Infection of *Saimiri boliviensis* Monkeys With *Plasmodium coatneyi*

Author(s): JoAnn S. Sullivan, Amy Bounngaseng, Ann Stewart, Sullivan James J, G. Gale Galland, Fleetwood Henry, and William E. Collins
Source: Journal of Parasitology, 91(2):479-481. 2005.
Published By: American Society of Parasitologists
DOI: http://dx.doi.org/10.1645/GE-3461RN
URL: http://www.bioone.org/doi/full/10.1645/GE-3461RN

RESEARCH NOTES

J. Parasitol., 91(?), 2005, pp. 458–461

Polymerase Chain Reaction Detection of *Cytauxzoon felis* From Field-Collected Ticks and Sequence Analysis of the Small Subunit and Internal Transcribed Spacer 1 Region of the Ribosomal RNA Gene

P. J. Bondy Jr., L. A. Cohn, J. W. Tyler, and A. E. Marsh*†, Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211; *Department of Veterinary Preventive Medicine, The Ohio State University, Columbus, Ohio 43210; †To whom correspondence should be addressed. *e-mail: marsh.2061@osu.edu*

ABSTRACT: *Cytauxzoon felis* produces a disease in domestic cats in the Midwest (U.S.A.), which often leads to a fatal outcome. Although the clinical disease process is well described, there are still many unanswered questions about this organism. For example, it is unknown whether species of ticks other than *Dermacentor variabilis* can serve as vectors for transmission. With recent reports of surviving cats from limited geographic areas, another relevant question is the potential for genetically less virulent organism strains. This study evaluated 352 individual or pooled tick samples (1,362 total ticks) for the presence of *C. felis* small subunit ribosomal RNA and internal transcribed spacer 1 (ITS-1) region genes using a polymerase chain reaction (PCR). These ticks were collected from dogs and cats in several Missouri counties, including 10 from cats diagnosed with cytauxzoonosis. Only 3 positive *C. felis* samples were identified in *Amblyomma americanum* nymphs, and there was very limited genetic variation noted in both genes. The small number of positive samples did not allow the study to determine which PCR analysis was more sensitive. This is the first known report of ITS-1 gene identification and sequencing for *C. felis*. It is also the first published investigation of genetic variation in *C. felis*.

The protozoan *Cytauxzoon felis* was first recognized as a cause of fatal disease in domestic cats by Wagner (1976). The course of disease is short, and most cats succumb within 5 days of the onset of clinical signs, regardless of attempted therapy (Ferris, 1979; Kier et al., 1987; Motzel and Wagner, 1990; Greene et al., 1999; Meinkoth et al., 2000). Diagnosis is most often based on identification of signet ring–shaped piroplasms in peripheral blood erythrocytes, although identification of schizonts within macrophages can confirm the diagnosis with premortem tissue fine needle aspirates or postmortem on necropsy (Kier et al., 1987; Hoover et al., 1994; Meinkoth et al., 2000). A polymerase chain reaction (PCR) for the detection of *C. felis* DNA in blood from infected cats has been reported (Meinkoth et al., 2000), but it is limited in that the target sequence is to a highly homologous region that has the potential to amplify other organisms, including mammalian DNA.

The domestic cat is assumed a dead-end host for *C. felis* because of the rapidly fatal course of disease in this species. The presumed reservoir host is the bobcat, *Lynx rufus rufus* (Kier, Wagner et al., 1982; Kier, Wightman et al., 1982). Infection of the bobcat with *C. felis* apparently leads to a brief, often nonlethal schizogonous phase, followed by lifelong maintenance of the intraerythrocytic parasites (Glenn et al., 1983; Kocan et al., 1985; Blouin et al., 1987). Close contact between ill, infected cats and healthy cats does not appear to transmit infection in the absence of vectors (Wagner et al., 1980). Transmission of the disease to the domestic cat can occur in 1 of 2 ways, either by the bite of a tick that carries the organism (Blouin et al., 1984) or by inoculation of schizonts from an infected animal (Wagner et al., 1980). Transfusion of blood containing erythrocytic piroplasms to the domestic cat results in apparent erythrocytic infection of the recipient without clinical disease and without protection from clinical disease after inoculation of schizonts (Kier, Wightman et al., 1982; Uilenberg et al., 1987). In experimental settings, *Dermacentor variabilis* infected as nymphs can later transmit the organism as adults when allowed to feed on an uninfected feline host (Blouin et al., 1984, 1987).

There is precedent for members of the Theileriidae to be transmitted by more than 1 species of Ixodidae (Chae et al., 1999). Often, the geographic restriction of tick vectors has a tremendous effect on the areas from which vector-borne disease is identified (Cupp, 1991). If more than 1 tick species is capable of transmitting *C. felis*, this may have implications on the potential for an expanding geographic area with susceptible domestic cats. The purpose of this study was to evaluate ticks from selected counties in the state of Missouri for the presence of *C. felis* DNA and compare resulting PCR-amplified DNA sequences for similarity of the small subunit ribosomal RNA (ssu-rRNA) gene and internal transcribed spacer 1 (ITS-1) gene.

Veterinarians from 7 counties (Camden, Franklin, Greene, Jackson, Phelps, Taney, and Vernon) across the state of Missouri collected ticks from dogs and cats that were presented to their clinics for any reason (Fig. 1). Previous cases of cytauxzoonosis confirmed by the University of Missouri–Columbia Veterinary Medical Diagnostic Laboratory (UMC-VMDL) had been reported from each of these counties. At least 100 ticks per county were collected from dogs and cats and then stored in 70% isopropyl alcohol. Ticks collected from cats suspected of having cytauxzoonosis were separately stored, and blood smears from these cats were stained with Wright–Giemsa for disease confirmation on the basis of the presence of piroplasms in erythrocytes. Because most clinical cases of cytauxzoonosis are present during the summer months, ticks were collected from the beginning of June to the end of July. Only entire hard-bodied ticks were included in this study. Ticks were identified and separated by life stage, i.e., adult, nymph, and larva (Bowman et al., 2003). Three species of laboratory-reared adult ticks and nymphs (*D. variabilis*, *Amblyomma americanum*, and *Rhipicephalus sanguineus*) were purchased from the Department of Entomology, Oklahoma State University, Stillwater, Oklahoma, to serve as negative controls.

