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Detection of *Babesia hongkongensis* sp. nov. in a Free-Roaming *Felis catus* Cat in Hong Kong

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Intraerythrocytic *Babesia*-like trophozoites were seen in postmortem kidney sections of a free-roaming cat in Hong Kong. DNA sequences of the 18S rRNA and mitochondrial cytochrome *b* genes had only 96.7% and 90.4% nucleotide identity with known *Babesia* sequences. We propose that this new species be named *Babesia hongkongensis.*

Babesiosis is the commonest vectorborne canine infection in Hong Kong, with 48% and 33% of the stray and pet dogs being infected, respectively (14). Human babesiosis is usually caused by *Babesia microti*, *B. divergens*, and some newly described strains such as the WA1, EU1, -2, and -3, CA1, -2, -3, and -4, and KO1 types (9). We incidentally observed *Babesia*-like organisms in the erythrocytes in feline kidney sections during a previous study (15).

Postmortem kidney tissues and peripheral EDTA blood were collected from euthanized free-roaming cats between March 2009 and February 2011 for a previous study on a morbillivirus associated with feline tubulointerstitial nephritis (15). DNA was extracted from EDTA whole-blood and kidney samples by the use of an EZ1 minikit (Qiagen, Hilden, Germany). The DNA was eluted in 60 μl of elution buffer and was used as the template for PCR and sequencing.

Blood and kidney tissues were screened using primers listed in Table 1. The PCR mixture (25 μl) contained DNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM $MgCl_2$, and 0.01% gelatin), 200 μM each deoxynucleoside triphosphates (dNTPs), and 1.0 U of *Taq* polymerase (Applied Biosystems, Foster City, CA). The mixtures were amplified in 60 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min in an automated thermal cycler (Applied Biosystems, Foster City, CA). A *Babesia gibsoni* strain found in our previous study was used as a positive control. PCR products were gel purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Both strands of the PCR products were sequenced twice using an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA). The sequences of the PCR products were compared with known sequences by BLAST analysis against the NCBI database.

Phylogenetic tree was constructed by the neighbor-joining method using Kimura's two-parameter correction with ClustalX 1.83, with bootstrap values calculated from 1,000 trees. The 1,368 and 364 bp of amplicons from the 18S rRNA and mitochondrial cytochrome *b* genes of the new *Babesia* species detected in this study were included in the analysis, using *Plasmodium* spp. as the outgroup.

The infected cat was clinically asymptomatic antemortem, but a full autopsy was not performed. A total of 457 blood samples and

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TABLE 1 Sequence of primers used in the study obtained by multiple alignments with CLUSTALW

Primer category or species name	Target gene	Primer name (sequence)	Target length (bp)	Primer design
Piroplasms	18S rRNA	P_18S1F (AAGATTAAGCCATGCATGTCTAA) P_18S1612R (AGTGATAAGGTTCACAAAACTT)	1,612	Consensus primers designed by multiple alignment of available 18S rRNA genes of known piroplasms and *Hepatozoon* spp.
Piroplasms	18S rRNA	P_18S1F (AAGATTAAGCCATGCATGTCTAA) P_18S522R (ATACGCTATTGGAGCTGGAATTA) P_18S500F (TAATTCCAGCTCCAATAGCGTAT) P_18S1071R (GTGTTGAGTCAAATTAAGCCGCA) P_18S1049F (TGCGGCTTAATTTGACTCAACAC) P_18S1612R (AGTGATAAGGTTCACAAAACTT)		Sequencing primers used for constructing the 18S rRNA gene of *Babesia hongkongensis*
Babesia hongkongensis (proposed name of the new *Babesia* species described in this study)	18S rRNA	BH_18S565F (CGTTTGGGCTTTTAGCTTT) BH_18S737R (TTAACCATTACTAAGGTTCCCA)	173	Screening primers designed specifically from the 18S rRNA gene of *Babesia hongkongensis.*
Piroplasms	mitochondrial cytochrome *b* (*cytb*)	P_cytbF (TGTTGCTCCCCAATAACTCATTT) P_cytbR (AGGAATTTAAATTCTAATTGGAATT)	359	Consensus primers designed by multiple alignment of available *cytb* gene of *Babesia bigemina*, *B. bovis*, *B. caballi*, *B gibsonii*, and *Theileria equi*.

