネズミ 270 匹から採取した腎臓をコルトフ培地で培養を行った結果，那覇空港で捕獲されたハツカネズミ 2 匹からレプトスピラが分離された(表 3)．分離株 2 株から染色体 DNA を抽出し，*flaB*-PCR，つづいて増幅された *flaB* の塩基配列を決定したところ，2 株の塩基配列は同一で，レプトスピラ種は *L. borgpetersenii* であると推定された．この塩基配列は，これまでに沖縄県で捕獲されたネズミから分離されたレプトスピラから見出されているものであった．またこれら分離株は増殖が非常に遅く，*flaB* 遺伝子の解析以外の性状解析は行えなかった．

レプトスピラ症は東南アジアや中南米では大規模な発生がみられており，これら地域から船舶などを介してレプトスピラ保有ネズミの侵入も考えられるため，今後とも港湾区域での侵入監視体制を確立していく必要がある．また港湾区域で捕獲されたレプトスピラが，国内に存在している型の菌であるか，あるいは海外から侵入した菌であるかを明らかにするためにも，国内でのレプトスピラ保有調査を強化するとともに，MLST などによるレプトスピラのより詳細な性状解析方法の確立も重要であり，今後の課題として取り組んでいく必要がある．

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三重県獣医師会

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沖縄県獣医師会

論文発表・著書

1. Koizumi N, Watanabe H. Leptospirosis. In: Vaccines for Biodefense and Emerging and Neglected Diseases (in press)
2. Koizumi N, Muto M, Yamamoto S, Baba Y, Kudo M, Tamae Y, Shimomura K, Takatori I, Iwakiri A, Ishikawa K, Soma H, Watanabe H. Investigation of reservoir animals of *Leptospira* in the northern part of Miyazaki Prefecture. Jpn J Infect Dis. 61(6):465-468, 2008
3. 小泉信夫，渡辺治雄．レプトスピラ抗体．新・臨床検査項辞典，2008

学会発表

1. 小泉信夫，武藤麻紀，山本正悟，下村高司，相馬宏敏，渡辺治雄．宮崎県におけるイヌのレプトスピラ症の発生状況及び分離株の性状解析．人と動物の共通感染症感染症研究会，東京，2008年11月．
2. 鈴木智之，高橋亮太，塩山陽子，山本正悟，岩切章，三浦美穂，石川幸治，相馬宏敏，小泉信夫，武藤麻紀，中島一敏，岡部信彦．宮崎県におけるレプトスピラ症の実地疫学調査．人と動物の共通感染症感染症研究会，東京，2008年11月
3. 塩山陽子，山本正悟，岩切章，三浦美穂，佐藤弘，中島一敏，大山卓昭，谷口清洲，岡部信彦，鈴木智之，高橋亮太，小泉信夫，武藤麻紀，渡辺治雄．宮崎県におけるレプトスピラ症の発生とその対応．衛生微生物技術協議会第29回研究会，東京，2008年6月
4. 塩山陽子，山本正悟，岩切章，佐藤弘，中島一敏，大山卓昭，谷口清洲，岡部信彦，鈴木智之，高橋亮太，小泉信夫．宮崎県におけるレプトスピラ症の発生とその対応．第82回日本感染症学会総会，島根，2008年4月
5. 坂本光男，加藤哲朗，佐藤文哉，堀野哲也，中澤靖，吉田正樹，小野寺昭一，河野緑，保科定頼，小泉信夫，渡辺治雄．当院で経験したレプトスピラ症の3例．第82回日本感染症学会総会，島根，2008年4月
6. 服部耕己，磯崎泰介，櫻井隆之，大曲貴夫，小泉信夫．急激な経過をたどったレプトスピラ症の一例．第82回日本感染症学会総会，島根，2008年4月

表 1. 宮崎県におけるイヌのレプトスピラ症強化サーベイランス陽性結果一覧

検体番号	居住地	種別	臨床症状	ワクチン接種歴	転帰	検査結果				
						分離結果[a]	分離株血清群	分離株 *flaB*[b]	血清抗体価	PCR（血しょう）[c]
08002	高原町	ペット	嘔吐，黄疸，腹痛	有	死亡	陽性（E, K）	Hebdomadis	*L. interrogans* ST2	陰性	＋
08004	国富町	ペット	嘔吐，粘膜の充出血，黄疸	無	死亡	陽性（E, K）	Autumnalis	*L. interrogans* ST2	陰性	－
08007	西都市	ペット	嘔吐，粘膜の充出血，黄疸	無	軽快	未実施			Autumnalis: 1,280 Pomona: 1,280 Rachmati: 1,280	－
08008	西都市	狩猟犬	粘膜の充出血，黄疸	無	死亡	陽性（E, K）	Hebdomadis	*L. interrogans* ST1	陰性	－
08009	えびの市	ペット	嘔吐，黄疸	無	死亡	陽性（E, K）	Australis	*L. interrogans* ST2	陰性	＋
08010	鹿児島県伊佐市	狩猟犬	嘔吐，黄疸	無	軽快	陰性			陰性	＋
08011	宮崎市	狩猟犬	粘膜の充出血，黄疸	無	死亡	陰性			Hebdomadis: 640	－
08012	西都市	狩猟犬	粘膜の充出血，黄疸	無	死亡	陽性（K）	Hebdomadis	*L. interrogans* ST2	Hebdomadis: 320	－
08013	宮崎市	狩猟犬	粘膜の充出血	不明	軽快	陰性			Hebdomadis: 320	－
08014	高城市	ペット	黄疸，吐血，起立困難	無	死亡	未実施			Autumnalis: 1,280 Pomona: 1,280	－
08015	西都市	狩猟犬	嘔吐，粘膜の充出血，黄疸	無	軽快	陽性（E, K）	Australis	*L. interrogans* ST2	Australis: 160	－
08017	西都市	狩猟犬	粘膜の充出血，黄疸	無	死亡	陽性（E）	Autumnalis	*L. interrogans* ST2	Autumnalis: 640 Pomona: 640	－
08019	西都市	狩猟犬	粘膜の充出血，黄疸	無	死亡	陽性（E, K）	Hebdomadis	*L. interrogans* ST2	Hebdomadis: 320 Kremastos: 320	－
08020	門川町	狩猟犬	発熱，嘔吐	無	死亡	陽性（E, K）	Hebdomadis	*L. interrogans* ST2	陰性	＋
08021	門川町	狩猟犬	嘔吐，粘膜の充出血	無	軽快	未実施			Copenhageni: 160	－
08023	都城市	ペット	元気・食欲なし，筋肉痛	無	軽快	陰性			Australis: 160 Autumnalis: 160 Pomona: 160	－

a) E: EMJH 培地，K: コルトフ培地
b) ST の数字がおなじ分離株の *flaB* 部分塩基配列は同一
c) PCR 産物の塩基配列の決定は未実施

表 2. 各県におけるイヌのレプトスピラ症サーベイランス陽性結果一覧

検体番号	居住地	種別	臨床症状	ワクチン接種歴	転帰	検査結果				
						分離結果[a]	分離株血清群	分離株 *flaB*[b]	血清抗体価	PCR
千葉 08005	千葉県 千葉市	ペット	嘔吐，黄疸，全身発赤，アンモニア口臭	不明	死亡	陰性			Copenhageni: 160 Ictero[c]: 160 Poi: 160	−(血液)
千葉 08013	千葉県 長柄市	ペット	食欲廃絶，元気低下，結膜充血	無	軽快	培養中			Canicola: 5,120 Castellonis: 5,120	+(尿) *L. interrogans* ST2
千葉 08015	千葉県 鴨川市	ペット	嘔吐，食欲低下	無	死亡	培養中			陰性	+(血液)[d]
三重 08002	三重県 亀山市	不明 (迷い犬)	嘔吐，黄疸，元気消失，多尿	無	軽快	未実施			Australis: 160	未実施
三重 08003	奈良県 山添村	ペット	嘔吐，血尿	無	死亡	陽性(K)	未実施	*L. interrogans* ST2	陰性	−(血液)
三重 08004	三重県 菰野町	ペット	嘔吐，黄疸	無	不明	陰性			Hebdomadis: 320	−(血液，尿)
福岡 08002	福岡県 福岡市	狩猟犬	発熱，嘔吐，粘膜の充出血	無	軽快	培養中			Australis: 320 Autumnalis: 320	−(血しょう)
佐賀 08002	佐賀県 鹿島市	狩猟犬	発熱，粘膜の充出血，元気・食欲なし	有	軽快	陰性			Hebdomadis: 640 Kremastos: 640	−(血しょう)
佐賀 08004	佐賀県 嬉野市	ペット	発熱，嘔吐	有	軽快	陰性			Castellonis: 160	−(血しょう)
熊本 08001	熊本県 人吉市	狩猟犬	嘔吐，黄疸	無	死亡	陰性			Hebdomadis: 160	−(血しょう)
鹿児島 08001	鹿児島県 霧島市内	ペット	黄疸，死産	不明	軽快	未実施			Hebdomadis: 160	未実施

a) E: EMJH 培地，K: コルトフ培地

b) ST の分類は表 1 と同一

c) Icterohaemorrhagiae

d) PCR 産物の塩基配列の決定は未実施

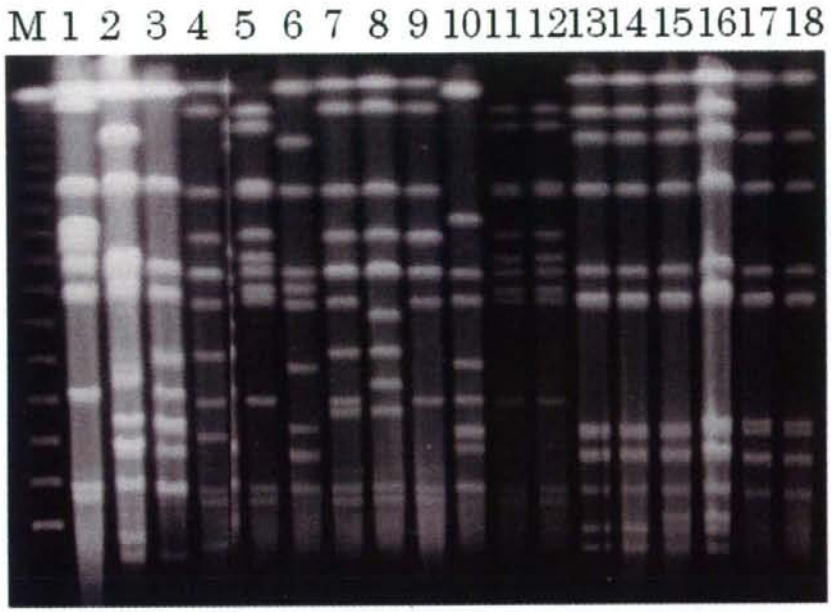

図 1. 宮崎県のイヌ分離株ゲノムのパルスフィールドゲル電気泳動による *Not* I 切断パターン.
レーン 1 ; Akiyami B (Hebdomadis 標準株), 2 ; Akiyami C (Australis 標準株), 3 ; Hond Utrecht IV (Canicola 標準株), 4 ; 宮崎ネズミ 07E9 (Hebdomadis), 5 ; 宮崎イヌ 07E2 (Hebdomadis), 6 ; 宮崎イヌ 07K5 (Australis), 7 ; 宮崎イヌ 07E6 (Hebdomadis), 8 ; 宮崎イヌ 07K6 (Hebdomadis), 9 ; 宮崎イヌ 07E7 (Hebdomadis), 10 ; 宮崎イヌ 07E10 (Canicola), 11 ; 宮崎イヌ 07E13 (Hebdomadis), 12 ; 宮崎イヌ 07K13 (Hebdomadis), 13 ; 宮崎イヌ 07E15 (Hebdomadis), 14 ; 宮崎イヌ 07K15 (Australis), 15 ; 宮崎イヌ 07E18 (Australis), 16 ; 宮崎イヌ 07K18 (Australis), 17 ; 宮崎イヌ 07E19 (Australis), 18 ; 宮崎イヌ 07K19 (Australis), M ; λマーカー(Bio-Rad)