The number of ticks per sample for DNA extraction and analysis depended on the size of the ticks. Adult ticks were grouped on the basis of species and size. Each heavily engorged tick was evaluated individually. Moderately engorged ticks were evaluated in groups of 2, and unengorged ticks were pooled in groups of 5. All nymphs were processed in groups of 5. Similar DNA extraction methods were performed using a DNeasy Tissue Kit (Qiagen, Valencia, California) for both the infected blood (the positive control) and tick samples; however, slight modifications were used to prepare the tick samples. Tick samples were placed in 6-mm-thick polyethylene bags and held at −70 C followed by liquid nitrogen emersion and pulverization with a hammer. The proteinase K treatment was modified to include incubation at 55 C in a rotating incubator for 24 hr. For the whole blood, 50-μl aliquots were used in each DNA extraction.

A previously published PCR for the gene encoding for the ssu-rRNA was evaluated for sequence homology to other tick-borne pathogens, mammalian, and tick DNA (Meinkoth et al., 2000) using a sequence alignment program (Vector NTI, Invitrogen, Carlsbad, California). The alignment indicated that the published primers could potentially amplify non–*C. felis* DNA; therefore, a new internal reverse primer, Cfelis-AEMRev, was designed (Fig. 2). Two different primer sets were used in this study, as summarized in Figure 2. To confirm that no inhibitors of the PCR were present in the extracted DNA, primer set I targeted an area of high sequence homology of the aligned ssu-rRNA gene sequences anticipated to amplify parasite, tick, or mammalian DNA. Primer set II (Fig. 2) was designed to be specific for the *C. felis* ssu-rRNA gene while avoiding amplification of tick and/or mammalian DNA. Specificity of the 2 different primer sets was tested against DNA samples extracted from laboratory-reared ticks, healthy cats, and other tick-borne pathogens (*Babesia bovis* and *Ehrlichia canis*).

DNA template of 10 μl was used per 50-μl reaction, which also

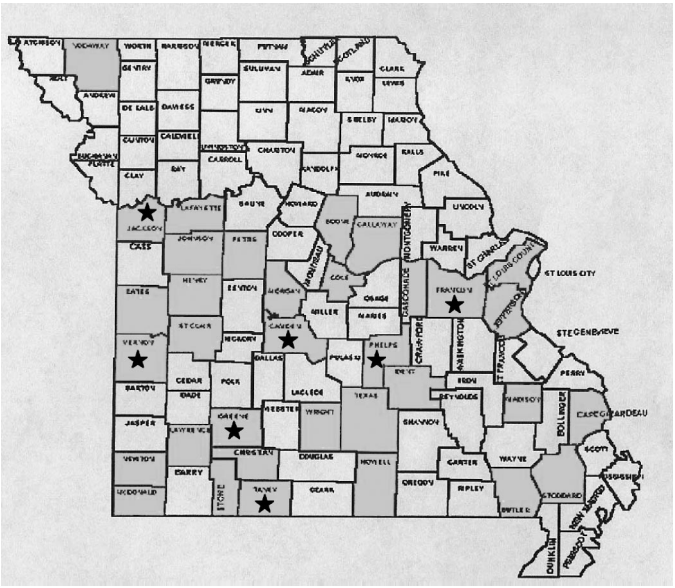


FIGURE 1. State map of Missouri, with shaded counties representing those with confirmed *Cytauxzoon felis* cases presented to the University of Missouri Veterinary Medical Teaching Hospital or Veterinary Medical Diagnostic Laboratory since 1999 and the counties (★) from which ticks were collected for this study.

contained 1.5 mM $MgCl_2$, 25 pmol of each primer for set I or set II, and other standard PCR components as recommended with the Taq enzyme kit (Promega, Madison, Wisconsin). Sterile water was used as a negative control, whereas whole-blood DNA extracted from a necropsy-confirmed *C. felis*–infected cat served as the positive control. The PCR products were evaluated by gel electrophoresis. Positive PCR products were purified using a PCR purification kit (Qiagen) as per the manufacturer's instructions.

Positive samples using the *C. felis*–specific primer set underwent further molecular analysis to target the ITS-1 gene of the rRNA gene. The 2 primers used, ITS-5 and ITS-2, have been described (White et al., 1990) and used to characterize closely related parasites (Marsh et al., 1998, 1999); therefore, similar methods were used as described, but the PCR cycling conditions differed in that 48 C was used for annealing; PCR primers were used for directional DNA sequencing.

Purified products were submitted for DNA sequencing to the DNA Core Facility at the University of Missouri–Columbia, Columbia, Missouri. The sequence analysis was facilitated using Vector NTI and compared with published sequences available through the National Library

```
Primer Set          Sequence
I/II  Forward       5'AACCTGGTTGATCCTGCCAGTAGTCATATGCTTG 3'
I     Reverse       5'CGCGGCTGCTGGCACCAGACTTGCCCTCC 3'
II    Reverse       5'TCACCAGAAAAAGCCACAAC 3'

          <1                                        <34
Cf: 5'AACCTGGTTGATCCTGCCAGTAGTCATATGCTTG…//…3'
Dm: 5'AACCTGGTTGATCCTGCCAGTAGTCATATGCTTG…//…3'
Hs: 5'TACCTGGTTGATCCTGCCAGTAG-CATATGCTTG…//…3'

               <540                                 <569
Cf: 5'…//…GGAGGGCAAGTCTGGTGCCAGCAGCCGCG…//…3'
Dm: 5'…//…GGAGGGCAAGTCTGGTGCCAGCAGCCGCG…//…3'
Hs: 5'…//…GGAGGGCAAGTCTGGTGCCAGCAGCCGCG…//…3'

               <670                                                 <689
Cf: 5'…//…GtTgtGgC-------------------------TttTtCtggTgA…//…3'
Dm: 5'…//…GCTCGGACT-GAACAT--CATGCCGGTCCTTTCTTGGTGCACTTCA…//…3'
Hs: 5'…//…GCCCGTCCCCGCCCCTTGCCTCTCGGCGCCCCCTC-GATGCTCTTA…//…3'
```

