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Detection of *Hepatocystis* sp. in Southeast Asian Flying Foxes (Pteropodidae) Using Microscopic and Molecular Methods

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ABSTRACT: Three species of flying fox (*Pteropus hypomelanus*, *P. vampyrus*, and *P. lylei*) from Malaysia and Vietnam were screened for apicomplexan parasites by thin blood smears and polymerase chain reaction. Only 1 of 16 bats sampled from 3 localities in southeast Asia was found to be infected (*P. hypomelanus* from Pulau Pangkor, Malaysia). We observed micro- and macrogametocytes, with morphology consistent with *Hepatocystis* sp. parasites, using light microscopy. Phylogenetic analysis of the cytochrome *b* gene showed that the parasite from *P. hypomelanus* groups with 2 published sequences from *Hepatocystis* spp., including one from *Cynopterus brachyotis*, another fruit bat in the Pteropodidae.

The study of malarial parasites in bats goes back to the nineteenth century (Dionisi, 1899); they were first observed in the Asian flying fox, *Pteropus natalis*, from Christmas Island by Durham less than a decade later (1908). In 2 independent reports, similar parasites of *Pteropus* species from Australia (Breinl, 1913) and India (Mackie, 1914) were described as *Plasmodium pteropi* and additional parasites were subsequently observed in Malaysian species of flying fox as well (Green, 1933; Bearup and Lawrence, 1947). Early workers reported only seeing gametocytes in the blood, but other parasitologists observed irregular erythrocytic schizonts (Rodhain, 1926) and even extracellular schizonts (Manwell, 1946). The parasites were later reclassified as species of *Hepatocystis* after Garnham (1950) described the asexual liver stages of *H. epomophori* and found them to resemble *H. kochi* from primates. These parasites show macroscopic exo-erythrocytic schizonts in the liver and Garnham (1953) speculated that the blood-stage schizonts observed previously were actually fragments from these large liver stages. Currently, *Hepatocystis* (Apicomplexa: Haemosporidia) contains 25 known species (Levine, 1988), and it is only known to infect Old World mammals, with bats as the second most prevalent hosts next to primates (Garnham, 1966).

Previous studies have documented a high prevalence of *H. pteropi* in *Pteropus* spp. from New Hebrides (Vanuatu) and Malaysia (McGhee, 1949; Garnham, 1966). More recently, the prevalence of *H. pteropi* was quantified for other species of fruit bats, i.e., *Cynopterus brachyotis* (39%) and *C. horsfieldi* (29%) from South Kalimantan, Indonesia (Masbar et al., 1981). It should be noted that another *Hepatocystis* species, *H. rodhain*, has been described from a Malaysian microchiropteran host, *Hipposideros galeritus* (Landau et al., 1976). The vectors for *Hepatocystis* spp. of flying foxes remain unknown. Experimental infections and wild collection of several arthropod species were both inconclusive in the search for *Hepatocystis* spp. vectors in primates and flying foxes (Bearup and Lawrence, 1947; Garnham, 1951). McGhee (1949) dissected >100 nycteribiid bat flies from *Pteropus* spp., also with negative results.

As part of a larger study examining the population structure of flying foxes and the ecology of Nipah virus, we have been surveying and capturing animals from across southeast Asia. Three species that occur in continental southeast Asia have been sampled. *Pteropus vampyrus*, the world's largest bat, has a wide geographic range from southern Vietnam, Cambodia, Thailand, Malaysia, Philippines, and through much of Indonesia to East Timor (Kunz and Jones, 2000). *Pteropus lylei* has a narrower range, and it is found in southern Vietnam, Cambodia, and Thailand (Koopman, 1989). *Pteropus hypomelanus*, the island flying fox, has a wide, but fragmented, distribution throughout the Indo-Australian region; it is primarily found on small off shore islands, often roosting in proximity to the coast (Jones and Kunz, 2000). These species overlap at some localities; *P. vampyrus*/*P. lylei* and *P. vampyrus*/*P. hypomelanus* have been found to roost together in the same trees in Cambodia and Malaysia, respectively (K. J. Olival, pers. comm.).