表 3. 日本の港湾区域で捕獲されたネズミのレプトスピラ保有状況（2008.2～2009.1）

調査機関	捕獲地	捕獲ネズミの種類および捕獲匹数							
		ドブネズミ	クマネズミ	ハツカネズミ	アカネズミ	エゾヤチネズミ	エゾアカネズミ	ムクゲネズミ	合計
小樽検疫所	網走港	1							1
	石狩港					2			2
	小樽港	10	14						24
	函館港					1			1
	花咲港	7							7
	紋別港	5							5
千歳空港検疫所支所	新千歳空港	2				2	4	1	9
	室蘭港	1				1			2
仙台検疫所	青森港	8							8
	石巻港	21		4					25
	大船渡港	2							2
	小名浜港	2							2
	気仙沼港	2							2
	塩釜港	13							13
	仙台港	5							5
	八戸港	14							14
	福島空港				1				1
	宮古港	1							1
仙台空港検疫所支所	仙台空港				9				9
成田空港検疫所	成田空港				5				5
東京検疫所	川崎港	2		3					5
	東京港	6							6
横浜検疫所	横浜港	2							2
名古屋検疫所	名古屋港	12		1					13
清水検疫所支所	清水港	9							9
中部空港検疫所支所	中部空港	1							1
大阪検疫所	大阪港			8					8
関西空港検疫所	関西空港			39					39
神戸検疫所	神戸港	16		3					19
福岡検疫所	博多港			7					7
福岡空港検疫所支所	福岡空港			1					1
那覇検疫所	那覇港	15							15
那覇空港検疫所支所	那覇空港		2	5 (2)					7 (2)
合計		157	16	71 (2)	15	6	4	1	270 (2)

（ ）内はレプトスピラが分離できたネズミの数

業績資料集

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PREVALENCE AND MOLECULAR PHYLOGENETIC CHARACTERIZATION OF *TRYPANOSOMA* (*MEGATRYPANUM*) *MINASENSE* IN THE PERIPHERAL BLOOD OF SMALL NEOTROPICAL PRIMATES AFTER A QUARANTINE PERIOD

Hiroshi Sato, Natalie Leo, Yuko Katakai*†, Jun-ichiro Takano*, Hirofumi Akari†, Shin-ichiro Nakamura‡, and Yumi Une‡
Laboratory of Veterinary Parasitology, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan.
e-mail: sato7dp4@yamaguchi-u.ac.jp

ABSTRACT: Neotropical primates of the Cebidae and Callitrichidae, in their natural habitats, are frequently infected with a variety of trypanosomes including *Trypanosoma cruzi*, which causes a serious zoonosis, Chagas' disease. The state of trypanosome infection after a 30-day quarantine period was assessed in 85 squirrel monkeys (*Saimiri sciureus*) and 15 red-handed tamarins (*Saguinus midas*), that were wild-caught and exported to Japan as companion animals or laboratory animals, for biomedical research, respectively. In addition to many microfilariae of *Mansonella* (*Tetrapetalonema*) *mariae* at a prevalence of 25.9%, and *Dipetalonema caudispina* at a prevalence of 3.5%, a few trypomastigotes of *Trypanosoma* (*Megatrypanum*) *minasense* were detected in Giemsa-stained thin films of blood from 20 squirrel monkeys at a prevalence of 23.5%. Although few *T. minasense* trypomastigotes were found in Giemsa-stained blood films from tamarins, a buffy-coat examination detected trypanosomes in 12 red-handed tamarins (80.0%), and PCR amplification of a highly variable region of the small subunit ribosomal RNA genes (SSU rDNA) for *Trypanosoma* spp. detected the infection in 14 of the 15 tamarins (93.3%). Nucleotide sequences of the amplicons were identical for trypanosomes from tamarins and squirrel monkeys, indicating a high prevalence but low parasitemia of *T. minasense* in imported Neotropical nonhuman primates. Based on the SSU rDNA and 5.8S rDNA, the molecular phylogenetic characterization of *T. minasense* indicated that *T. minasense* is closely related to trypanosomes with *Trypanosoma theileri*-like morphology and is distinct from *Trypanosoma* (*Tejeraia*) *rangeli*, as well as from *T. cruzi*. Using some blood samples from these monkeys, amplification and subsequent sequencing of the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene fragments detected 4 trypanosome genotypes, including 2 types of *T. cruzi* clade, 1 type of *T. rangeli* clade, and 1 *T. rangeli*-related type, but failed to indicate its phylogenetic position based on the gGAPDH gene. Furthermore, species ordinarily classified in the *Megatrypanum* by morphological criteria do not form a clade in any molecular phylogenetic trees based on rDNA or gGAPDH genes.

Neotropical primates of the Cebidae and Callitrichidae, such as squirrel monkeys (*Saimiri* spp.), marmosets (*Callithrix* and *Cebuella* spp.), and tamarins (*Saguinus* and *Leontopithecus* spp.), in their natural habitats are frequently infected with a variety of trypanosomes. In addition to 2 zoonotic trypanosome species, i.e., *Trypanosoma* (*Tejeraia*) *rangeri* Téjera, 1920 and *Trypanosoma* (*Schizotrypanum*) *cruzi* Chagas, 1909, Neotropical nonhuman primates are variably infected with *T.* (*Megatrypanum*) *minasense* Chagas, 1909, *T.* (*M.*) *devei* Leger and Porry, 1918, and *T.* (*M.*) *lambretchi* Marinkelle, 1968 (Dunn et al., 1963; Hoare, 1972; Ziccardi et al., 2000). These monkeys are exported to other continents to be kept in research facilities as laboratory animals for biomedical research, e.g., vaccine development for human malaria (WHO, 1988), and in zoo facilities, sometimes as accessible exhibition animals. In addition, small Neotropical monkeys have currently become one of the favorite exotic companion animals in developed countries, including Japan. Although wild-caught monkeys are imported, in most cases, and may have a variety of parasites (Yamashita, 1963; Orihel, 1970; Kuntz and Meyers, 1972; Potkay, 1992; Sullivan et al., 1993), no special attention has been paid to their parasitic infections during the imported-animal quarantine or in sequential animal care under any usages. Exceptionally, an acanthocephalan infection caused by *Prosthenorchis elegans*, which perforates the gut wall, as well as an intestinal nematode infection with *Ptergodermatites nycticebi*, are well known to zoo veterinarians due to a high mortality of the host monkeys (Sato et al., 2003).

The present study reports on the occurrence and prevalence of blood parasites, after a 30-day quarantine period in Japan, in common squirrel monkeys (*Saimiri sciureus*) imported as companion animals and in red-handed tamarins (*Saguinus midas*) imported for use in biomedical research. By examination of stained blood films, we found neither *T. cruzi* nor *T. rangeli* infection, but a high prevalence of *T. minasense* infection, in these monkeys, in addition to microfilariae. Nucleotide sequences of the small and large subunit (SSU/LSU) ribosomal RNA genes (rDNA) of *T. minasense* were determined for the first time. Amplification of the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) detected *T. cruzi* and *T. rangeli* infections in some squirrel monkeys and tamarins, by nested PCR, and a reliable sequence of the *T. minasense* gGAPDH gene was obtained in the present study.

MATERIALS AND METHODS

Parasitological survey

Animals examined in the present study included 85 squirrel monkeys and 15 red-handed tamarins. All of them were wild-caught; the ages of the monkeys were unknown. Furthermore, no information on the original sources, or the periods of captivity, of these monkeys was provided. To quarantine imported primates, they were kept for 30 days in a special facility in the Narita Airport Quarantine. Squirrel monkeys were checked just after this quarantine period, and tamarins were examined after transfer to an experimental animal facility.

The peripheral blood was drawn from squirrel monkeys under inhalation anaesthesia using isoflurane. The main purpose for blood collection at that time was to check antibody levels to viral zoonoses. After making a thin blood film, individual blood samples collected in special tubes for serum separation were centrifuged at 1,300 *g* for 10 min at ambient temperature. After removing the serum, the tubes were then sent, under cool conditions, to the parasitology laboratory. Each tube

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* The Corporation for Production and Research of Laboratory Primates, 1 Hachimandai, Tsukuba 305-0843, Japan.
† Laboratory of Disease Control, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1 Hachimandai, Tsukuba 305-0843, Japan.
‡ Laboratory of Veterinary Pathology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagamihara 229-8501, Japan.

contained a coagulated blood mass and a small volume of uncoagulated blood. Parasite DNA extraction was attempted using the latter portion. Initially, samples from tamarins arrived in a similar manner to the ones from squirrel monkeys; however, EDTA-treated blood was later sent to the parasitology laboratory. In addition to preparing thin blood films, EDTA-treated blood was individually aspirated in a capillary hematocrit tube, centrifuged at 1,300 *g* for 10 min at 4 C, and the buffy-coat layer was examined using inverted light microscopy. When trypanosomes were found in the buffy-coat layer, the fine tube was cut to make a smear on a glass slide. Simultaneously, individual blood was used for parasite DNA extraction, as described below.

Morphological examination

Blood films were stained in Giemsa's solution and observed thoroughly at ×200 magnification using light microscopy. Positive blood films with trypanosomes were further observed under oil immersion at ×1,000 magnification. Arbitrarily selected, but undistorted, well-stained trypanosomes, 21 in number from squirrel-monkeys and 6 from tamarins, were photographed at this magnification, transformed into photographs using Adobe® Photoshop® v.5.0 (Adobe Systems, Inc., San Jose, California), and printed out at higher magnification. Measurements were made using printed photographs (×1,413 of the original size on the paper) by use of a digital curvimetre type S (Uchida-yoko, Chuoh-ku, Tokyo, Japan), when necessary. All measurements were expressed in μm and included total length, maximum width, posterior end to middle kinetoplast (PK), kinetoplast to middle of nucleus (KN), middle of nucleus to anterior end (NA), free flagellum (FF), and sizes of kinetoplast and nucleus. Nuclear index (NI) and kinetoplast index (KI) were calculated as follows: NI = (PK + KN)/NA; and KI = (PK + KN)/KN, following Hoare (1972). For comparison with *T. minasense* measurements performed by Ziccardi and Lourenço-de-Oliveira (1999), KI values were also calculated according to Deane and Damasceno (1961), i.e., KI = PK/KN.