FIGURE 2. Polymerase chain reaction primers and partial sequence alignment of the small subunit ribosomal RNA gene of *Cytauxzoon felis* (*Cf*), *Dermacentor marginatus* (*Dm*), and *Homo sapiens* (*Hs*) to show areas of primer binding. Intervening gene sequences not shown are represented by . . . //. . . , and the dashes (—) represent the absence of a base in the aligned sequences. Sequence alignment is based on GenBank L19080, *C. felis*. Top alignment contains a segment where the forward primer of both primer set I and II would anneal. Middle alignment shows a nonspecific reverse primer annealing site of primer set I. Bottom alignment shows a *C. felis* sequence target, which the internal reverse primer would specifically anneal for primer set II. The lower case letters are bases that differ between *C. felis* and *D. marginatus*. Cycling conditions for both primer sets used an annealing step of 53 C for 1 min.

Medicine GenBank site. The aligned sequences were trimmed to the limits of the shortest sequence, and this data set was used for all the group comparisons. The confidence interval (CI) for the percent prevalence in the tick samples was calculated using the method as described in Daniel (1999).

Only 3 species of ticks, *D. variabilis*, *A. americanum*, and *R. sanguineus*, were collected as summarized in Table I. A total of 10 *C. felis* suspected cases were identified and confirmed by observation of piroplasms in erythrocytes on a peripheral blood smear. Ticks collected from these *C. felis*–infected hosts originated from 4 of the 7 counties (Camden, Franklin, Phelps, and Taney) (Fig. 1). *Dermacentor variabilis*, *A. americanum*, and *R. sanguineus* were collected from the 10 cats with cytauxzoonosis, and more than 1 tick species was found on some cats.

Extracted DNA was successfully amplified from 323/352 (91.8%) individual or tick-pooled samples as determined through the positive

TABLE I. Overview of total number of tick samples (individual or pooled) from which DNA was extracted.

Tick spp. and life stage	Total samples* (total ticks†)	Total samples‡ (total ticks)	Positive samples	Counties of positive ticks
Amblyomma americanum				
Adults	210 (764 ticks)	3 (4 ticks)	0	
Nymphs	16 (65 ticks)	4 (6 ticks)	3	Camden, Franklin, Phelps
Dermacentor variabilis				
Adults	79 (293 ticks)	3 (5 ticks)	0	
Nymphs	0	0	0	
Rhipicephalus sanguineus				
Adults	35 (157 ticks)	2 (3 ticks)	0	
Nymphs	0	0	0	

* Total number of samples that includes a subset representing pooled ticks into DNA samples for analysis purposes.
† Total number of ticks, individual and enumerated into pooled samples.
‡ From infected hosts.

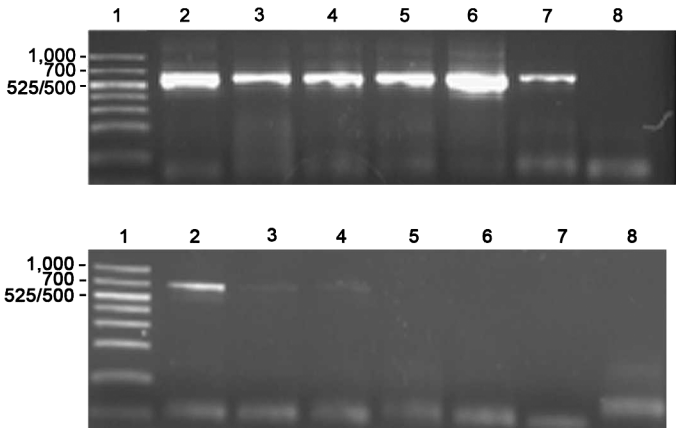


FIGURE 3. (Top panel) Ethidium bromide–stained agarose gel showing polymerase chain reaction (PCR) products produced by using primer set I (nonspecific to *Cytauxzoon felis*). 1: molecular size ladder; 2: positive control from a confirmed feline case; 3: *Amblyomma americanum* nymph from a confirmed case in Franklin County; 4: *A. americanum* nymph from a confirmed case in Phelps County; 5: *Dermacentor variabilis* adult collected from a healthy animal; 6: *A. americanum* adult collected from a healthy animal; 7: *Rhipicephalus sanguineus* adult collected from a healthy animal; 8: water blank as negative control. (Bottom panel) Ethidium bromide–stained gel of *C. felis* small subunit ribosomal RNA PCR amplified gene product by using primer set II. 1: molecular size ladder; 2: positive control, blood-derived DNA extracted from a confirmed feline case; 3: *A. americanum* nymph from a confirmed case in Franklin County; 4: *A. americanum* nymph from a confirmed case in Phelps County; 5: *Dermacentor variabilis* adult collected from a healthy animal; 6: *A. americanum* adult collected from a healthy animal; 7: *R. sanguineus* adult collected from a healthy animal; 8: water blank as negative control.

reaction using primer set I (Fig. 3). The DNA from a healthy cat, laboratory-reared ticks, *B. bovis*, and *E. canis* (all approximately 550 bp) were also amplified using primer set I. For primer set II (*C. felis* specific), there was an approximately 700-bp product using DNA extracted from whole blood of a confirmed case of feline cytauxzoonosis, and no PCR product was identified when tested on DNA extracted from whole blood of a healthy cat, laboratory-reared ticks, *B. bovis*, or *E. canis*. Of the 323 tick samples from which DNA was successfully extracted (positive reaction with primer set I), 3 samples (0.93%; 95% CI: 0%, 1.98%) were PCR positive (Table I) using the *C. felis*–specific primers. All the positive samples were from *A. americanum* nymphs from each of the following counties: Camden, Franklin, and Phelps. Two of the 3 *C. felis* PCR-positive samples from ticks were also positive when using the primer set for the ITS-1 gene.