From January to March 2006, blood samples were collected from 8 *P. hypomelanus* on a small islet off the west coast of Pulau Pangkor, Malaysia (04.23593°N, 100.54056°E), 6 *P. vampyrus* from Soc Trang (09.60108°N, 105.97665°E) and Ca Mau (09.15258°N, 104.91347°E) Vietnam, and 2 *P. lylei* from Soc Trang, Vietnam (09.57849°N, 105.97201°E). In Malaysia, bats were captured in mist nets set near diurnal roosting trees, and they were immediately removed from the net upon capture and held in cloth bags for ~1 hr before sampling. In Vietnam, local hunters and trappers captured the sampled bats in mist nets near feeding sites. Bats were anesthetized using ketamine:xylazine (8:1.6 mg/kg) administered intramuscularly. The health of each individual was assessed by physical examination while under anesthesia. A nominal body condition score, from excellent, good, fair, to poor, was assigned based on palpation of pectoral muscles and weight. Two milliliters of blood was drawn from the brachial vein of anesthetized individuals. Thin blood smears were collected in the field, air-dried, and stored in plastic bags with silica desiccant. Several drops of blood were also collected on Whatman no. 4 filter paper for DNA assays. When present, nycteribiid bat flies (*Cyclopodia* spp.) were collected and preserved in ethanol. Animals were released at the site of capture after recovery from anesthesia.

Slides were fixed and stained with Hema 3® staining kit (Biochemical Sciences, Inc., Swedesboro, New Jersey) in the lab after being transported from the field. Slides were scanned for parasites at ×1,000 with a light microscope for 7 min each. DNA was extracted from blood dots (n = 15) using QIAGEN (Valencia, California) DNeasy extraction kits per the protocol for animal tissues. The extraction protocol was modified slightly so that 2 aliquots of AE Buffer (preheated to 55 C) were combined for a total elution volume of 100 µl. DNA extracted from blood dots were screened with cytochrome *b* primers DW2 (TAATGC CTAGACGTATTCCTGATTATCCAG) and 3932R (GACCCCAAGG TAATACATAACCC) and puReTaq polymerase chain reaction (PCR) beads (GE Healthcare, Piscataway, New Jersey). After an initial denaturation period of 4 min at 94 C, conditions were 35 cycles of 94 C for 20 sec, 50 C for 10 sec, and 68 C for 45 sec. PCR products were cleaned using Agencourt Bioscience (Beverly, Massachusetts) AmPure magnetic beads, cycle sequenced with Big Dye version 2.0 terminator mix (Applied Biosystems, Foster City, California), and DNA was precipitated using Agencourt Bioscience CleanSeq magnetic beads. All sequencing was performed on ABI 3730xl capillary sequencers at the

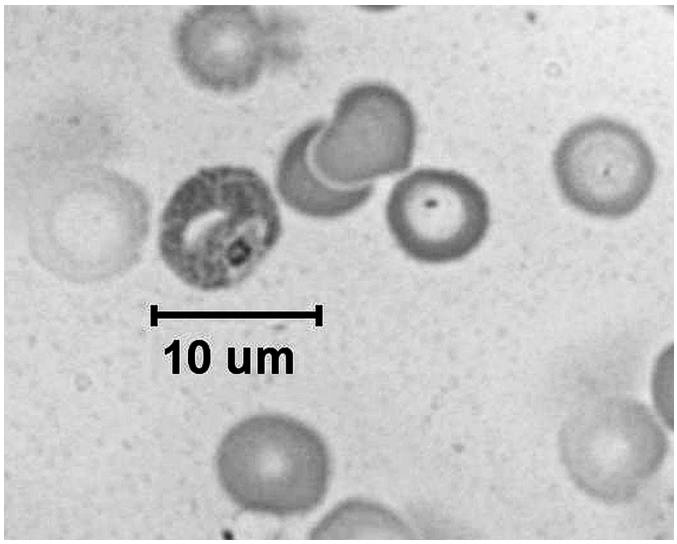

FIGURE 1. Light microscopy (×1,000) of infected red blood cell from *Pteropus hypomelanus* (PPH 008) showing macrogametocyte of *Hepatocystis* sp.

