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CASE REPORT

Emergence of human babesiosis along the border of China with Myanmar: detection by PCR and confirmation by sequencing

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Babesiosis is a tick-borne, zoonotic disease caused by *Babesia* spp. Two cases of babesiosis were detected by nested polymerase chain reaction (PCR) in Yunnan province, China, and further confirmed by molecular assay. The blood smears showed intraerythrocytic ring form and tetrads typical of small *B. microti*. In both cases, the rapid diagnostic test (RDT) ruled out the possibility of co-infections with malaria. Neither case was initially diagnosed because of the low *Babesia* parasitemia. These two cases of babesiosis in areas along the Myanmar–China border pose the question of the emergence of this under recognized infection in countries or areas where malaria is endemic.

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CASE REPORT

Patient 1 was a 60-year-old man who was hospitalized on 5 August 2013, because of two weeks of fever, arthralgias and malaise. The rapid diagnostic test (RDT) for malaria was negative. Furthermore, no *Plasmodium* parasite was observed on the Giemsa stained blood smears obtained on the first day of hospitalization. The patient had a temperature of 38.5℃, low platelet count (6.9×10^{10}/L) and hematocrit (39.0 %), but other routine blood examinations were normal. He also had increased levels of direct bilirubin (6.10 μmol/L) and alanine aminotransferase (100 U/L). The ultrasonography scan revealed splenomegaly. The patient recalled that he had travelled to Myanmar and back to China frequently, but had never received any blood transfusions or blood products. He also recalled multiple tick bites about 2 months prior to diagnosis, particularly after outdoor activities in the jungle along border areas of China–Myanmar.

Patient 2 was a 30-year-old man who was hospitalized on 9 April 2013, because of two days of fever, diaphoresis, myalgias, progressive dyspnea and fatigue. The patient had a temperature of 38.4℃, a slightly elevated leukocyte count (1.11×10^{10}/L), but normal levels of other routine blood parameters. The RDT for malaria was negative. Originally from Tengchong village, he had travelled to Myanmar 2 months ago, returning home at the beginning of April. He recalled multiple tick bites in the recent past and had also received blood transfusion and blood products for the treatment of renal-malaria caused by infection with *Plasmodium falciparum* during the summer of 2012.

A nested polymerase chain reaction (PCR) with two sets of *Babesia microti* specific primers for the small subunit ribosomal RNA (SSU rRNA) was conducted on blood samples.[1,2] Positive samples were tested using another set of nested PCR primers to detect the beta-tubulin gene of *B. microti*.[3] To identify the clades to which the isolates belong, a more accurate analysis of the sequence encoding the 18S rRNA gene of the *Babesia* parasite from the patients was applied with the nested PCR primers Piro1F/rRNA-3′ and BablA/Prio6R as described by Medlin *et al.*[4] Second round PCR products that were full-length cDNAs for the 18S rRNA gene (~1700 bp) and the beta-tubulin gene (~590 bp) were sequenced. The sequences were entered in the BLAST database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The blood smears showed intraerythrocytic ring form and tetrad typical of small *Babesia* parasites (Figure 1).

Sequences from SSU rRNA and beta-tubulin gene obtained by direct sequencing of PCR products from the two patients were aligned by using the ClustalW method (EMBL-EBI, Hixton, Cambridge, UK). Those sequences were deposited in GenBank with accession NOs KF410825, KJ128385 and KJ128387, respectively. The sequences of our two isolates and other sequences were clustered following the pattern described by Hunfeld *et al.*[5] to identify the group of our isolates.

Phylogenetic trees were produced by using the neighbor joining method in MEGA version 5.1 (http://mega.software.informer.com/5.1b/). The case samples (denoted as Yunnan China) clustered with other *B. microti* isolates. *B. divergens* and *B. rodhaini* were set as the outgroups (Figure 2).