Sequence data were determined from the 3 positive PCR tick samples as well as from the PCR product of the naturally infected feline whole blood used as a positive control. Sequence analysis of the products using primer set II indicated >99.8% sequence similarity between each sequence and the previously published sequence (Meinkoth et al., 2000) as well as to each other. There was only 1 nucleotide that was not identical with the alignment of 620 bases (GenBank L19080).

The sequence data from the products using the primers for the ITS-1 gene also exhibited very high similarity when compared with each other. Three of 4 samples, 2 from ticks and 1 from the positive control, gave satisfactory sequence data for *C. felis*–derived ITS-1 PCR product. Cloning of the PCR products was not pursued for any of the products including the fourth, which did not yield results using direct sequencing. There were 2 bases of the 474 bases sequenced from the ITS-1 gene that could not be definitively identified from the sequence data of 1 of the samples. Except for the ambiguous bases of the single sample (GenBank AY669146), there was 100% homology for 3 (2 tick and 1 blood derived) of the samples. The ITS-1 gene sequence based on the blood-derived parasites (GenBank AY158898) determined in this study was 75% similar to *Toxoplasma gondii*, 52% similar to *B. bovis*, and 15% similar to *Theileria parva*.

In this study, all the cases of suspected feline cytauxzoonosis were confirmed by observation of piroplasms in erythrocytes on peripheral blood smears. Previous work presented a PCR methodology used to identify the ssu-rRNA gene of *C. felis* from whole blood of an infected cat (Meinkoth et al., 2000), which suggests the potential for identifying the organism in other samples, i.e., infected ticks, based on molecular methods. However, to the authors' knowledge, there has been no published work identifying ticks that have been naturally infected with *C. felis*; there is only experimental evidence of a tick's capability, specifically *D. variabilis*, of transmitting the organism to a domestic cat (Blouin et al., 1984). Therefore, our study's goal was to use a PCR-based assay to detect *C. felis* in ticks collected from domestic dogs and cats. The primers described in Meinkoth et al. (2000) were targeting an area of high sequence homology, which could potentially amplify DNA from *C. felis* as well as other organisms, e.g., mammalian whole blood, various tick species, or other tick-borne organisms. This required modification of the published methods of Meinkoth et al. (2000) and evaluation of the assay before application for testing field-collected samples.

The development of a new reverse primer for a more unique area of the *C. felis* ssu-rRNA gene was shown to be specific for this organism while avoiding this same gene sequence of other organisms. The use of 2 separate primer sets increased the time required to evaluate the DNA samples; however, the additional use of primer set I allowed us to eliminate 8.5% of the DNA samples that might have provided false-negative results for *C. felis* DNA due to absence of any DNA extracted.

From the processed and evaluated tick samples, there were only 3 (0.93%) positive samples identified, and each of these was removed from an infected cat. This suggests a very low prevalence of *C. felis* in ticks from the counties included in this study. However, it is also possible that an infection level in ticks could be lower than the detection limit of the PCR protocol established. Increased sensitivity may have been achieved through nested PCR or *C. felis*–specific probe hybridization of the PCR products; however, these additional procedures were not designed into this initial survey.

The obtained sequences of the positive samples (3 tick samples and 1 infected whole blood from a domestic cat) had no evidence of variation with the exception of 1 nucleotide base for each gene evaluated. A single nucleotide base differed from the others in the ssu-rRNA gene sequence of 1 sample, whereas a single nucleotide base of 1 of the ITS-1 gene sequences differed in another sample. We were unable to clearly identify a single nucleotide base for either of these sites and used ambiguous International Union of Biochemistry (IUPAC) code base for this sequence. Because the DNA sequencing was based on a PCR pool, it could reflect polymorphisms in the different gene cassettes as have been noted in *Babesia* spp. (Dalrymple, 1990; Reddy et al., 1991).

There were limitations present in this study. First, only 6 of 114 Missouri counties were included, leaving >20 counties with confirmed cases of feline cytauxzoonosis unsurveyed. Furthermore, although the counties chosen for this study were those reporting the highest number of cases of feline cytauxzoonosis in previous years, it is possible during the year of tick collection that other counties not included in the study had greater numbers of cases and inclusion of these counties could have increased the prevalence of infected ticks.

Previous experimental work demonstrated the ability of the *D. variabilis* adult tick to transmit the *C. felis* organism to a domestic cat (Blouin et al., 1984). Although *D. variabilis* adults were collected in this study from infected cats, none was identified as containing *C. felis* DNA using the PCR developed. However, the number collected from infected cats was very small (Table I). It is also possible that the infection was below the established detection level because these ticks were not heavily engorged. It is probable that none of these ticks was infected because of inadequate length of attachment to the host before removal. Because clinical illness is delayed until 2 wk after the bite of an infected tick (Blouin et al., 1984), the tick responsible for infection of the cats presented for veterinary care may have already fed and detached from the host before the onset of illness.

This is the first report of *C. felis* detection in field-collected ticks. Although not proven a competent vector, *A. americanum* has now been identified as at least a potential vector that should be further pursued. Furthermore, this study was able to identify and sequence an additional subunit of the rRNA gene, the ITS-1 region, which has not been previously published for *C. felis*.

This work was made possible by grants from Merial Companion An-

imal Global Enterprises and the University of Missouri–Columbia, College of Veterinary Medicine Phi Zeta Chapter. We would also like to acknowledge the excellent technical assistance of PollyAnna Higgins. The *Ehrlichia canis* DNA was extracted from culture-derived organisms as described above, whereas *Babesia bovis* DNA was generously provided by C. A. Carson.