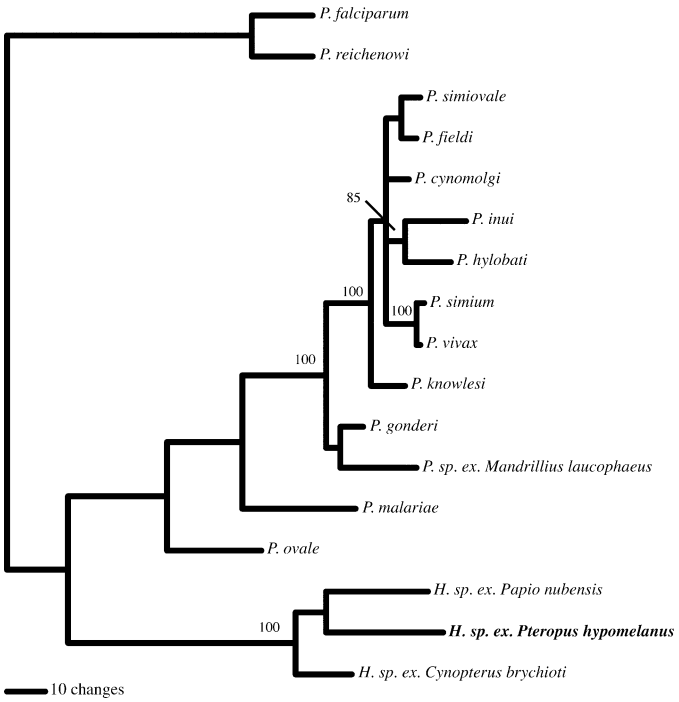

FIGURE 2. Phylogenetic tree (strict consensus) of *Plasmodium* and *Hepatocystis* cytochrome *b* sequences, with new sample indicated in bold.

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Sequences were edited in Sequencher version 4.6 (Gene Codes Corporation, Ann Arbor, Michigan) and manually corrected for ambiguous base calls. In total, 1,038 base pairs were aligned with MAFFT version 5.8 (Katoh et al., 2005). Alignments were exported to PAUP version 4.0b10 (Swofford, 2002). Parsimony searches were performed with a heuristic search criteria and equal weighting of characters with gaps treated as missing data. Support values for nodes were determined using jackknife resampling with 100 replicates of the full heuristic search and deletion of 37% of characters in each search.

Of the 16 individuals screened using thin blood smears and 15 using PCR assays of extracted blood (8 *P. hypomelanus*, 6 *P. vampyrus*, and 2 *P. lylei*), only 1 specimen of *P. hypomelanus* (PPH 008) was positive for *Hepatocystis* sp. via both light microscopy and PCR. This individual was a young adult male (forearm = 137 mm, weight = 290 g) with a body condition score of fair. Light microscopy of thin blood smears revealed both micro- and macrogametocytes (Fig. 1). Phylogenetic analysis of the cytochrome *b* sequence (GenBank EF587238) and those of published primate and rodent malaria parasites (Escalante et al., 1998; Perkins and Schall, 2002) showed that the parasite from *P. hypomelanus* groups as a sister to the *Hepatocystis* sp. from *Cynopterus brachyotis*, another fruit bat in the Pteropodidae (Fig. 2). Thus, based on morphological and molecular evidence presented here, it is likely that the observed parasite is *H. pteropi*. However, it is possible that cryptic diversity in the *Hepatocystis* spp. of flying foxes exists, and our observation was of a morphologically similar, but genetically distinct, species. A lack of comparable *Hepatocystis* spp. DNA sequences from the highly diverse fruit bat family Pteropodidae precluded us from assigning this parasite to the species level.