Infection of human babesiosis has only been reported as a few sporadic cases in China.[6] Because malaria RDT was negative for each case,[7] and the blood smears failed to find any protozoan parasites, neither patient was treated with any anti-protozoa drugs. Atovaquone,

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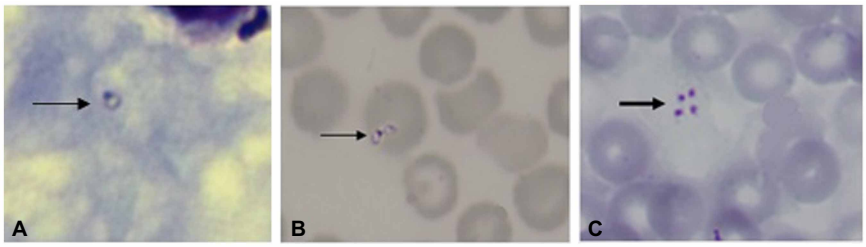

Figure 1 Microscopic evidence of babesiosis in a patient from the China–Myanmar border area. Giemsa stained thick blood smears (**A**) and thin blood smears (**B** and **C**) obtained on the first day of hospitalization for patient show an intraerythrocytic trophozoite (thin arrow). The lacking of hemozoin deposits distinguishes *Babesia* spp. from *Plasmodium* spp. The tetrad (thick arrow) is pathognomonic of small *Babesia* spp. Original magnification, ×1000.

the drug of first choice for treatment of babesiosis is not available in China. However, a combination of penicilin, quinolone and other symptomatic treatments, such as oral paracetamol tablets for the high temperature and supplemental vitamin C and vitamin B1, were administered to these two patients. Both patients had no significant immune deficiency; *Babebsia* infections were relatively benign since fever and other symptoms resolved within 1 month of therapy.

DISCUSSION AND CONCLUSION

Babesiosis is a tick-borne, zoonotic disease caused by the *Babesia* protozoa. The severity of human babesiosis depends on the immune status of the host and on the *Babesia* species causing the infection, ranging from an asymptomatic infection to a severe life threatening disease.[6] In some areas, *B. microti* is known to elicit no symptoms in about half of children and a quarter of adults.[8] Cases caused by *B. duncani* infections have ranged from asymptomatic to fatal.[9] Most cases reported on *B. divergens* infection are severe and most of these severe babesiosis cases occurred in people who lack a spleen.[5,10] The first case of babesiosis infection in an immunocompetent person was reported in 1970.[11] Babesiosis is now classified as a notifiable disease in the United States and is recognized as an emerging health risk in other parts of the world.[6,12,13] In Asia, *B. microti*-like organisms have caused illness in Japan, Taiwan and Zhejiang province of China.[2,14–16] Importantly, human babesiosis has sometimes been diagnosed initially as malaria because of the similarity between the two diseases or the two parasites,[17] but little is known about the prevalence of *Babesia* spp. infections in malaria-endemic areas, where misidentification as *Plasmodium* infection is most probable. We report two cases of ignored babesiosis in China–Myanmar border areas of Yunnan, China, where is endemic area for malaria, suggesting that babesiosis and malaria co-exist in this region. Both cases presented with malaria-like symptoms; babesia infections were confirmed by blood smears, PCR amplification and amplicon sequencing. These two cases were first detected by PCR. Upon review, their blood smears showed intraerythrocytic ring forms and tetrads, the latter forms being an uncommon finding that is considered pathognomonic of small *Babesia* spp., such as *B. microti* and *B. duncani*.[18]

These two cases reveal that human babesiosis caused by *B. microti* occurs in areas along the China–Myanmar border that are otherwise known to be highly endemic for malaria. The lessons learnt from this finding are two-fold:

First, diagnosis of babesiasis is usually detected by blood smear, but the ring forms of both *P. falciparum* and *Babesia* spp. are difficult to distinguish from each other under clinical microscopy. Previous study showed that at least 300 microscopical fields need to be reviewed to detect low level parasitemia.[6] The confirmation of two cases by PCR indicated that human babesiosis can be distinguished from malaria with the assistance of PCR-based diagnostics. *B. microti* was regarded as a single species before, but then regarded as a genetically diverse species complex. Clade 1 of *B. microti* contained mostly rodent parasites and also the majority of strains thought to be zoonotic. Clade 2 contained carnivore parasites, and Clade 3 contained rodent parasites that are probably not zoonotic.[19] As expected, the *B. microti* isolates reported herein belong to the clade that contains the majority of zoonotic strains.