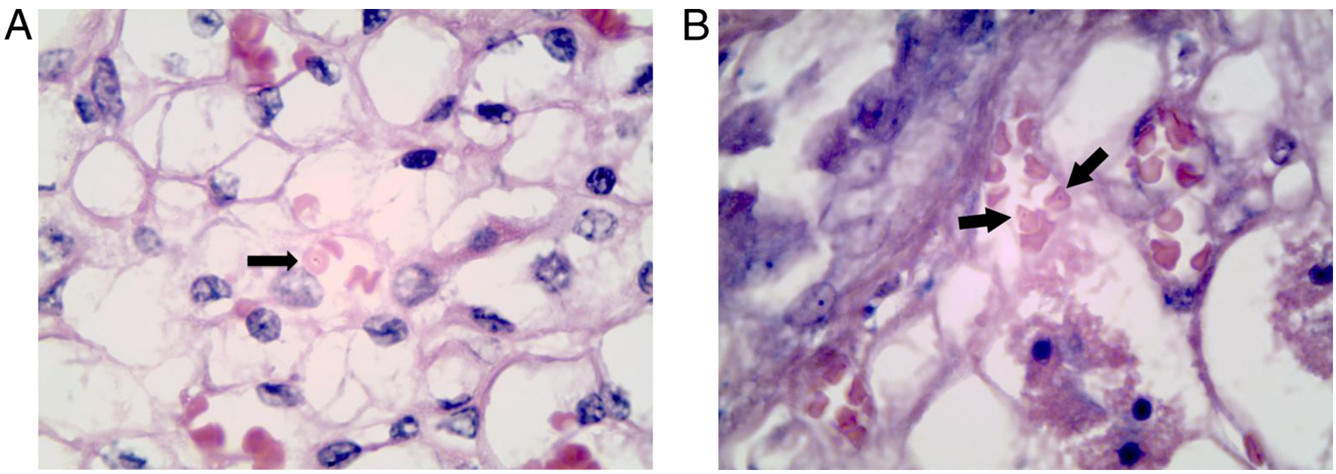

FIG 1 Photomicrographs of kidney sections showing *Babesia*-like organisms. (A) Single, small, round-to-oval intracellular organism with light-blue cytoplasm and an eccentric purple nucleus (signet ring-shaped) in an erythrocyte within the vasa recta of formalin-fixed renal tissue from a free-roaming cat in Hong Kong (hematoxylin and eosin [H&E] staining; original magnification, ×1,000). (B) Single, small, round-to-oval intracellular organism with light-blue cytoplasm in two erythrocytes within the vasa recta of formalin-fixed renal tissue (Giemsa staining; original magnification, ×1,000).

TABLE 2 Taxonomy, GenBank accession numbers, hosts, geographical regions of isolation, and percentages of sequence identity of *Babesia hongkongensis* sp. nov. with the 38 piroplasms and *Hepatozoon* spp. used as operational taxonomic units in the phylogenetic analysis