LITERATURE CITED

BLOUIN, E. F., A. A. KOCAN, B. L. GLENN, K. M. KOCAN, AND J. A. HAIR. 1984. Transmission of *Cytauxzoon felis* (Kier, 1979) from bobcats, *Lynx rufus* (Schreber), to domestic cats by *Dermacentor variabilis* (Say). Journal of Wildlife Diseases **20:** 241–242.

———, ———, K. M. KOCAN, AND J. A. HAIR. 1987. Evidence of a limited schizogonous cycle for *Cytauxzoon felis* in bobcats following exposure to infected ticks. Journal of Wildlife Diseases **23:** 499–501.

BOWMAN, D. D., R. C. LYNN, J. R. GEORGI, AND M. L. EBERHARD. 2003. Georgi's parasitology for veterinarians, 8th ed. Saunders Publishing Company, St. Louis, Missouri, 422 p.

CHAE, J., M. LEVY, J. HUNT JR., J. Schlater, G. Snider, S. D. Waghela, P. J. Holman, and G. G. Wagner. 1999. *Theileria* sp. infections associated with bovine fatalities in the United States confirmed by small-subunit rRNA gene analyses of blood and tick samples. Journal of Clinical Microbiology **37:** 3037–3040.

CUPP, E. W. 1991. Biology of ticks. Veterinary Clinics of North America Small Animal Practice **21:** 1–26.

DALRYMPLE, B. P. 1990. Cloning and characterization of the rRNA genes and flanking regions from Babesia bovis: Use of the genes as strain discriminating probes. Molecular and Biochemical Parasitology **43:** 117–124.

DANIEL, W. W. 1999. Biostatistics: A foundation for analysis in the health sciences, 7th ed. John Wiley and Son Inc., New York, 755 p.

FERRIS, D. H. 1979. A progress report on the status of a new disease of American cats: Cytauxzoonosis. Comparative Immunology Microbiology and Infectious Diseases **1:** 269–276.

GLENN, B. L., A. A. KOCAN, AND E. F. BLOUIN. 1983. Cytauxzoonosis in bobcats. Journal of the American Veterinary Medical Association **183:** 1155–1158.

GREENE, C. E., K. LATIMER, E. HOPPER, G. SHOEFFLER, K. LOWER, AND F. CULLENS. 1999. Administration of diminazene aceturate or imidocarb dipropionate for treatment of cytauxzoonosis in cats. Journal of the American Veterinary Medical Association **215:** 497–500.

HOOVER, J. P., D. B. WALKER, AND J. D. HEDGES. 1994. Cytauxzoonosis in cats: Eight cases (1985–1992). Journal of the American Veterinary Medical Association **205:** 455–460.

KIER, A. B., J. E. WAGNER, AND D. A. KINDEN. 1987. The pathology of experimental cytauxzoonosis. Journal of Comparative Pathology **97:** 415–432.

———, ———, AND L. G. MOREHOUSE. 1982. Experimental transmission of *Cytauxzoon felis* from bobcats (*Lynx rufus*) to domestic cats (*Felis domesticus*). American Journal of Veterinary Research **43:** 97–101.

———, S. R. WIGHTMAN, AND J. E. WAGNER. 1982. Interspecies transmission of *Cytauxzoon felis*. American Journal of Veterinary Research **43:** 102–105.

KOCAN, A. A., E. F. BLOUIN, AND B. L. GLENN. 1985. Hematologic and serum chemical values for free-ranging bobcats, *Felis rufus* (Schreber), with reference to animals with natural infections of *Cytauxzoon felis* Kier, 1979. Journal of Wildlife Diseases **21:** 190–192.

MARSH, A. E., B. C. BARR, A. E. PACKHAM, AND P. A. CONRAD. 1998. Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). Journal of Parasitology **84:** 983–991.

———, ———, L. TELL, D. D. BOWMAN, P. A. CONRAD, C. KETCHERSIDE, AND T. GREEN. 1999. Molecular characterization and differentiation of *Sarcocystis falcatula* and *Sarcocystis neurona* isolates. Journal of Parasitology **85:** 750–757.

MEINKOTH, J., A. A. KOCAN, L. WHITWORTH, G. MURPHY, J. C. FOX, AND J. P. WOODS. 2000. Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997–1998). Journal of Veterinary Internal Medicine **14:** 521–525.

MOTZEL, S. L., AND J. E. WAGNER. 1990. Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. Veterinary Parasitology **35:** 131–138.

REDDY, G. R., D. CHAKRABARTI, C. A. YOWELL, AND J. B. DAME. 1991. Sequence microheterogeneity of three small subunit ribosomal RNA genes of *Babesia bigemina*: Expression in erythrocyte culture. Nucleic Acids Research **19:** 3641–3645.

UILENBERG, G., F. F. FRANSSEN, AND N. M. PERIE. 1987. Relationships between *Cytauxzoon felis* and African piroplasmids. Veterinary Parasitology **26:** 21–28.

WAGNER, J. E. 1976. A fatal cytauxzoonosis-like disease in cats. Journal of the American Veterinary Medical Association **168:** 585–588.

———, D. H. FERRIS, A. B. KIER, S. R. WIGHTMAN, E. MARING, L. G. MORHOUSE, AND R. D. HANSEN. 1980. Experimentally induced cytauxzoonosis-like disease in domestic cats. Veterinary Parasitology **6:** 305–311.

WHITE, T. J., T. BRUNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols: A guide to methods and applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.). Academic Press, San Diego, California, p. 315–322.