DNA extraction, polymerase chain reaction (PCR), and sequencing

Parasite DNA was extracted from 0.2 ml of each blood sample from selected trypanosome- or microfilaria-positive squirrel monkeys, and from all tamarins, using a GFX genomic blood DNA purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden). PCR amplification of a special fragment of the SSU rDNA, used for detecting stercorarian trypanosomes of terrestrial vertebrates, was conducted using a primer pair of TRY927F and TRY927R, and SSU561F and SSU561R (Noyes et al., 1999). Furthermore, amplification of overlapping fragments of the SSU/LSU rDNA of trypanosomes, using multiple primer combinations, was performed using some parasite DNA extracts as described previously (Sato et al., 2005). After direct sequencing of PCR amplicons (Sato et al., 2005), sequences were assembled manually with the aid of the CLUSTRAL W multiple alignment program (Thompson et al., 1994). The SSU/LSU rDNA construction of the trypanosome was determined as described previously (Sato et al., 2005). The gGAPDH gene as amplified with degenerate primers G3 (5′-TTYGCCGYATYGGY CGCATGG-3′) and G5 (5′-ACMAGRTCCACCACRCGGTG-3′) with the following program: 3 min at 95 C followed by 35 cycles of 60 sec at 95 C, 30 sec at 55 C, and 60 sec at 72 C, then a final extension at 72 C for 7 min, according to Hamilton et al. (2004). As sequencing primers, G6 (5′-GYGGTKTCSVTSAAGGACTG-3′) and G7 (5′-CSC CTGTBGTGCTBGGTATG-3′) (Hamilton et al., 2004) were also used. In addition, the gGAPDH gene of *T. cruzi* (Y strain), *T. grosi* (SESUJI and HANTO isolates), and *T. kuseli* (Sato et al., 2005, 2007) were newly sequenced in this study as technical controls. To obtain more gGAPDH genes of trypanosomes from blood samples of squirrel monkeys and tamarins, nested PCR, using a primer pair of G3 and G5 in the first round, and G1 (5′-CGCGGATCCASGGYCTYMTCGGBAM KGAGAT-3′) and G4a (5′-GTTYTGCAGSGTCGCCTTGG-3′) or G1 and G4b (5′-CCAMGASACVAYCTTGAAGAA-3′) primers in the second round, was conducted according to Hamilton et al. (2004). Amplicons were cloned into a plasmid vector pTA2 (Target Clone®; Toyobo, Osaka, Japan) and transformed into *Escherichia coli* JM109 (TOYOBO) according to the instructions of the manufacturer. After propagation, plasmid DNA was extracted by use of a NucleoSpin® Plasmid kit (Macherey-Nagel GmbH, Düren, Germany), and inserts (from at least 3 independent clones per amplicon) were sequenced using M13 forward and reverse primers.

For microfilariae, internal transcribed spacer (ITS) 1 was amplified using the forward primer S.r.ITS1-NC5/F1 (5′-TTACGTCCCTGCCC TTTGTA-3′) and the reverse primer NC13R (5′-GCTGCGTTCTTCA TCGAT-3′) with the following program: 3 min at 95 C followed by 35 cycles of 45 sec at 94 C, 60 sec at 65 C, and 90 sec at 72 C, then a final extension at 72 C for 7 min (Zhu et al., 2000; Sato et al., 2006). PCR products were separated by electrophoresis in 1.3% agarose gels in 0.5 × TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light. To estimate the molecular sizes, 100-bp DNA Ladder (Promega, Madison, Wisconsin) was used. Molecular sizes of PCR products of *T. cruzi* and *T. rangeli* by use of a primer pair of TRY927F and TRY927R were calculated using deposited sequences in the DDBJ/EMBL/GenBank databases under the accession numbers AF228685, AF232214, AF239980, AF239981, AF245380–AF245383, AF288600, AF288601, AF292942, AF301912, AF303659, AF303660, AF359461–AF359496, AJ009147–AJ009150, AJ620544, AY491762, AY785561–AY785586, M31432, and X53917 (84 *T. cruzi* SSU rDNA sequences), and AF065157, AJ009160, AJ012412–AJ012417, AY491766, and AY491767 (10 *T. rangeli* SSU rDNA sequences).

Phylogenetic analysis

For phylogenetic analyses, nucleotide sequences of SSU rDNA, 5.8S rDNA, and gGAPDH gene were examined. The newly obtained SSU rDNA sequence of the trypanosome, and those of related trypanosome species obtained from DDBJ/EMBL/GenBank, 1,828-bp to 2,233-bp long, were aligned using the CLUSTRAL W multiple alignment program, with subsequent manual adjustment. An insect trypanosomatid species, *Crithidia fasciculata*, was used as an outgroup. Regions judged to be poorly aligned, and characters with a gap in any sequence, were excluded from subsequent analyses; 1,703 characters, of which 312 were variable and 173 were parsimony-informative, remained for subsequent analyses. Phylogenetic analyses of the alignment were conducted using neighbor-joining (NJ), minimum evolution (ME), and maximum parsimony (MP) methods, as implemented in the MEGA program version 3.1 (Kumar et al., 2004). For all 3 methods, 1,000 bootstrap replicates were calculated.

To compare 5.8S rDNA, available sequences of *T. theileri* (AY773698–AY773700, AB007814), *T. cruzi* (M63701, L22334, AF362827–AF362829), *T. rangeli* (AY230232–AY230240, AF362832), *T. grosi* (AB175622–AB175624), *T. otospermophili* (AB175625, AB190228), *T. kuseli* (AB175626); 9 *Leishmania* spp. (AJ000306, AJ000311, AJ000313, AJ000314, AJ300484, AJ300485, AJ634371, AJ634372, AM502245, AJ634378, AY283793, DQ300180, DQ316038, DQ316039, DQ316052); and *Crithidia fasciculata* (Y00055) were obtained from the DDBJ/EMBL/GenBank and aligned by the CLUSTRAL W multiple alignment program. Regions judged to be poorly aligned, and characters with a gap in any sequence, were excluded from subsequent analyses; 163 characters, of which 12 were variable and 7 were parsimony-informative, remained for subsequent analyses. Based on the structural association between 5.8S and 28S rDNA (Hwang and Kim, 2000; Gottschling and Plötner, 2004), a putative secondary structure of the 5.8S rDNA of *T. minasense* was drawn, and base sites at which species-, subgenus-, or genus-specific substitutions constantly occur (parsimony-informative sites) were plotted on this 5.8S rDNA model to understand the structural basis for these changes.

For phylogenetic analyses based on the nucleotide sequences of the gGAPDH gene, the newly obtained sequence of the trypanosome, and those of related trypanosomatids obtained from DDBJ/EMBL/GenBank, were aligned using the CLUSTRAL W multiple alignment program, with subsequent manual adjustment. To convert nucleotide to amino acid sequences using the universal genetic code, the "translation tool" on the internet (http://ca.expasy.org/tools/dna.html) was used. According to Hamilton et al. (2004) and Hamilton, Stevens, Gildley et al (2005), the third codon position was excluded for analyses. Regions with a gap in any sequence were excluded from subsequent analyses; 387 characters, of which 107 were variable and 75 were parsimony-informative, remained for subsequent analyses. Phylogenetic analyses of the alignment were conducted as 18S rDNA analyses, mentioned above.

RESULTS

Prevalence of trypanosomes and their morphology

Peripheral blood of squirrel monkeys was drawn on 25 April 2005. A few trypanosomes were found in Giemsa-stained blood smears from 20 of the 85 squirrel monkeys (23.5%) by intensive microscopic examinations; in most cases, only a single trypomastigote was observed on a slide. As shown in Figure 1, trypanosomes from squirrel monkeys were pleiomorphic. They were, however, characterized by broad bodies with the anteriorly positioned kinetoplasts, coincident with the subgenus *Megatrypanum* (Hoare, 1972). Based on the host species, morphology, and morphometric data (Table I), the trypanosomes from squirrel monkeys were identified as *Trypanosoma* (*Megatrypanum*) *minasense* Chagas, 1909. PCR amplification of a highly variable region of the SSU rDNA was attempted with several blood samples using 4 primer combinations of TRY927F, TRY927R, SSU561F, and SSU561R; the primer pair of TRY927F and SSU561R succeeded in amplifying 650-bp long sequences of the trypanosomes in the blood of 4 squirrel monkeys. Except for 1 nucleotide, the sequence of these amplicons (DDBJ/EMBL/GenBank AB362412) was absolutely identical with that of trypanosomes from tamarins mentioned below.

On 11 November 2005, peripheral blood was taken from 15 tamarins, and buffy-coat examinations detected trypanosomes in 10 EDTA-treated blood samples (nos. 1–4, 6, 8, 10, 13, 15, and 16). On 6 January 2006, blood samples were collected from 15 tamarins, and buffy-coat examinations detected trypanosomes in 4 samples (nos. 8, 11, 12, and 15). On both occasions, trypanosomes were very few in number. PCR amplification of a highly variable region of trypanosome SSU rDNA detected the infection in 14 of the 15 tamarin blood samples taken on both occasions. As shown in Figure 2, PCR products of blood trypanosomes in 14 tamarins had identical molecular size, but smaller-than-calculated molecular sizes of the homologous region of 84 available *T. cruzi* sequences (999 bp–1,040 bp) or that of 10 available *T. rangeli* sequences (1,004 bp or 1,008 bp). Sequences of 4 arbitrarily chosen amplicons of 931-bp length by the primer pair of TRY927F and TRY927R were identical, indicating that infection by a single trypanosome species was prevalent in these tamarins, as in the squirrel monkeys mentioned above.

SSU/LSU rDNA of *T.* (*M.*) *minasense* and its molecular phylogenetic relationship with other trypanosomes

The rDNA sequences of *T. minasense* examined in the present study had 2,204-bp 18S, 449-bp ITS1, 172-bp 5.8S, more than 577-bp ITS2, 1,772-bp 28Sα, 49-bp ITS3, 214-bp 28Sγ, 78-bp ITS4, 1,484-bp 28Sβ, 52-bp ITS5, 183-bp 28Sδ, 193-bp ITS6, and more than 68-bp 28Sζ (DDBJ/EMBL/GenBank AB362411).

BLAST analysis of the newly obtained 2,196-bp sequence of 18S rDNA found *T. grayi* (a crocodile trypanosome in Africa) and *T. bennetti* (a kestrel trypanosome in North America) with the closest 18S sequences, followed by a variety of trypanosomes of the subgenus *Herpetosoma*, as well as trypanosome species having broad bodies, such as *T. avium* (an avian trypanosome) and *T. theileri* (a cattle trypanosome).