Species	GenBank accession no.	Host	Geographical region of isolation	Yr of isolation	Percent sequence identity with *B. hongkongensis*
Babesia sp. SAP#091	AB251609.1	Feral raccoon	Japan	2009	96.7
Babesia capreoli	GQ304526	Deer	France	2011	95.1
Babesia divergens	GQ304525.1	Deer	France	2011	95.1
Babesia odocoilei	AY237638.1	Reindeer	United States	2004	95.1
Babesia gibsoni	AB478329	Dog	Japan	2010	95.3
Babesia canis rossi	DQ111760	Dog	Japan	2005	94.6
Babesia canis canis	AY072926	Dog	Europe	2002	94.5
Babesia canis vogeli	AY072925	Dog	Europe	2002	94.3
Babesia caballi	EU642513	Horse	South Africa	2009	94.3
Babesia kiwiensis	EF551335.1	Brown kiwi	Australia	2008	94.5
Babesia major	GU194290.1	Cattle	France	2009	93.5
Babesia orientalis	HQ840969.1	Water buffalo	China	2011	93.6
Babesia bigemina	DQ785311.1	Cattle	Spain	2007	93.6
Babesia crassa	AY260176	Sheep	Germany	2004	92.9
Babesia motasi	AY533147.1	Sheep	Spain	2004	93.2
Babesia occultans	HQ331479.1	*Hyalomma* ticks	Tunisia	2011	93.4
Babesia ovis	AY150058.1	Goat	Spain	2006	90.7
Babesia duncani	HQ285838.1	Human	United States	2011	88.8
Babesia leo	AF244911.1	Lion	South Africa	2004	88.6
Babesia felis	AY452707.1	Cat	South Africa	2004	88.8
Babesia lengau	GQ411417.1	Cheetah	South Africa	2010	88.4
Babesia conradae	AF158702	Dog	United States	2008	88.1
Babesia rodhaini	AB049999.1	Mouse	Japan	2008	87.8
Babesia microti	U09833.1	Mouse	United States	1994	87.7
Babesia microti JM1	AB576641.1	Monkey	Japan	2011	87.4
Babesia bovis	L31922.1	Cattle	Mexico	2001	85.1
Theileria velifera	AF097993.1	Cattle	Tanzania	1999	89.9
Theileria ovis	FJ603460.1	Goat	China	2011	89.9
Theileria cervi WU11	HQ184411.1	Sika deer	China	2010	89.9
Theileria sinensis	EU277003.1	*Bos grunniens*	China	2008	89.8
Thseileria orientalis	AB520957.1	Cattle	Australia	2011	89.6
Theileria buffeli	HQ840968.1	Water buffalo	China	2011	89.6
Theileria bicornis	AF499604.1	Black rhinoceros	South Africa	2003	88.8
Theileria equi	EU642511.1	Horse	South Africa	2009	88.7
Cytauxzoon felis	AF399930.1	Cat	United States	2002	87.4
Hepatozoon felis	AY628681.1	Cat	Spain	2006	87.4
Hepatozoon americanum	AF176836.1	Dog	United States	2001	86.1
Hepatozoon canis	DQ111754.1	Dog	Japan	2005	85.3
Plasmodium falciparum	M19172.1	Human	Africa	1993	79.4