J. Parasitol., 91(2), 2005, pp. 461–463

Observations on the Vietnam Palo Alto Strain of *Plasmodium vivax* in Two Species of *Aotus* Monkeys

William E. Collins, JoAnn S. Sullivan, G. Gale Galland, Allison Williams, Douglas Nace, Tyrone Williams*, and John W. Barnwell
Division of Parasitic Diseases and Animal Resources Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, 30341. *e-mail: wec1@cdc.gov*; *Atlanta Research and Education Foundation, Atlanta, Georgia 30333

ABSTRACT: Thirty-three splenectomized *Aotus lemurinus griseimembra* monkeys with no previous experience with malaria were infected with the Vietnam Palo Alto strain of *Plasmodium vivax*. The median maximum parasite count was 280,000/μl. Nine splenectomized monkeys with previous infection with *Plasmodium falciparum* had median maximum parasite counts of 120,000/μl. Splenectomized *Aotus nancymai* monkeys supported infections at a lower level. Transmission via the bites of *Anopheles dirus* mosquitoes was obtained in a splenectomized *A. lemurinus griseimembra*, with a prepatent period of 31 days. It is estimated that between 1.5×10^8 and 1.6×10^9 parasites can be removed from an infected animal for molecular or diagnostic antigenic studies.

The adaptation of human malaria parasites to New World monkeys has allowed for the testing of candidate antimalarial drugs and vaccines in addition to the production of malarial antigens. Until *P. vivax* is routinely produced in vitro, nonhuman primates serve as a major source of antigen for molecular and diagnostic studies with this species. Many different isolates of *P. vivax* have been adapted to develop in New

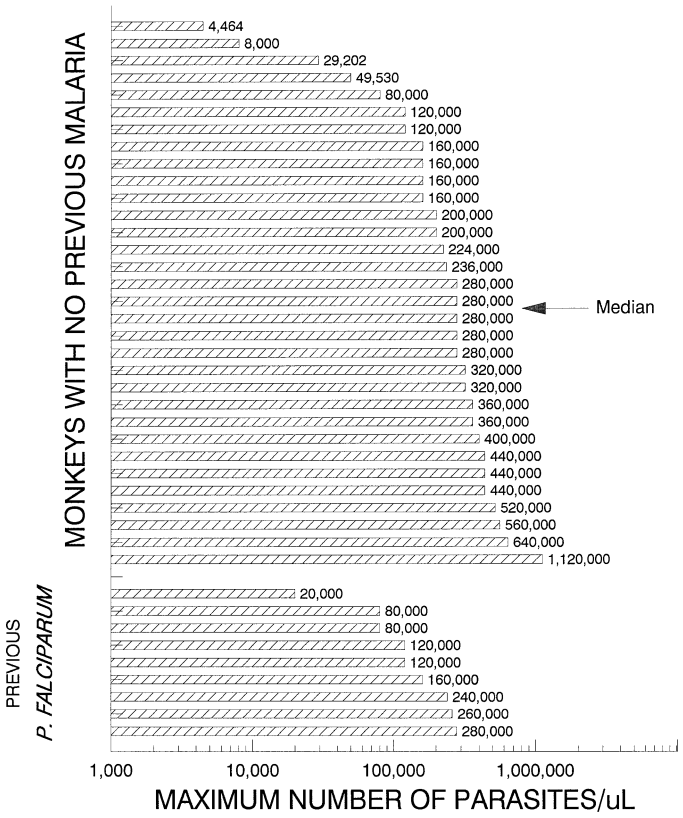


FIGURE 1. Maximum parasite counts with the Vietnam Palo Alto strain of *Plasmodium vivax* in 33 splenectomized *Aotus lemurinus griseimembra* monkeys with no previous malaria and 9 with previous infection with *Plasmodium falciparum*.

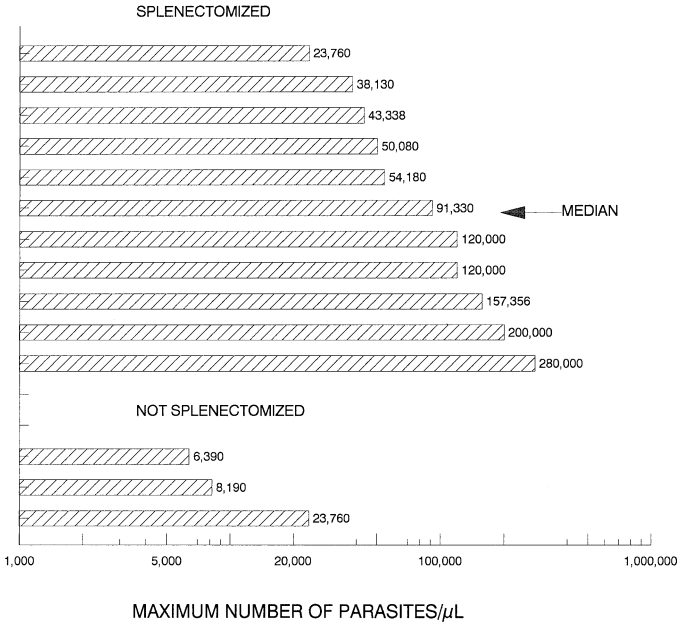


FIGURE 2. Maximum parasite counts with the Vietnam Palo Alto strain of *Plasmodium vivax* in 14 *Aotus nancymai* monkeys.

World monkeys. One of the earliest was Vietnam Palo Alto (Geiman and Meagher, 1967). Highly predictable parasite counts were obtained following extensive passages in intact *A. lemurinus griseimembra* monkeys (Schmidt, 1976). In the CDC laboratories, studies are made on the development of human malaria parasites primarily in splenectomized monkeys. The results of these observations are reported here.

Most animals were obtained commercially; some were laboratory born. *Aotus lemurinus griseimembra* originated in Colombia and *A. nancymai* originated in Peru; a few of the monkeys were laboratory born. Animals were fed a diet of animal chow, fruits, and vegetables considered suitable for their maintenance in captivity. Splenectomy was performed under sterile conditions by a qualified veterinarian using standard procedures. Some of the animals were initially infected with different strains of *P. falciparum* before being infected with the Vietnam Palo Alto strain of *P. vivax*. The strain was initially obtained from Dr. Leon Schmidt. Monkeys were infected by the intravenous inoculation of various concentrations of infected erythrocytes, either freshly collected from a donor animal or that had been stored frozen. In one instance, a monkey was infected by allowing infectious mosquitoes to feed directly on a tranquilized animal. After feeding, the salivary glands of the mosquitoes were dissected and examined to confirm the presence of sporozoites. Beginning 1 day after injection of parasitized erythrocytes, or 14 days after the mosquito feeding, thick and thin blood films were prepared by the method of Earle and Perez (1932), stained with Giemsa, and examined microscopically. Parasite counts were recorded per microliter of blood.