Bootstrap supports for partial topology were low in phylogenetic trees based on the SSU rDNA alignment of 21 named and several unnamed *Trypanosoma* spp., by either NJ, ME, or MP methods. There were apparently 4 groups in the constructed phylogenetic trees (Fig. 3): (1) *Megatrypanum*-type trypanosomes containing *T. theileri* clade, *T. avium* clade, and other trypanosomes of *T. theileri*-like morphology, (2) South American trypanosome clade containing *T. cruzi* and *T. rangeli*, (3) insectivore trypanosomes exhibiting *Megatrypanum* morphology, and (4) rodent trypanosome (the subgenus *Herpetosoma*) clade containing *T. lewisi* and trypanosomes with *T. lewisi*-like morphology. *Trypanosoma minasense* detected in the present study was in the *Megatrypanum*-type trypanosome group, close to *T. bennetti* and *T. grayi*.

5.8S rDNA of *T.* (*M.*) *minasense* and its molecular phylogenetic relationship with other trypanosomes

BLAST analysis of the newly obtained 172-bp sequence of 5.8S rDNA found *T. theileri* with the closest sequence, followed by *T. cruzi* rather than by *T. rangeli* or *Herpetosoma* trypanosomes. Limited numbers of deposited sequences of trypanosomes, other than SSU rDNA in the DDBJ/EMBL/GenBank, hampered further analyses.

The 5.8S rDNA alignment of 7 *Trypanosoma* spp. and 9 *Leishmania* spp. contained 7 parsimony-informative characters, which were mainly located at unpaired regions such as loop tips (Fig. 4). Table II compares nucleotides of different species at base positions of parsimony-informative or *Leishmania*-specific characters. The 5.8S rDNA sequences were rather consistent for a broad range of *Trypanosoma* spp. and *Leishmania* spp., but nucleotides unique to respective species (shown in Table II) were well conserved by multiple isolates, as determined from available sequences in the DDBJ/EMBL/GenBank. This suggested that *T. minasense* analyzed in the present study was distinct from *T. rangeli*.

gGAPDH genes of trypanosomes, in some blood samples, and their molecular phylogenetic relationships with reported trypanosome gGAPDH gene sequences

In a single round of PCR using a primer pair of G3 and G5, a specific band of 971-bp length was generated by a single blood sample from a tamarin (No. 15). Using the nested PCR after the first-round PCR, specific bands were obtained in blood samples of 2 squirrel monkeys (nos. 45, 54) and 3 tamarins (nos. 6, 10, 16). BLAST analyses of the 6 newly obtained sequences of gGAPDH gene (1 sequence of 971-bp length, 4 sequences of 883-bp length, and 1 sequence of 842-bp length) revealed either *T. rangeli* or *T. cruzi* as the closest sequence, rather than other trypanosome species.

The phylogenetic trees were constructed based on a g-GAPDH gene alignment (the first and second codons) of fish, reptile, bird, and mammalian trypanosomes (Fig. 5). Relationships between different groups of trypanosomes were easily changed by the method used (NJ, ME, and MP), as evidenced by low bootstrap supports for partial topology in the proximal part of the phylogenetic tree. Regardless of tree construction, 2 sequences from 2 tamarins (nos. 10 and 16) grouped firmly with *T. cruzi*; 1 sequence from 1 tamarin (no. 6) and 2 squirrel monkeys (nos. 45 and 54) grouped with the LSTH isolate of *T.*

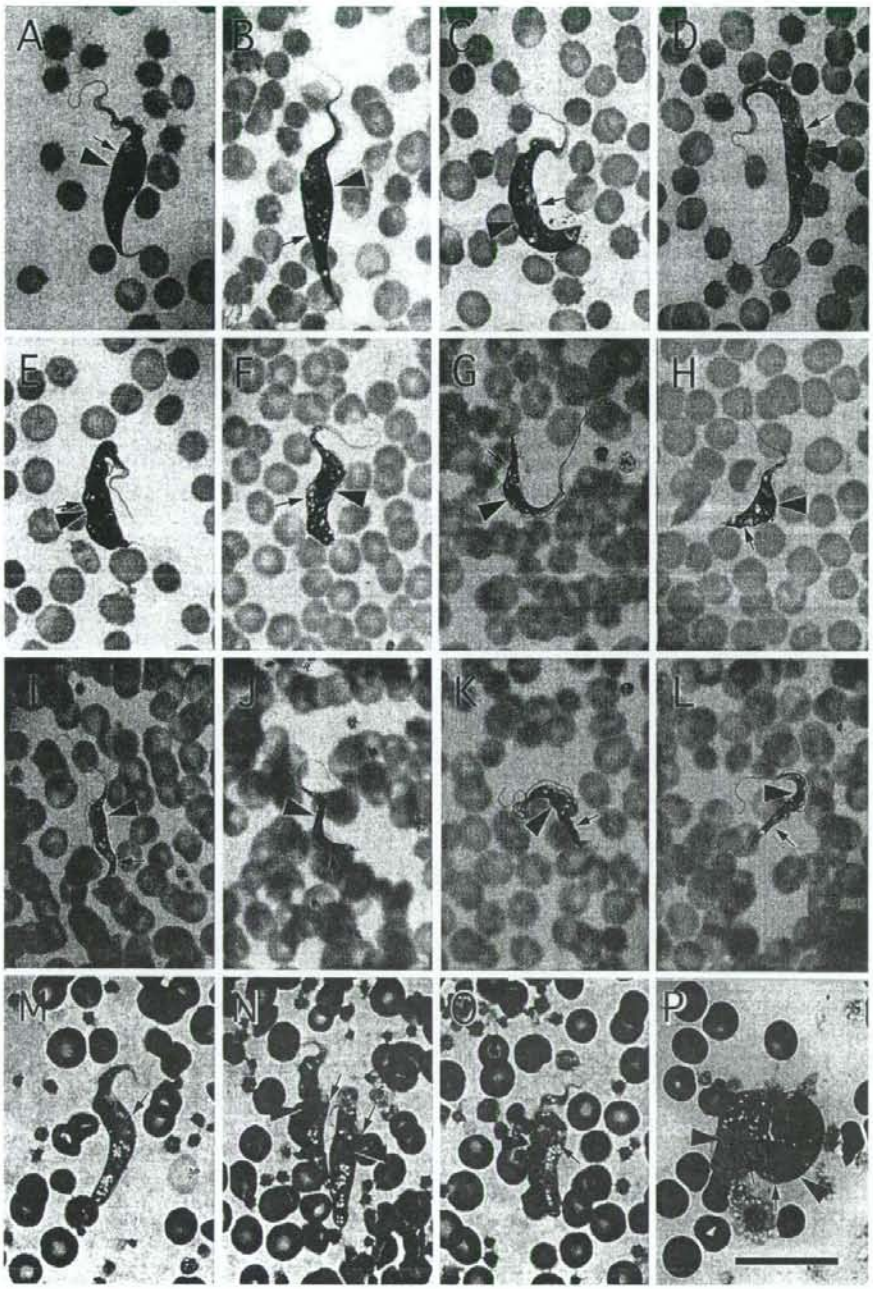

FIGURE 1. *Trypanosoma minasense* in the peripheral blood of squirrel monkeys (**A–L**) and red-handed tamarins (**M–P**). Arrow head indicates the nucleus and small arrow indicates the kinetoplast. The specimens have been stained with Giemsa's solution. The scale bar in (P) applies to all parts of figure and = 20 μm; all photographs are at the same magnification.

TABLE I. Morphometric comparison of *T. minasense* with previously recorded species of the subgenus *Megatrypanum* and *T. rangeli* in small neotropical monkeys.*

Parasite	Host	Number of samples	Reference	L
T. minasense	*Saimiri sciureus*	17	The present study (Natural infection)	29.7–46.7 (40.0 ± 4.8)
T. minasense	*Saimiri sciureus*	12	Ziccardi and Lourenço-de-Oliveira (1999) (Experimental infection)	26–40 (32.3 ± 1.33)
T. minasense	*Callithrix penicillata*	19	Ziccardi and Lourenço-de-Oliveira (1999) (Experimental infection)	30–40 (38.9 ± 0.8)
T. devei	*Midas midas* *Midas rufimanus* *Leontocebus tamarin*	? (multiple sources)	Hoare (1972)	38.7–45.5
T. lambrechti	*Cebus griseus* *Cebus albifrons*	?	Marinkelle (1968) cited by Hoare (1972)	30.1–43.2 (34.9)
T. rangeli	*Saimiri sciureus* *Saimiri ustus* *Callithrix penicillata*	17 (multiple sources)	Ziccardi and Lourenço-de-Oliveira (1998) (Experimental infection in marmosets and natural infection in squirrel monkeys)	29–36 (30.9 ± 0.49)

* Expressed as range, followed by mean or mean ± SD in parentheses.
† Calculated after Deane and Damasceno (1961), i.e., KI = PK/KN.
‡ Calculated after Hoare (1972), i.e., KI = (PK + KN)/KN.

rangeli; and the other 1 sequence from 1 tamarin (no. 15) situated near *T. rangeli*, *T. conorhini*, and *T. vespertilionis*, far from *T. theileri* and other trypanosome species with *T. theileri*-like morphology.

Microfilariae in the blood

Many microfilariae of *Mansonella* (*Tetrapetalonema*) *mariae* Petit, Bain and Roussihon, 1985, 319 ± 18 (range 295–357) μm in length and 2.7 ± 0.2 (range 2.2–2.8) μm in width (n = 10), were found in Giemsa-stained thin blood films from 22 squirrel monkeys (prevalence, 25.9%) (Fig. 6A). Several sheathed microfilariae of *Dipetalonema caudispina* (Molin, 1858) Diesing, 1861, 237 ± 17 (range 192–269) μm in length and 4.6 ± 0.3 (range 4.0–5.0) μm in width (n = 14), were found in Giemsa-stained thin blood films from 3 squirrel monkeys (prevalence, 3.5%) (Fig. 6B); 2 monkeys were infected with the former species. Microfilariae morphology of these 2

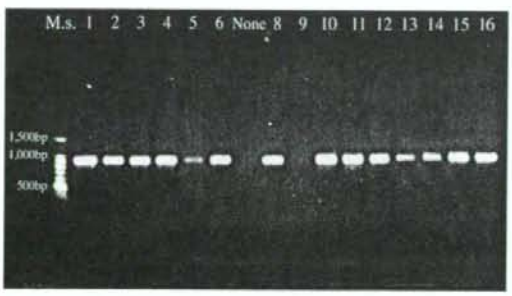

FIGURE 2. Ethidium bromide-stained SSU rDNA amplicons using a primer pair of TRY927F and TRY927R. Amplicons using respective blood DNA extracts of red-handed tamarins, nos. 1–16 (except for no. 7), and no template control (None) were separated by electrophoresis with molecular size markers (M.s.) on the left. Note that all amplicons (tamarin nos. 1–6, 8, and 10–16) had an identical molecular size less than 1,000 bp.

species were consistent with the description by Petit et al. (1985) and Eberhard et al. (1979), respectively. Partial sequences of ITS1 of *M. mariae*, newly obtained using the peripheral blood microfilariae from 1 infected squirrel monkey (DDBJ/EMBL/GenBank AB362562; 163bp 3′-end of 18S rDNA and 301bp 5′-end of ITS1), were closer to, but different from, available sequences of the corresponding region of *Mansonella ozzardi* (AF228559–AF228564), as well as from *Mansonella perstans* (DQ995498); these were the only species with currently available sequences among species of *Mansonella* in the database (Morales-Hojas et al., 2001). Tamarins had no microfilariae in their blood, probably due to injections of ivermectin at the time of transfer to a laboratory animal facility.