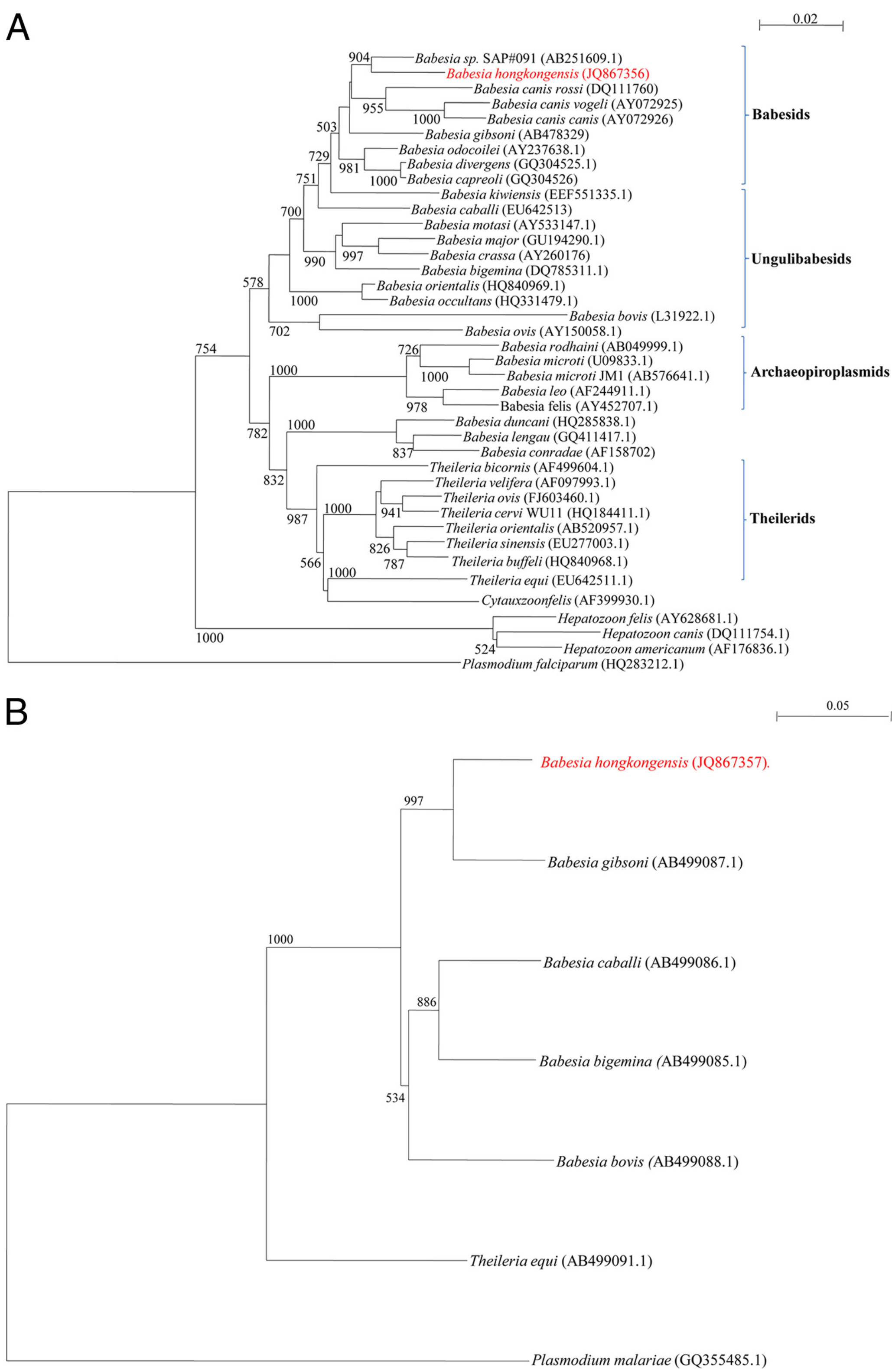

FIG 2 Phylogenetic study of *Babesia hongkongenesis.* (A) Phylogenetic analysis of nucleotide sequences of the 1,368-bp fragment of the 18S rRNA gene of *Babesia hongkongensis* sp. nov. identified from a free-roaming cat in the present study. The tree was constructed by the neighbor-joining method using Kimura-2 correction and bootstrap values calculated from 1,000 trees. The scale bar indicates the estimated number of substitutions per 50 nucleotides. *Plasmodium falciparum* (HQ283212.1) was used as the outgroup. (B) Phylogenetic analysis of nucleotide sequences of the 364-bp fragment of the mitochondrial cytochrome *b* gene of *Babesia hongkongensis* sp. nov. identified from the free-roaming cat in the present study. The tree was constructed by the neighbor-joining method using Kimura-2 correction and bootstrap values calculated from 1,000 trees. The scale bar indicates the estimated number of substitutions per 20 nucleotides. *Plasmodium malariae* (GQ355485.1) was used as the outgroup.

48 kidney samples were obtained. One of the 48 kidney sample sections showed intraerythrocytic *Babesia*-like trophozoites (Fig. 1A). The trophozoites were round to oval, with a light-blue cytoplasm and an eccentric purple nucleus. Single rings were slightly more often found to be located near the center of the erythrocyte. The organism resembles a small *Babesia* species, with ring forms measuring 1.4 to 1.6 μm in diameter. Similar trophozoites at various stages of development were also seen in Giemsa-stained sections of the kidney (Fig. 1B). No organisms were seen within the leukocytes in the sections.