In the DPD/CDC insectary, *Anopheles freeborni* (from California), *Anopheles quadrimaculatus* (from the southeastern United States), *Anopheles maculatus* (from Malaysia), and *An. dirus* (from Thailand) were maintained. The procedures for feeding, handling, and dissection of the mosquitoes have been reported previously (Collins et al., 1966).

Thirty-three splenectomized *A. lemurinus griseimembra* with no history of previous infection were infected with the Vietnam Palo Alto strain of *P. vivax*. Maximum parasite counts ranged from 10,484 to 1,120,000/μl, with a median of 280,000/μl (Fig. 1). Nine additional animals that had been infected previously with *P. falciparum* were infected with this strain. Maximum parasite counts ranged from 20,000 to 280,000/μl, with a median of 120,000/μl. The day of maximum parasite count ranged from day 6 to day 35 of patent parasitemia (median = day 12), depending primarily on the number of parasites inoculated.

Eleven splenectomized *A. nancymai* with no previous infection were infected. Maximum parasite counts ranged from 23,808 to 280,000/μl, with a median of 91,330/μl. Three intact *A. nancymai* were infected; maximum parasite counts ranged from 6,390 to 23,760/μl.

The Vietnam Palo Alto strain had been passaged by blood repeatedly before attempts were made to passage via sporozoites. Thus, gametocyte production was minimal; actually, gametocytes were rarely observed even on the few days when mosquitoes were infected. However, there were 19 occasions when *An. freeborni* were infected by feeding on 4 of the monkeys when at least 1 of the other species of mosquitoes was also fed (Table I). *Anopheles dirus* was infected on 5 days; on 4 of these, the number of oocysts per infected gut was equal to or higher than that of *An. freeborni*. In all but one other comparison (number 12 with *An. maculatus*), the number of oocysts present in *An. freeborni* exceeded that of the other mosquitoes. Only the salivary glands of *An. dirus* and *An. maculatus* mosquitoes were found to be heavily infected with sporozoites. *Anopheles dirus* mosquitoes were infected by feeding on a splenectomized *A. lemurinus griseimembra* monkey. Subsequently, 13 heavily infected mosquitoes were allowed to bite another splenectomized *A. lemurinus griseimembra* monkey with no previous history of malaria; the prepatent period was 31 days.

These monkeys normally weigh an average of 800–1,000 g, and collection of blood is restricted to a total of 6 ml per mo over the course the infection with *P. vivax*. It was apparent that the Vietnam Palo Alto strain of *P. vivax* would produce higher density parasite counts in splenectomized *A. lemurinus griseimembra* monkeys for the production of parasitic material from these relatively small animals. Monkeys that had been previously infected with *P. falciparum* also supported the production of high-density parasitemia. Nonetheless, with the high-density parasite counts, parasite counts produced by the Vietnam Oak Knoll strain in *A. nancymai*, often between 1.5×10^8 and 1.6×10^9 parasites can be obtained by the extraction of 4–6 μl of blood during each infection for molecular and diagnostic antigen preparation and still be within the normal guidelines for accepted animal management practices.

Mosquito infection is rarely obtained with this strain of *P. vivax*. However, once mosquitoes were infected, the 2 Asian species, *An. dirus*

TABLE I. Comparative infectivity of *Anopheles freeborni, Anopheles maculatus, Anopheles quadrimaculatus,* and *Anopheles dirus* mosquitoes fed on monkeys infected with the Vietnam Palo Alto strain of *Plasmodium vivax.*

Feeding no.	*An. freeborni*			*An. maculates*			*An. quadrimaculatus*			*An. dirus*		
	P/D*	%	Oo/+gut	P/D	%	Oo/+gut	P/D	%	Oo/+gut	P/D	%	Oo/+gut
1	5/5	100	7.20	4/5	80	6.75				12/15	80	18.50
2	17/25	68	4.25	10/20	50	2.40				1/25	4	1.00
3	8/15	53	3.28	2/25	8	1.00	1/25	4	1.00			
4	1/2	50	3.00	4/25	31	2.00	1/25	4	1.00			
5	8/12	68	2.83	4/25	16	1.50						
6	3/12	25	2.68	4/25	16	1.25	1/15	4	1.00			
7	3/12	25	2.68	2/25	8	1.00	2/25	8	1.00			
8	6/10	60	2.50	4/25	16	1.50	1/25	4	1.00			
9	4/22	18	2.00							2/10	20	1.00
10	7/25	28	1.86	4/25	16	1.50						
11	3/4	75	1.67	4/10	40	1.00	0/25	0	0			
12	6/25	24	1.58	7/25	28	2.43				4/5	80	5.50
13	1/25	4	1.00	0/25	0	0				7/28	28	1.12
14	2/25	8	1.00	0/25	0	0	0/25	0	0			
15	2/25	8	1.00	0/25	0	0	0/25	0	0			
16	1/25	4	1.00	0/25	0	0	0/25	0	0			
17	2/25	8	1.00	0/25	0	0						
18	1/25	4	1.00	0/25	0	0						
19	1/25	4	1.00	0/25	0	0						

* P/D = positive mosquitoes/mosquitoes dissected and examined; Oo/+gut = number of oocysts per positive mosquito gut examined.

and *An. maculatus*, supported salivary-gland infection with sporozoites, and transmission via bites was obtained using *An. dirus* to a splenectomized *A. lemurinus griseimembra*.

Our previous studies with many different isolates of *P. vivax* in splenectomized *A. lemurinus griseimembra* and *An. nancymai* have failed to demonstrate maximum parasite counts approaching that obtainable with the Vietnam Palo Alto strain. In most instances, average parasite counts are at least 1 log lower in density. Thus, this strain has distinct advantages where large numbers of parasites are required for molecular studies or for antigen preparation.