DISCUSSION

Due to the highly pleiomorphic nature of *T.* (*Megatrypanum*) *minasense* in the bloodstream, an accurate identification of the species based only upon morphology is often elusive (Ziccardi and Lourenço-de-Oliveira, 1997, 1998, 1999; Ziccardi et al., 2000, 2005). In an experimental infection using squirrel monkeys and marmosets (*Callithrix penicillata*), *T. minasense* sometimes looked like *T.* (*Tejeraia*) *rangeli* (Ziccardi and Lourenço-de-Oliveira, 1999). Our observations indicate that kinetoplasts of trypanosomes detected in the present study are located more anteriorly than *T. rangeli* (Table I). *Trypanosoma minasense*, and at least 2 other species of the subgenus *Megatrypanum*, i.e., *T.* (*M.*) *devei* and *T.* (*M.*) *lambretchi*, were recorded in Neotropical nonhuman primates (Deane and Damasceno, 1961; Lambrecht, 1965; Marinkelle, 1968; Hoare, 1972; Lanham et al., 1984; Ziccardi et al., 2000). *Trypanosoma devei* is a slender trypanosome, much narrower than *T. minasense*. *Trypanosoma lambretchi* has a nucleus in the posterior part of the body. Although the extreme scantiness of the infection in individual hosts, and polymorphic forms by individual hosts (Fig. 1), made the species identification difficult, the trypanosomes found in both squirrel monkeys and tamarins were closer to *T. minasense* than to *T. devei* or *T. lambretchi* (Hoare, 1972).

TABLE I. Extended.

BW	PK	KN	NA	F	N	K	KI†	KI‡	NI
1.8–5.0 (3.2 ± 1.0)	5.7–13.5 (7.7 ± 2.2)	2.8–10.6 (6.9 ± 1.9)	8.5–17.7 (11.9 ± 3.1)	7.8–19.8 (13.7 ± 3.4)	2.5–5.0 (3.3 ± 0.6)	0.7–1.4 (1.1 ± 0.2)	0.8–3.0 (1.3 ± 0.7)	1.8–4.0 (2.3 ± 0.7)	0.6–1.9 (1.3 ± 0.4)
1.8–2.3 (2 ± 0.04)	2.5–11 (5.8 ± 0.97)	5–8 (6.2 ± 0.34)	6–14 (9.4 ± 0.73)	6–10 (8.8 ± 0.37)	1.2–2 (1.6 ± 0.11)	0.8–1.1 (1 ± 0.03)	0.4–2.2 (1 ± 1.20)	—	0.7–2.3 (1.4 ± 0.14)
2–4 (3 ± 0.16)	3–12 (10 ± 0.64)	3–11 (5.8 ± 0.44)	9–14 (11.3 ± 0.35)	6–10 (8.6 ± 0.26)	1–2.5 (1.8 ± 0.09)	0.8–1 (1 ± 0.02)	0.4–3.5 (1.9 ± 0.22)	—	1–2 (1.4 ± 0.06)
2.0–3.0	8.7–16.5	4.0–9.5	—	6.5–7.2	—	—	—	ca. 3.0	1.2–1.5
ca. 2.5–4.0	—	3.0	—	ca. 11.6–13.2	—	—	—	—	—
1.2–3.0 (1.9 ± 0.12)	2–6 (3.8 ± 0.30)	7–9 (7.8 ± 0.18)	6–13 (8.6 ± 0.49)	5–11 (9.2 ± 0.41)	1.5–2 (2 ± 0.03)	0.5–1.1 (0.9 ± 0.03)	0.3–0.9 (0.5 ± 0.04)	—	0.8–2 (1.4 ± 0.09)

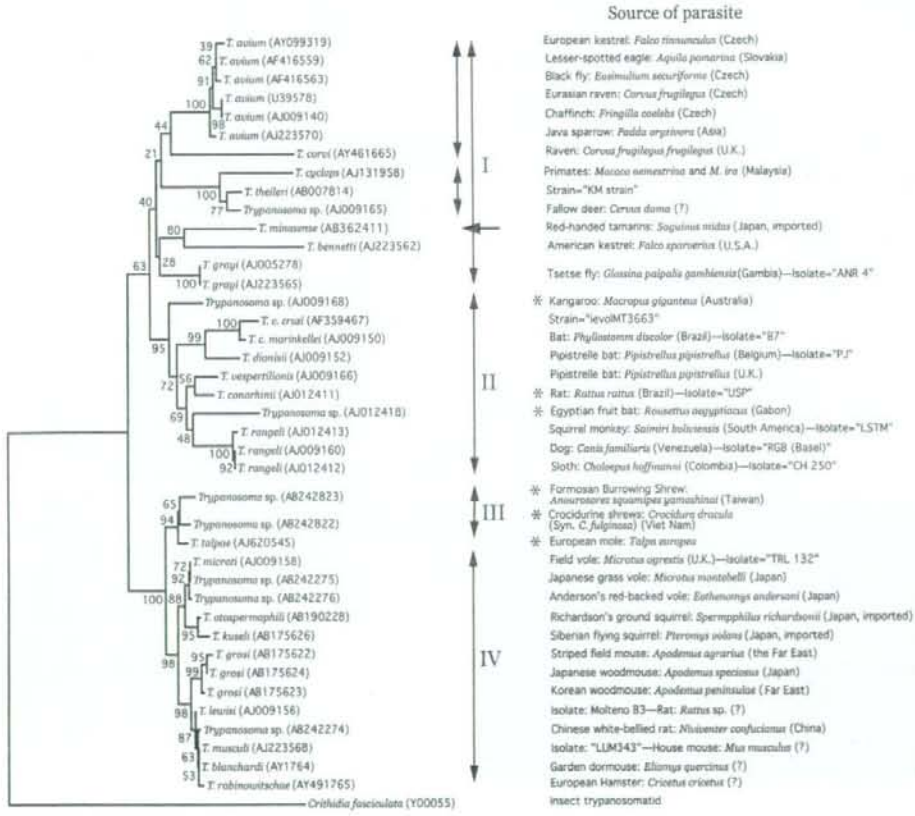

FIGURE 3. Neighbor-joining phylogenetic tree based on alignment of SSU rDNA sequences. Bootstrap scores, expressed as percentages of 1,000 replicates, are given at nodes. The *T. minasense* examined in the present study is indicated by a closed arrow. The sources of the parasites were checked with reference to Votypka et al. (2004), Sato et al. (2005, 2007), Hamilton, Stevens, Gidley et al. (2005), and Hamilton, Stevens, Holz et al. (2005).

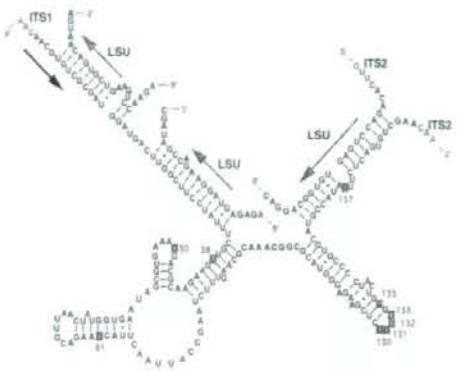

FIGURE 4. Putative secondary structure of the 5.8S rDNA of *T. minasense* with the associated 28S rDNA structure. Shaded nucleotides, with position numbers from the beginning of the 5.8S rDNA (DDBJ/EMBL/GenBank AB362411), are highly variable between trypanosomatid species or genus but absolutely conserved by species or genus, as shown in Table II.

Ziccardi et al. (2005) conducted sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on several strains of *T. rangeli*, *T. saimirii*-like trypanosomes isolated from squirrel monkeys, and *T. minasense*, and found that *T. saimirii*-like trypanosomes shared more than 80% of 20 polypeptides, ranging between 18 and 200 kDa, with *T. rangeli*, whereas *T. minasense* shared only 9 of 20 polypeptide bands with *T. rangeli* isolates. Based on the SDS-PAGE profiles mentioned above, as well as on no morphological or biological differences, the infectivity to triatomine bugs, successful axenic cultures, and successful PCR amplification of a randomly-amplified DNA fragment (Tra625) of *T. rangeli* and squirrel-monkey-driven trypanosomes (Ziccardi and Lourenço-de-Oliveira, 1998, 1999; Da Silva, Rodrigues et al., 2004), Ziccardi et al. (2005) concluded that *T. saimirii* is a junior synonym of *T. rangeli*. Moreover, *T. minasense* is an independent species from *T. rangeli*. The 2,184-bp sequence of SSU rDNA from the "LSTM" isolate of South American trypanosomes from a squirrel monkey (DDBJ/EMBL/GenBank AJ012413) was previously thought to be the sequence of *T. minasense*, but was recently concluded to be that of *T. rangeli* (Stevens et al., 1998, 1999; Da Silva, Noyes et al., 2004; Hamilton et al., 2007). The 2,176-bp SSU rDNA sequence of *T. minasense* in the present study was closer to those of *T. theileri* and other trypanosome species with *T. theileri*-like morphology and was distinct from *T. rangeli* (Fig. 3). Furthermore, the 5.8S rDNA sequence of *T. minasense* examined in the present study was again closer to *T. theileri* than to *T. cruzi* or *T. rangeli* (Table II). Although considerable variations were noted in 5.8S rDNA sequences of *T. rangeli* as single base insertion–deletion events or substitutions (Beltrame-Botelho et al., 2005), no nucleotide substitutions were seen at 9 highly variable base positions (shown in Table II), by species or higher taxa, in isolates of *T. rangeli*; however, 3 base substitutions existed between *T. rangeli* and *T. minasense*, as analyzed in the present study, indicating that these 2 were distinct species. Further analyses should be done on the present finding that the phylogenetic trees, based on a 18S rDNA alignment, found *T. bennetti* (kestrel trypanosome in North America) as the closest trypanosome species; these analyses should be performed after clarifying the 5.8S rDNA sequence of *T. bennetti* and after accumulation of more rDNA sequences of a variety of trypanosome spp. from mammals, birds, reptiles, and amphibians.