Three hundred randomly selected archival blood specimens were screened for *Babesia* 18S rRNA PCR. Only the aforementioned cat's specimen was positive. The *Babesia*-positive cat's blood and the kidney tissue were both PCR positive using consensus 18S rRNA primers for *Babesia*.

Nearly the full ~1,700 bp of the 18S rRNA gene of the new *Babesia* species were built by consensus primer PCR and sequencing (Table 1). The DNA sequences from the kidney section and peripheral blood were identical. BLAST analyses of the sequence did not fully match with any of the sequences in GenBank. It was most closely related (94.3% to 96.7% nucleotide identity with 98% to 100% coverage) to various *Babesia* sequences found in feral raccoon and dogs (Table 2). By using the ClustalW option of BioEdit, we aligned 1,368 bp of the *B. hongkongensis* 18S rRNA gene sequence to 38 sequences (Table 2) of other members of the *Piroplasmida* and *Hepatozoon* spp. representative of the 5 groups identified within this order as previously defined (8). A representative tree is shown in Fig. 2A. *B. hongkongensis* falls into a distinct branch of the Babesiidae. The phylogenetic tree is consistent with the topology of previously reported analyses based on 18S rRNA gene sequences of piroplasmids (8). Internal branches of the trees were statistically supported by high bootstrap values. Phylogenetic analysis of a 364-bp region of the mitochondrial cytochrome *b* gene (Fig. 2B) was most closely related to *B. gibsoni*. Although only a few *Babesia* mitochondrial cytochrome *b* gene sequences have been reported to date, our strain's sequence has only 90.4% identity with that of *B. gibsonii*. As with the 18S rRNA gene sequence analysis, the Ungulibabesids and Theilerides were grouped separately. We propose that this new *Babesia* strain, genetically and geographically distinct from all other previously described species, be tentatively named *Babesia hongkongensis* sp. nov.

Feline babesiosis has been described in domestic cats and wild felines (lions, leopards, panthers, cougars, and cheetahs) and is caused by *B. felis*, *B. cati*, *B. leo*, *B. canis presentii*, *B. canis canis*, *B. canis vogeli*, *B. pantherae*, *B. herpalluri*, and *B. microti*-like spp. (*Theileria annae*) (2–6, 10, 11, 12). Few studies have addressed the prevalence of *Babesia* in domestic and urban free-roaming cats. In Pakistan, a prevalence of 3.14% was found in pet cats as detected by light microscopy (1). Using PCR to supplement light microscopy, *Babesia* was found in 1.4% of stray cats in Bangkok (12). Molecular studies contribute to the identification of new species which may have similar microscopic appearances and to diagnosing novel infections caused by environmental species which may initially be misdiagnosed as babesiosis (16). Accurate species identification is important in that different species may have different clinical manifestations and antiparastic drug susceptibilities (2).

The prevalence of *B. hongkongensis* appears to be low (0.3%) among free-roaming cats. Its prevalence and pathogenicity in pet cats have to be explored. Pet ownership is common is most countries. In Hong Kong, 12.6% of the households were keeping pet animals, with 22.3% of them having cats (7). If *B. hongkongensis* causes disease in pet animals, this could represent a significant veterinary problem.

Thorough examination of the peripheral blood is important to confirm the absence of a schizogony cycle in leukocytes, which would classify the organism as a *Theileria* (13). However, our current phylogenetic analysis suggests that this is unlikely, since it is closely clustered with other *Babesia* species. The vector for *B. hongkongensis* is unknown, but all known *Babesia* species utilize hard ticks as the arthropod vector for transmission. Further study should be performed to understand the epidemiology, life cycle, host vector, pathogenicity, and drug susceptibility of this new feline *Babesia* species. The pathogenicity and zoonotic potential of this new feline *Babesia* species remain to be determined by further studies.