The identification of malarial parasites is dependent on one's adherence to a morphological, genetic, or phylogenetic species concept, and it is not clear whether modern species concepts can be applied to all of the taxonomic groups of Plasmodiidae (Martinsen et al., 2006). This preliminary study is among the first to use molecular and morphological data in distinguishing species of *Hepatocystis* parasites from chiropteran hosts. Morphological characters have been used for over a century to identify species of Plasmodiidae, but their reliability as indicators to phylogeny has been disputed (Perkins, 2000). To resolve this problem, reliable systematic characters must be found and used. The advent of molecular techniques has made these characters available. In many cases, subpatent infections, undetectable by light microscopy, will be detected by PCR (e.g., Perkins et al., 1998; Vardo et al., 2005). In our relatively limited sample, we found these independent measures of parasite prevalence to be entirely congruent, i.e., the only individual identified as positive via molecular detection techniques for *Hepatocystis* sp. was also identified as positive using light microscopy. In surveying the malarial parasites of southeast Asian flying foxes, we have combined both light microscopy and modern phylogenetic analyses, and the continued use of these combined methods will be necessary to provide increased resolution and confidence to the taxonomy of the Plasmodiidae.

The 1 *P. hypomelanus* found positive for *Hepatocystis* sp. was the only individual sampled to receive a body condition score of ''fair.'' All other individuals were rated ''good'' or ''excellent.'' This finding is of interest as very little is known about the effect of these parasites on their native host species, and may suggest that bats suffer some degree of morbidity from infection with *Hepatocystis* spp. However, confounding factors such as coinfection with other pathogens, diet, and the limited sample size must be considered. Experimental infections in a controlled environment will be necessary to really assess the effects of *Hepatocystis* spp. on the health of these native hosts.

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Seroprevalence of *Toxoplasma gondii* in Pigs From Slaughterhouses in Taiwan

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ABSTRACT: From May 2003 to April 2004, blood samples from 395 feeder pigs in Taiwan were examined for antibodies to *Toxoplasma gondii* using the latex agglutination test; antibodies (titer 1:32 or higher) were found in 10.1% of 395 pigs. The results indicate a high prevalence of infection in pigs in Taiwan destined for human consumption.

Pigs are considered an important source of *Toxoplasma gondii* infection in many countries (Dubey and Beattie, 1988; Tenter et al., 2001). Infection by the protozoan parasite *T. gondii* is prevalent in animals and humans worldwide. Humans become infected with *T. gondii* usually by ingesting oocysts in food and water contaminated by cat feces or by consuming tissue cysts in undercooked meat (Dubey and Beattie, 1988). Consumption of raw or undercooked pork was considered as the main risk factor for *T. gondii* infection in people in Taiwan (Fan et al., 2002), where pigs are considered an important food commodity. In a survey conducted more than 25 yr ago, *T. gondii* antibodies were found in 27.7% of 3,880 pigs slaughtered in 8 counties of Taiwan (Chang et al., 1991). In another survey conducted in 1998, *T. gondii* antibodies were found in 28.8% of 111 pigs (Fan et al., 2004). Here, we report that the *T. gondii* infection in pigs has decreased in the last decade.

From October 2003 to October 2004, blood samples were collected from 395 pigs in slaughterhouses located in the north of Taiwan (Shulin city, Taipei County; Lujhu Township, Taoyuan County; Jhubei city, Hsinchu County). Sera were stored at −20 C until tested for *T. gondii* antibodies using the latex agglutination test (Toxo Test-MT, Eiken Co. Ltd., Tokyo, Japan). Sera were diluted 2-fold from 1:32 to 1:512. A 1:32 titer was considered as indicative of *T. gondii* infection (Chang et al., 1991). We analyzed seroprevalence in terms of seasonal relationships using a chi-square test, where a P value of <0.05 was considered as significant (SPSS Inc., Chicago, Illinois) (Chen, 1993).

Antibodies were found in 10.1% of 395 pigs with titers of 1:32 in 18 pigs, 1:64 in 14 pigs, 1:128 in 6 pigs, and 1:256 in 2 pigs. Distributions by month were 8.3% (4/48) in April, 21.3% (10/47) in May, 15.4% (6/39) in June, 6.7% (3/45) in July, 5.6 % (2/36) in August, 29.4% (10/34) in September, 3.7% (1/27) in November, 3.3% (1/30) in December, and 16.7% (3/18) in February. The results of the present study indicate that the prevalence of *T. gondii* infection in pigs has decreased in the last decade. Nonetheless, a thorough cooking of pork is recommended before human consumption.

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