SSU rDNA sequence analyses give vastly different, and poorly supported, positions for species of *Trypanosoma* in kinetoplastid evolutionary trees, depending on the out-group taxon and the analysis method (Lukes et al., 1997; Stevens et al., 1998, 1999, 2001; Hughes and Piontkivska, 2003; Hamilton et al., 2004; Hamilton, Stevens, Gidley et al., 2005; Simpson et al., 2006). Recent use of cytoplasmic heat shock protein 90 (hsp90) genes (Simpson et al., 2002) or gGAPDH genes (Hamilton et al., 2004; Hamilton, Stevens, Gidley et al., 2005; Hamilton et al., 2007) for phylogenetic trees resolves the aforementioned problem with robust statistical support, resulting in rather different phylogenetic trees from those inferred from SSU rDNA sequences. According to this viewpoint, we have attempted to characterize the gGAPDH nucleotide sequence of *T. minasense*, but all 6 sequences fell into either *T. cruzi* or *T. rangeli* clades (Fig. 5). Of these, 5 gGAPDH sequences which had been amplified by the nested PCR are apparently parts of

TABLE II. Comparison of 5.8S rDNA sequences of some representative trypanosomatid species.

Trypanosomatid species	No. of isolates examined	Length (bp)	Position of base substitution*								
			38	50	81	130	131	132	133	135	157
T. minasense	1	172	U	C	C	U	U	C	G	A	U
T. rangeli	10	172–173	•	•	U	•	•	U	C	•	•
T. theileri	4	172	•	•	U	C	•	•	•	•	•
T. cruzi	5	172	•	•	U	C	•	•	A	•	•
T. grosi	3	172	A	•	U	•	•	U	U	•	A
T. otospermophili	2	172	A	•	U	•	•	U	U	•	A
T. kuseli	1	172	A	•	U	•	•	U	U	•	A
Leishmania spp. (9 species†)	12	169–171	•	U	U	•	A	U	U	U	–
Crithidia fasciculata	1	171	•	U	U	•	•	U	U	•	–

* Corresponding to the base position of *T. minasense* (DDBJ/EMBL/GenBank AB362411). Dot (•) indicates the identical base with *T. minasense*, dash (–) indicates a deletion of base.

† *Leishmania donovanii, L. ethiopica, L. infantum, L. major, L. tropica, L. amazonensis, L. braziliensis, L. chagasi, and L. mexicana.*

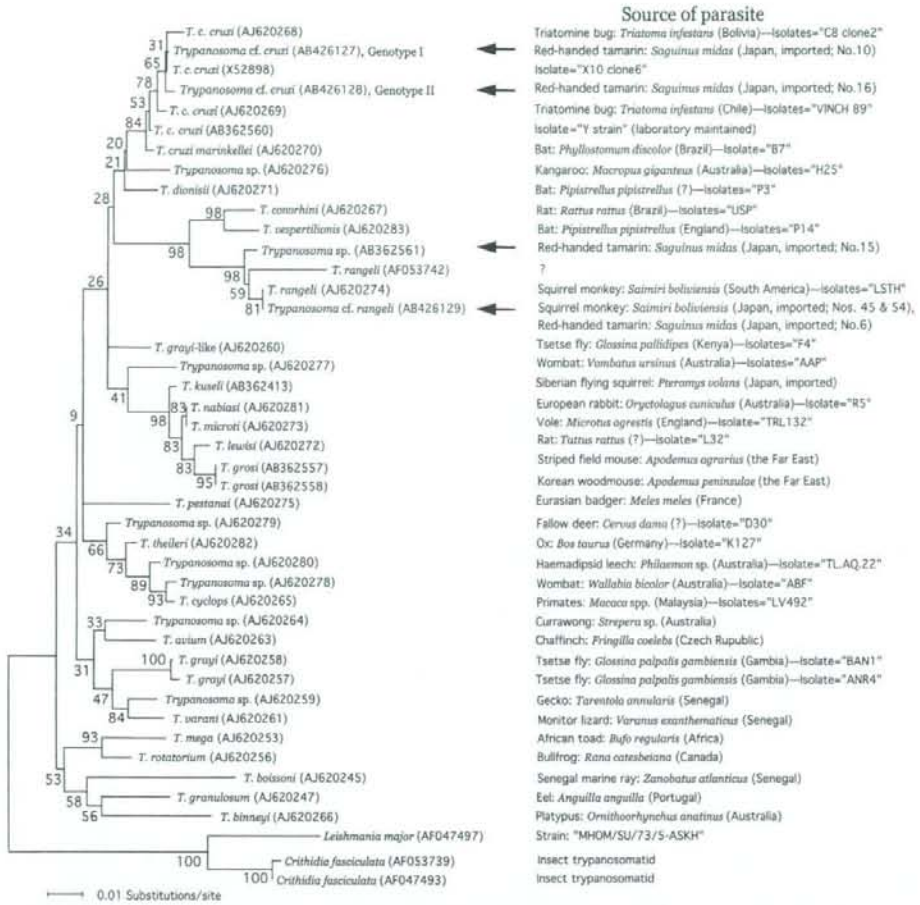

FIGURE 5. Neighbor-joining phylogenetic tree, based on alignment of gGAPDH gene sequences. Sequences obtained in the present study are indicated by arrows. Bootstrap scores, expressed as percentages of 1,000 replicates, are given at nodes. The sources of the parasites were checked as described in the legend for Figure 2.

the genes of *T. cruzi* or *T. rangeli*, whereas the 971-bp long sequence (DDBJ/EMBL/GenBank AB362561) from the blood of a tamarin (no. 15) situates near a group of *T. rangeli* isolates, but branches off from it (Fig. 5). At present, we could not determine whether or not this gGAPDH sequence is that of *T. minasense*, thus failing to identify its proper phylogenetic position based on the gGAPDH gene.

As shown in Figure 2, specific bands amplified by the primer pair of TRY927F and TRY927R, universal primers for the highly variable region of trypanosome 18S rDNA, seem to be single for all 14 positive tamarins, and by this primer pair are clearly less than 1,000 bp in size and the calculated sizes of *T. cruzi* and *T. rangeli* 18S rDNA amplicons. To the contrary, the gGAPDH primers used in the present study preferentially amplified the *T. rangeli* and *T. cruzi* gGAPDH genes. To clarify the gGAPDH gene sequence of *T. minasense*, and to construct the proper phylogenetic tree based on it, for the special amplification we should select primers using an adequate trypanosome sample collected in the endemic regions and specifically diagnosed by multiple criteria (Ziccardi et al., 2005).

Stevens et al. (1999) proposed that use of the names *Herpetosoma* and *Megatrypanum* should be discontinued until their status is clarified because these subgenera are clearly polyphyletic and lack evolutionary and taxonomic relevance. As shown in Figure 3, clade I, which we refer to as "*Megatrypanum*-type trypanosome clade" and which contains *T. avium* and *T. theileri*, was poorly supported by bootstraps. In addition, several species, e.g., *T. conorhinii* and *T. talpae*, classically defined in the subgenus *Megatrypanum*, are positioned in different clades. Hoare (1972) stated that the subgenus *Megatrypanum* is a rather heterogeneous group of large mammalian trypanosomes, and may be regarded, phylogenetically, as the most primitive representative of the genus *Trypanosoma* in mammals because it shows affinities with some corresponding parasites of amphib-

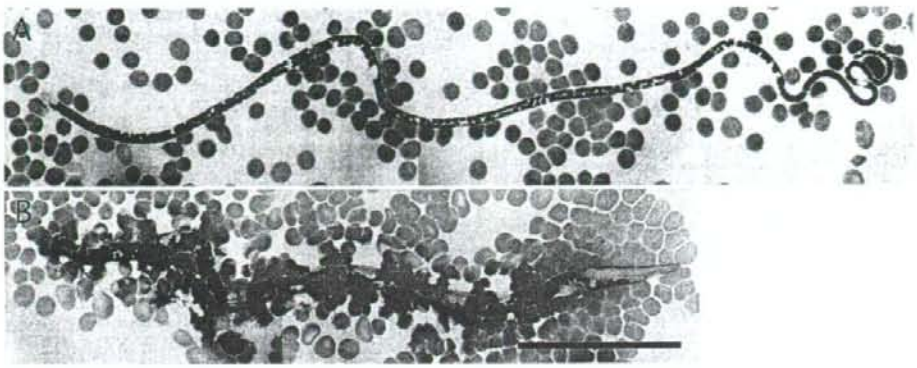

FIGURE 6. Microfilariae of *Mansonella* (*Tetrapetalonema*) *mariae* (**A**) and *Dipetalonema caudispina* (**B**) in the peripheral blood of squirrel monkeys. The specimens have been stained with Giemsa's solution. The scale bar in (B) applies to both A and B and = 50 μm; the 2 photographs are at the same magnification.

ians and reptiles. Accumulation of more molecular data is currently needed about trypanosomes having morphological characters of the subgenus *Megatrypanum*; such studies on trypanosomes of indigenous Australian vertebrates (Noyes et al., 1999; Hamilton, Stevens, Gidley et al., 2005) provided fascinating insights on the issue. Similarly, as seen in Figure 2, *T. talpae* from moles, along with 2 unnamed trypanosomes from shrews of the genera *Anourosores* and *Crocidura*, formed a distinct clade of insectivore trypanosomes exhibiting *Megatrypanum* morphology.

Trypomastigotes of *T. minasense* have been recorded in 32 species or subspecies of Neotropical nonhuman primates, primarily in monkeys of the Cebidae and Callitrichidae (Dunn et al., 1963; Deane et al., 1974; De Resende et al., 1994; Ziccardi et al., 1996). This species is the only trypanosome for which a circadian rhythm of parasitemia was noted (Deane et al., 1974). Furthermore, scanty parasitemia by *T. minasense* was sustained for at least 17 or 18 mo in marmosets (*Callithrix penicillata*) during captivity (De Resende et al., 1994; Ziccardi et al., 1996). Ziccardi and Lourenço-de-Oliveira (1997) examined 165 squirrel monkeys (70 *Saimiri sciureus* and 95 *S. ustus*) caught in the Brazilian Amazon basin, and found *T. rangeli* in 58 (35.2%), *T. minasense* in 55 (33.3%), and *T. cruzi* in 58 (35.2%) monkeys. Another study by the same group reported parasitemia by *T. minasense* in 4.3% (2 tamarins) of 46 nonhuman primates at a different locality in Brazil (Ziccardi et al., 2000). Due to apparent effects caused by the source of primates, the timing of blood aspiration for examination, and the applied techniques for examination, the reported prevalence (23.5%) of *T. minasense* in 85 squirrel monkeys, examined only by Giemsa-stained blood smears, might be an underestimation. More sensitive methods such as hematocrit and PCR-based detection techniques, applied in this study for tamarins only, as well as hemoculture, would most likely increase the prevalence. As mentioned above, we should note that the use of universal primers for a broad range of *Trypanosoma* spp. often detect only a single species, even though the animal has a mixed infection with multiple species.