Nucleotide sequence accession numbers. Partial nucleotide sequences of the 18S rRNA and mitochrondrial cytochrome b genes obtained in this study have been deposited in the GenBank sequence database under accession numbers JQ867356 to JQ867357.

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We declare no conflict of interest.

REFERENCES

1. **Ahmad SS, Khan MS, Khan MA, Ahmad N.** 2011. Prevalence of babesiosis in cats in Lahore, Pakistan. J. Animal Plant Sci. **21**(2 Suppl.):354–357.
2. **Ayoob AL, Hackner SG, Prittie J.** 2010. Clinical management of canine babesiosis. J. Vet. Emerg. Crit. Care (San Antonio) **20**:77–89.
3. **Ayoob AL, Prittie J, Hackner SG.** 2010. Feline babesiosis. J. Vet. Emerg. Crit. Care (San Antonio) **20**:90–97.
4. **Baneth G, et al.** 2004. Infection with a proposed new subspecies of *Babesia canis*, *Babesia canis* subsp. *presentii*, in domestic cats. J. Clin. Microbiol. **42**:99–105.
5. **Bosman AM, Oosthuizen MC, Peirce MA, Venter EH, Penzhorn BL.** 2010. *Babesia lengau* sp. nov., a novel *Babesia* species in cheetah (*Acinonyx jubatus*, Schreber, 1775) populations in South Africa. J. Clin. Microbiol. **48**:2703–2708.
6. **Bosman AM, Venter EH, Penzhorn BL.** 2007. Occurrence of *Babesia felis* and *Babesia leo* in various wild felid species and domestic cats in Southern Africa, based on reverse line blot analysis. Vet. Parasitol. **144**:33–38.
7. **Census and Statistics Department Hong Kong Special Administrative Region.** 2005. Thematic household survey report—report No. 26. Census and Statistics Department, Hong Kong Special Administrative Region. http://www.censtatd.gov.hk/products_and_services/products/publications/statistical_report/social_data/index_cd_B1130226_dt_detail.jsp.
8. **Criado-Fornelio A, Martinez-Marcos A, Buling-Saraña A, Barba-Carretero JC.** 2003. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part II. Phylogenetic analysis and evolutionary history. Vet. Parasitol. **114**:173–194.

9. **Gray J, Zintl A, Hildebrandt A, Hunfeld KP, Weiss L.** 2010. Zoonotic babesiosis: overview of the disease and novel aspects of pathogen identity. Ticks Tick Borne Dis. **1**:3–10.
10. **Luaces I, et al.** 2005. First report of an intraerythrocytic small piroplasm in wild Iberian lynx (*Lynx pardinus*). J. Wildl. Dis. **41**:810–815.
11. **Penzhorn BL, Kjemtrup AM, López-Rebollar LM, Conrad PA.** 2001. *Babesia leo* n. sp. from lions in the Kruger National Park, South Africa, and its relation to other small piroplasms. J. Parasitol. **87**:681–685.
12. **Simking P, Wongnakphet S, Stich RW, Jittapalapong S.** 2010. Detection of *Babesia vogeli* in stray cats of metropolitan Bangkok, Thailand. Vet. Parasitol. **173**:70–75.
13. **Uilenberg G.** 2006. *Babesia*—a historical overview. Vet. Parasitol. **138**: 3–10.
14. **Wong SS, et al.** 2011. Comparative evaluation of a point-of-care immunochromatographic test SNAP 4Dx with molecular detection tests for vector-borne canine pathogens in Hong Kong. Vector Borne Zoonotic Dis. **11**:1269–1277.
15. **Woo PC, et al.** 2012. Feline morbillivirus, a previously undescribed paramyxovirus associated with tubulointerstitial nephritis in domestic cats. Proc. Natl. Acad. Sci. U. S. A. **109**:5435–5440.
16. **Yuan CL, et al.** 2012. *Colpodella* spp.-like parasite infection in woman, China. Emerg. Infect. Dis. **18**:125–127.