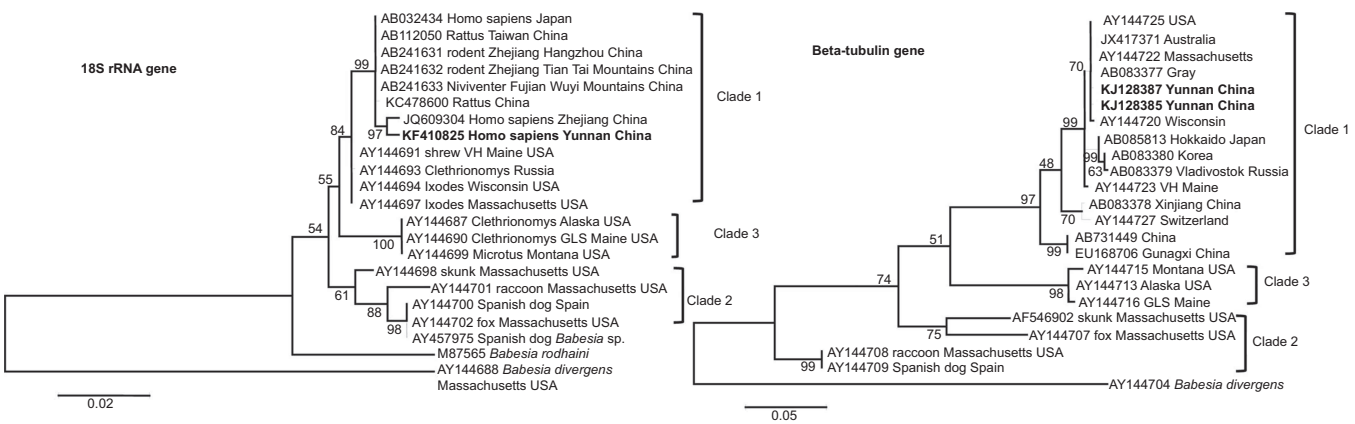

Figure 2 Phylogenetic trees of the SSU rRNA and beta-tubulin gene sequences for the *B. microti* isolates obtained from the two patients in the China–Myanmar border area. Phylogenetic analysis produced by the neighbour-joining method using MEGA version 5.1 software. SSU rRNA and beta-tubulin gene sequences of our case study samples were denoted as Yunnan China in bold face. *B. divergens* and *B. rodhaini* were set as the outgroups. Scale bar indicates nucleotide substitutions per site.

Second, babesiosis does not respond to chloroquine which might cause its misidentification as drug-resistant malaria, especially in syndemic areas. Both cases were treated with penicillin and quinolone, although these drugs have not been shown to be effective against babesiosis. It must also be noted that most antimalarial drugs, such as chloroquine and mefloquine have no effect on babesiosis.[20,21] The first regimen effective against babesiosis consists of atovaquone and azithromycin whereas the second consists of clindamycin and quinine.[6] From the treatment standpoint, the diagnosis and treatment of *Babesia* infection in malaria syndemic areas deserves more attention. Particularly, artemisinin-resistant *P. falciparum* malaria has emerged in Cambodia and Thailand–Myanmar border.[22] *B. microti* appears to be responsive to artemisinin derivatives, as per the study on a mouse model.[23] But there is still not enough data or studies to confirm that artemisinin derivatives are effective to eliminate *Babesia* infection in humans.

In conclusion, babesiosis and malaria are caused by pathogens with their similar morphology and overlapping symptoms, but require different treatments. Improved diagnostic approaches would undoubtedly improve case management of these diseases in syndemic areas.

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