Along with trypanosomes, microfilariae of *Dipetalonema* (*D. obtusa*, *D. gracile*, *D. caudispina*, *D. graciliforme*, and *D. robini*) and *Mansonella* (*M.* [*Tetrapetalonema*] *marmosetae*, *M.* [*T.*] *tamarinae*, and *M.* [*T.*] *mariae*) are the most frequently encountered helminth infections in New World monkeys (Dunn and Lambrecht, 1963; Esslinger and Gardiner, 1974; Petit et al., 1985). Prevalence often exceeds 70%, and multiple infections with 2–4 species are common in endemic areas for Neotropical nonhuman primates. The prepatent periods for species of *Mansonella* were estimated to be of moderate length, on the order of 5–6 mo (Orihel et al., 1981; Eberhard and Lowrie, 1987), and those for species of *Dipetalonema* were longer, 9–10 mo (Travie et al., 1985). Again, the prevalences of microfilariae reported in this study (25.9% for *M. mariae* and 3.5% for *D. caudispina*) may be underestimations. The squirrel monkey was refractory to experimental infection with the human filarial worm, *Mansonella ozzardi* (Orihel et al., 1981). Therefore, it is not unusual to refer to a newly obtained nucleotide sequence of the ITS region of microfilariae from our squirrel monkeys (DDBJ/EMBL/GenBank AB362562) as *M. mariae*, although ITS nucleotide sequences of these 2 species closely resembled each other.

Sullivan et al. (1993) discussed, in detail, the possibility that the natural trypanosome or filarial infections may modify the experimental infection of these monkeys, confounding the variables that these infections can induce; they also discussed the potential risk for animal handlers and laboratory staff to infection by *T. cruzi*. Detection of scanty *T. minasense*, *T. rangeli*, and *T. cruzi* parasitemia is difficult, particularly by Giemsa-stained blood films, and sustained infection must have been commonly overlooked to date. We do not know the effects of this background infection to the aimed experimental analyses using Neotropical nonhuman primates. Furthermore, the vector transmitting *T. minasense* is still unknown at present (Dunn et al., 1963; Hoare, 1972). During transportation and laboratory maintenance, possible transmission of the trypanosomes from infected Neotropical nonhuman primates to uninfected individuals remains a possibility. The partial molecular characterization of *T. minasense* in the present study enables us to identify the vector(s) for this species, to survey the accurate prevalence of this trypanosome infection in natural habitats, or to diagnose the infection in captivity. Accumulation of more data on the

biology of *T. minasense*, as well as on trypanosome species of the subgenus *Megatrypanum* in Neotropical nonhuman primates, is required.

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LITERATURE CITED

BELTRAME-BOTELHO, I. T., D. GASPAR-SILVA, M. STEINDEL, A. M. R. DÁVILA, AND E. C. GRISARD. 2005. Internal transcribed spacers (ITS) of *Trypanosoma rangeli* ribosomal DNA (rDNA): A useful marker for inter-specific differentiation. Infection, Genetics and Evolution **5:** 17–28.

DA SILVA, F. M., H. NOYES, M. CAMPANER, A. C. JUNQUEIRA, J. R. COURA, N. ANEZ, J. J. SHAW, J. R. STEVENS, AND M. M. TEIXEIRA. 2004. Phylogeny, taxonomy and grouping of *Trypanosoma rangeli* isolates from man, triatomines and sylvatic mammals from widespread geographical origin based on SSU and ITS ribosomal sequences. Parasitology **129:** 549–561.

———, A. C. RODRIGUES, M. CAMPANER, C. S. A. TAKATA, M. C. BRIDIDO, A. C. V. JUNQUEIRA, J. R. COURA, G. F. TAKEDA, J. J. SHAW, AND M. M. G. TEIXEIRA. 2004. Randomly amplified polymorphic DNA analysis of *Trypanosoma rangeli* and allied species from human, monkeys and other sylvatic mammals of the Brazilian Amazon disclosed a new group and species-specific marker. Parasitology **128:** 283–294.

DEANE, L. M., AND R. G. DAMASCENO. 1961. Tripanosomídeos de mamíferos da região amazônica II. Tripanosomas de macacos da Zona do Salgado, Estado do Pará. Revista do Instituto de Medicina Tropical de São Paulo **3:** 149–160.

———, J. E. SILVA, AND L. F. LOURES. 1974. Nycthemeral variation in the parasitaemia of *Trypanosoma minasense* in naturally infected marmosets of the genus *Callithrix* (Primates, Callithricidae). Revista do Instituto de Medicina Tropical de São Paulo **16:** 1–16.

DE RESENDE, D. M., L. H. PEREIRA, AND A. LÔBO. 1994. Long-term patency of blood parasitism by *Trypanosoma minasense* and microfilariae in *Callithrix penicillata* marmosets (Primates, Callitrichidae), caught at the wild and maintained in captivity. Memórias do Instituto Oswaldo Cruz **89:** 127–128.

DUNN, F. L., AND F. L. LAMBRECHT. 1963. On some filarial parasites of South American primates, with a description of *Tetrapetalonema tamarinae* n. sp. from the Peruvian tamarin marmoset, *Tamarinus nigricollis* (SPIX, 1823). Journal of Helminthology **37:** 261–286.

———, ———, AND R. DU PLESSIS. 1963. Trypanosomes of South American monkeys and marmosets. American Journal of Tropical Medicine and Hygiene **12:** 524–534.

EBERHARD, M. L., AND R. C. LOWRIE, JR. 1987. Laboratory studies on *Mansonella marmosetae* in the squirrel monkey, *Saimiri sciureus*. Journal of Parasitology **73:** 233–234.

———, ———, AND T. C. ORIHEL. 1979. Development of *Dipetalonema gracile* and *D. caudispina* to the infective stage in *Culicoides hollensis*. Journal of Parasitology **65:** 89–95.

ESSLINGER, J. H., AND C. H. GARDINER. 1974. *Dipetalonema barbascalensis* sp. n. (Nematoda: Filarioidea) from the owl monkey, *Aotus trivirgatus*, with a consideration of the status of *Parlitomosa zakii* Nagaty, 1935. Journal of Parasitology **60:** 1001–1005.

GOTTSCHLING, M., AND J. PLÖTNER. 2004. Secondary structure models of the nuclear internal transcribed spacer regions and 5.8S rRNA in Calciodinelloideae (Peridiniaceae) and other dinoflagellates. Nucleic Acids Research **32:** 307–315.

HAMILTON, P. B., J. R. STEVENS, M. W. GAUNT, J. GIDLEY, AND W. C. GIBSON. 2004. Trypanosomes are monophyletic: Evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. International Journal for Parasitology **34:** 1393–1404.

———, ———, AND W. C. GIBSON. 2007. Patterns of co-evolution between trypanosomes and their hosts deduced from ribosomal RNA and protein-coding gene phylogenies. Molecular Phylogenetics and Evolution **44:** 15–25.

———, ———, J. GIDLEY, P. HOLZ, AND W. C. GIBSON. 2005. A new lineage of trypanosomes from Australian vertebrates and terrestrial bloodsucking leeches (Haemadipsidae). International Journal for Parasitology **35:** 431–443.

———, ———, P. HOLZ, B. BOAG, B. COOKE, AND W. C. GIBSON. 2005. The inadvertent introduction into Australia of *Trypanosoma nabiasi*, the trypanosome of the European rabbit (*Oryctolagus cuniculus*), and its potential for biocontrol. Molecular Ecology **14:** 3167–3175.

HOARE, C. A. 1972. The trypanosomes of mammals: A zoological monograph. Blackwell Scientific Publications, Oxford, U.K., p. 749.

HUGHES, A. L., AND H. PIONTKIVSKA. 2003. Molecular phylogenetics of Trypanosomatidae: Contrasting results from 18S rRNA and protein phylogenies. Kinetoplastid Biology and Disease 2: 15 [available from: http://www.kinetoplastids.com/content/2/1/15].

HWANG, S.-K., AND J.-G. KIM. 2000. Secondary structural and phylogenetic implications of nuclear large subunit ribosomal RNA in the ectomycorrhizal fungus *Tricholoma matsutake*. Current Microbiology **40:** 250–256.

KUMAR, S., K. TAMURA, AND A. NEI. 2004. MEGA3: Intergrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics **5:** 150–163.

KUNTZ, R. E., AND B. J. MEYERS. 1972. Parasites of South American primates. International Zoo Yearbook **12:** 61–68.

LAMBRECHT, F. L. 1965. An unusual trypanosome in *Cebus griseus* F. Cuvier, 1819, from Colombia, South America. Revista do Instituto de Medicina Tropical de São Paulo **7:** 89–98.

LANHAM, S. M., M. A. MILES, A. A. A. SOUZA, AND M. M. PÓVOA. 1984. Anion-exchange separation for neotropical trypanosomes: A preliminary trial and a description of *Trypanosoma devei* from the tamarin *Saguinus midas niger*. Zeitschrift für Parasitenkunde **70:** 311–319.

LUKES, J., M. JIRKU, D. DOLEZEL, I. KRAL'OVÁ, L. HOLLAR, AND D. A. MASLOV. 1997. Analysis of ribosomal RNA genes suggests that trypanosomes are monophyletic. Journal of Molecular Evolution **44:** 521–527.

MARINKELLE, C. J. 1968. *Trypanosoma lambrechti* n. sp. aislado de micos (*Cebus albifrons*) de Colombia. Caldasia **10:** 155–164.

MORALES-HOJAS, R., R. J. POST, A. J. SHELLEY, M. MAIA-HERZOG, S. COSCARÓN, AND R. A. CHEKE. 2001. Characterization of nuclear ribosomal DNA sequences from *Onchocerca volvulus* and *Mansonella ozzardi* (Nematoda: Filarioidea) and development of a PCR-based method for their detection in skin biopsies. International Journal for Parasitology **31:** 169–177.

NOYES, H. A., J. R. STEVENS, M. TEIXEIRA, J. PHELAN, AND P. HOLZ. 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. (Corrigendum: International Journal for Parasitology 30: 228 [2000]).

ORIHEL, T. C. 1970. The helminth parasites of nonhuman primates and man. Laboratory Animal Care **20:** 395–401.

———, R. C. LOWRIE, JR., M. L. EBERHARD, C. RACCURT, W. J. KOZEK, M. A., TIDWELL, AND M. TIDWELL. 1981. Susceptibility of laboratory primates to infection with *Mansonella ozzardi* from man. American Journal of Tropical Medicine and Hygiene **30:** 790–794.

PETIT, G., O. BAIN, AND C. ROUSSILHON. 1985. Deux nouvelles filaires chez un singe, *Saimiri sciureus*, au Guyana. Annales de Parasitologie Humaine et Comparée **60:** 65–81.

POTKAY, S. 1992. Diseases of the Calltrichidae: A review. Journal of Medical Primatology **21:** 189–236.

SATO, H., B. H. AL-ADHAMI, Y. UNE, AND H. KAMIYA. 2007. *Trypanosoma* (*Herpetosoma*) *kuseli* sp. n. (Protozoa: Kinetoplastida) in Siberian flying squirrels (*Pteromys volans*). Parasitology Research **101:** 453–461.

———, K. MATSUO, H. KAMIYA, T. ISHIKAWA, S. OKABAYASHI, N. KISHI, AND Y. UNE. 2003. *Ptergodermatites nycticebi* (Nematoda: Rictulariidae): Accidental detection of encapsulated third-stage larvae in the tissue of a white-fronted marmoset. Journal of Parasitology **89:** 1163–1166.

———, A. OSANAI, H. KAMIYA, Y. OBARA, W. JIANG, Q. ZHEN, J. CHAI, Y. UNE, AND M. ITO. 2005. Characterization of SSU and LSU rRNA

genes of three *Trypanosoma* (*Herpetosoma*) *grosi* isolates maintained in Mongolian jirds. Parasitology **130:** 157–167.

———, K. Suzuki, A. Osanai, H. Kamiya, and H. Furuoka. 2006. Identification and characterization of the threadworm, *Strongyloides procyonis*, from feral raccoons (*Procyon lotor*) in Japan. Journal of Parasitology **92:** 63–68.

Simpson, A. G. B., J. Lukes, and A. J. Roger. 2002. The evolutionary history of kinetoplastids and their kinetoplasts. Molecular Biology and Evolution **19:** 2071–2083.

———, H. Noyes, and W. Gibson. 1998. The evolution of trypanosomes infecting human and primates. Memórias do Instituto Oswaldo Cruz **93:** 669–676.

———, J. R. Stevens, and J. Lukes. 2006 The evolution and diversity of kinetoplastid flagellates. Trends in Parasitology **22:** 168–174.

Stevens, J., H. A. Noyes, C. J. Schofield, and W. Gibson. 2001. The molecular evolution of Trypanosomatidae. Advances in Parasitology **48:** 1–56.

———, M. M. G. Teixeira, L. E. H. Bingle, and W. C. Gibson. 1999. The taxonomic position and evolutionary relationships of *Trypanosoma rangeli*. International Journal for Parasitology **29:** 749–757.

Sullivan, J. J., F. Steurer, G. Benavides, R. L. Tarleton, M. L. Eberhard, and S. Landry. 1993. Trypanosomes and microfilariae in feral owl and squirrel monkeys maintained in research colonies. American Journal of Tropical Medicine and Hygiene **49:** 254–259.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTRAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research **22:** 4673–4680.

Travi, B. L., M. L. Eberhard, and R. C. Lowrie, Jr. 1985. Development of *Dipetalonema gracile* in the squirrel monkey (*Saimiri sciureus*), with notes on its biology. Journal of Parasitology **71:** 17–19.

Votypka, J., J. Lukeš, and M. Oborník. 2004. Phylogenetic relationship of *Trypanosoma corvi* with other avian trypanosomes. Acta Protozoologica **43:** 225–231.

WHO. 1988. Role of nonhuman primates in malaria vaccines development. Bulletin de lOrganisation Mondiale de la Sant **66:** 686–687.

Yamashita, J. 1963. Ecological relationships between parasites and primates, I. Helminth parasites and primates. Primates **4:** 25–49.

Zhu, X., R. B. Gasser, D. E. Jacobs, G.-C. Hung, and N. B. Chilton. 2000. Relationships among some ascaridoid nematodes based on ribosomal DNA sequence data. Parasitology Research **86:** 738–744.

Ziccardi, M., and R. Lourenço-de-Oliveira. 1997. The infection rates of trypanosomes in squirrel monkeys at two sites in the Brazilian Amazon. Memórias do Instituto Oswaldo Cruz **92:** 465–470.

———, and ———. 1998. Morphological features of trypanosomes from squirrel monkeys from the Brazilian Amazon. Memórias do Instituto Oswaldo Cruz **93:** 45–55.

———, and ———. 1999. Polymorphism in trypomastigotes of *Trypanosoma* (*Megatrypanum*) *minasense* in the blood of experimentally infected squirrel monkey and marmosets. Memórias do Instituto Oswaldo Cruz **94:** 649–653.

———, ———, M. C. Alves, and M. F. F. da Cruz. 2005. *Trypanosoma saimirii* Rodhain, a junior synonym of *Trypanosoma rangeli* Tejera. Journal of Parasitology **91:** 653–656.

———, ———, R. Lainson, M. C. O. Brígido, and J. A. P. C. Muniz. 2000. Typanosomes of nonhuman primates from the national centre of primates, Ananindeua, State of Pará, Brazil. Memórias do Instituto Oswaldo Cruz **95:** 157–159.

———, ———, and R. Nogueira. 1996. The haemoculture of *Trypanosoma minasense* Chagas, 1908. Memórias do Instituto Oswaldo Cruz **91:** 501–505.

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Short communication

Virulence characteristics of *Yersinia pseudotuberculosis* isolated from breeding monkeys in Japan

Taketoshi Iwata [a], Yumi Une [b], Alexandre Tomomitsu Okatani [b], Yukio Kato [b], Aya Nakadai [a], Ken-Ichi Lee [a], Maiko Watanabe [a], Takahide Taniguchi [a], AbdelAzim Elsayed Elhelaly [a], Yoshikazu Hirota [a], Hideki Hayashidani [a,*]

[a] *Division of Animal Science, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*

[b] *School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan*

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Abstract

Between April 2001 and 2007, 18 *Yersinia pseudotuberculosis* outbreaks occurred in breeding monkeys at 12 zoological gardens in Japan, and 28 monkeys of 8 species died. A total of 18 *Y. pseudotuberculosis* strains from the dead monkeys, comprising one strain per outbreak, were examined for serotype and the presence of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB* and *ypmC*) and *irp2*. Of the 18 *Y. pseudotuberculosis* strains, 7 (38.9%) were serotype 4b, 7 (38.9%) were serotype 1b, and there was one each of serotypes 2b, 3, 6 and 7. All the 18 strains examined harbored *virF* and *inv*. Sixteen (88.9%) strains, including the strain of serotype 7, harbored *ypmA*. However, no strain harbored *ypmB*, *ypmC* and *irp2*.

This study demonstrated that among other pathogenic factors, almost all the *Y. pseudotuberculosis* isolated from the outbreaks had the *ypm* gene encoding the superantigenic toxin, YPM. As most of the monkeys who died in those outbreaks originated from South America and other regions, where the presence of the *ypm* gene have not been reported, YPM might be the cause, or at least the most important factor for, the high mortality of the breeding monkeys infected by *Y. pseudotuberculosis* in Japan. This is also the first report of a fatal case due to *Y. pseudotuberculosis* serotype 7 infection in the world.

Keywords: *Yersinia pseudotuberculosis*; Breeding monkey; Virulence genes; YPM

1. Introduction

Yersinia pseudotuberculosis is known to be an important causal agent of zoonosis. Monkey species are especially sensitive to *Y. pseudotuberculosis*, and many fatal cases of *Y. pseudotuberculosis* infection in

* Corresponding author. Tel.: +81 42 367 5775; fax: +81 42 367 5775.
E-mail address: eisei@cc.tuat.ac.jp (H. Hayashidani).

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breeding monkeys have been reported throughout the world, including in Japan (Buhles et al., 1981; Hirai et al., 1974; Kageyama et al., 2002; MacArthur and Wood, 1983; Maruyama et al., 1983; Murata and Hama, 1992; Rosenberg et al., 1980; Sasaki et al., 1996; Taffs and Dunn, 1983; Une et al., 2003). Affected monkeys may die unexpectedly or after a very short illness, and at the present time there is no effective preventive method against *Y. pseudotuberculosis* infection. Therefore, monkey *Y. pseudotuberculosis* infection poses a serious problem for zoological gardens engaged in monkey breeding.

The pathogenicity of *Y. pseudotuberculosis* is associated with several virulence factors. Pathogenic strains of *Y. pseudotuberculosis* harbor 70-kb virulence plasmid (pYV), which encodes a number of important virulence and virulence-associated proteins. Additionally, a high-pathogenicity island (HPI), encoding an iron uptake system represented by its siderophore yersiniabactin (Carniel, 1999), and *Y. pseudotuberculosis*-derived mitogen (YPM), which is a superantigenic toxin, are known to play important roles in causing severe systemic infection (Abe et al., 1997). However, it remains unclear which virulence factor is connected with the high mortality of monkeys in *Y. pseudotuberculosis* infection. In the present study, we investigated the characteristics of *Y. pseudotuberculosis* isolated from dead breeding monkeys in Japan.

2. Materials and methods

2.1. Bacterial strains

Eighteen *Y. pseudotuberculosis* strains isolated from monkeys that died in 18 outbreaks (one strain per outbreak) were analyzed. These outbreaks occurred between April 2001 and 2007 at 12 zoological gardens (A–L) in Japan, and a total of 28 monkeys of 8 species, comprising 19 squirrel monkeys (*Saimiri sciureus*), 2 hamadryas baboons (*Papio hamadryas*), 2 white-faced sakis (*Pithecia pithecia*), 1 agile gibbon (*Hylobates agilis*), 1 dusky leaf monkey (*Presbytis obscurus*), 1 orangutan (*Pongo pygmaeus*), 1 ring-tailed lemur (*Lemur catta*) and 1 ruffed lemur (*Varecia variegata*), died (Table 1). Pathological findings such as swelling of the Peyer's patch and abscesses in the spleen and liver were typical of yersiniosis. Outbreaks occurred two, three and four times in the zoological gardens C, H and G, respectively.

Table 1
Sources of *Y. pseudotuberculosis* isolated from breeding monkeys in Japan

No.	Strain	Institution	Region	Isolation month year	Source (number and species of other monkeys dead in the same outbreak)
1	NP011001	A	Kanto	April 2002	Squirrel monkey
2	NP031103	B	Kanto	November 2003	Orangutan
3	NP031101	C	Kanto	November 2003	Squirrel monkey (1 squirrel monkey)
4	NP050101	C	Kanto	January 2005	Squirrel monkey
5	NP070401	D	Kanto	April 2007	Dusky leaf monkey
6	NP031201	E	Kinki	December 2003	Squirrel monkey (2 squirrel monkeys)
7	NP040301	F	Chugoku	March 2004	Squirrel monkey
8	NP010401	G	Sikoku	April 2001	Squirrel monkey
9	NP030401	G	Sikoku	April 2003	Squirrel monkey
10	NP050102	G	Sikoku	January 2005	Squirrel monkey
11	NP051201	G	Sikoku	December 2005	Squirrel monkey
12	NP020501	H	Kyusyu	May 2002	Squirrel monkey
13	NP030601	H	Kyusyu	June 2003	Squirrel monkey
14	NP070201	H	Kyusyu	February 2007	Squirrel monkey
15	NP030701	I	Kyusyu	July 2003	Squirrel monkey (1 squirrel monkey)
16	NP050201	J	Kyusyu	February 2005	Hamadryas baboon (1 hamadryas baboon and 1 agile gibbon)
17	NP050301	K	Kyusyu	March 2005	Squirrel monkey (1 squirrel monkey)
18	NP050303	L	Kyusyu	March 2005	White-faced saki (1 white-faced saki, 1 ruffed lemur and 1 ring-tailed lemur)

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