This work was supported in part by an interagency agreement between the U.S. Agency for International Development, Malaria Vaccine Development Program, and the Centers for Disease Control and Prevention, Project 936-6001, and the Atlanta Research and Education Foundation of the Veterans Affairs Medical Center of Atlanta.

LITERATURE CITED

COLLINS, W. E., E. G. GUINN, AND J. R. HELD. 1966. Studies on the transmission of two strains of *Plasmodium inui* by *Anopheles maculatus* and *A. stephensi*. Journal of Parasitology **52:** 664–668.

EARLE, W. C., AND M. PEREZ. 1932. Enumeration of parasites in the blood of malarial patients. Journal of Laboratory and Clinical Medicine **17:** 1124–1130.

GEIMAN, Q. M., AND M. J. MEAGHER. 1967. Susceptibility of a New World monkey to *Plasmodium falciparum* from man. Nature **215:** 437–439.

SCHMIDT, L. H. 1978. *Plasmodium falciparum* and *Plasmodium vivax* infections in the owl monkey (*Aotus trivirgatus*). I. The course of untreated infections. American Journal of Tropical Medicine and Hygiene **43:** 671–702.

J. Parasitol., 91(2), 2005, pp. 463–465

Molecular Characterization of Human Pathogen *Babesia* EU1 in *Ixodes ricinus* Ticks From Slovenia

Darja Duh, Miroslav Petrovec, and Tatjana Avsic-Zupanc, Institute of Microbiology and Immunology, Medical Faculty, SI-1000 Ljubljana, Slovenia. *e-mail: tatjana.avsic@mf.uni-lj.si*

ABSTRACT: New cases of human babesiosis were recently reported in Europe. The etiological agent was identified as *Babesia* EU1, a zoonotic pathogen with previously unreported molecular characteristics. On the basis of a comparison of the complete babesial 18S rRNA gene, we have generated strong molecular evidence that *Ixodes ricinus* ticks from Slovenia are infected with EU1.

Introduction of molecular methods, including PCR and sequence analysis, in the field of parasitology has provided new insights into babesiosis. Caused by the intraerythrocytic parasites of *Babesia* sp., babesiosis is an emerging tick-transmitted disease of animals, including humans (Kjemtrup and Conrad, 2000). Most research regarding molecular methods has been done for *Babesia microti*, a parasite of small mammals and the causative agent of human babesiosis in the United States (Persing et al., 1992; Homer et al., 2000). Although human babesiosis in Europe does not have an emerging status, the disease is nonetheless dangerous and, without aggressive treatment, is usually fatal. On the basis of morphological characters and antigenic reactivity, the bovine parasite *Babesia divergens* has usually been identified as the agent of this zoonosis in Europe (Kjemtrup and Conrad, 2000). However,

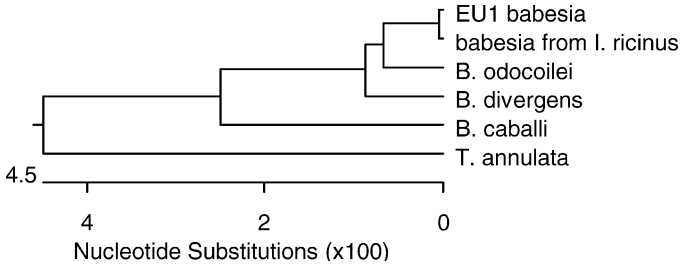


FIGURE 1. Phylogenetic relationships of babesial parasites from *Ixodes ricinus* ticks from Slovenia and those deposited in GenBank inferred from multiple sequence alignment of the complete 18S rRNA gene with the ClustalW algorithm. Accession numbers: *Babesia* from *I. ricinus* ticks (AY553915), EU1 (AY046575), *B. divergens* (AY046576), *B. odocoilei* (AY046577), *B. caballi* (Z15104), and *Theileria annulata* (M64243). Units at the bottom of the phylogenetic tree indicate the percentage of nucleotide substitutions.

molecular data for *B. divergens* are scarce, with only a single, recently deposited, DNA sequence of this parasite from humans on the mainland of Europe (Centeno-Lima et al., 2003). The vector of *B. divergens* is the European sheep tick *Ixodes ricinus*. Extensive studies, mostly in Ireland and France, revealed a correlation of the bovine babesiosis with the distribution and seasonal activity of *I. ricinus* ticks (Gray, 1980, 1985; L'Hostis and Chauvin, 1999). Furthermore, experimental data showed that the human strain of *B. divergens* can be transmitted to cattle and gerbils by *I. ricinus* (Lewis and Young, 1980).

Recently, we conducted a study to identify babesiae infecting *I. ricinus* ticks with molecular techniques based on the detection of partial babesial 18S rRNA gene sequences (Duh et al., 2001). However, 3 of the 13 detected babesial parasites we isolated were not identical to any known babesiae described to date. They were closely related to, but clearly distinct from, *B. divergens*. A novel *Babesia*, named EU1, was recently identified as an etiological agent for 2 cases of human babesiosis in Europe (Herwaldt et al., 2003). Molecular analysis of the parasite showed that EU1 is indistinguishable from babesiae from Slovenian ticks in the analyzed 364–base pair (bp) portion of the 18S rRNA gene.

To characterize the detected babesiae in *I. ricinus* ticks from Slovenia and to clarify further its identity with EU1, we cloned and sequenced the complete 18S rRNA gene of babesiae. DNA from *I. ricinus* ticks infected with babesiae from the aforementioned study was the source for further molecular characterization of the unknown babesial parasite (Duh et al., 2001). For cloning purposes, DNA was successfully amplified with CRYPTO F (5′AAC CTG GTT GAT CCT GCC AGT AGT CAT) and CRYPTO R (5′GAA TGA TCC TTC CGC AGG TTC ACC TAC) primers yielding a PCR product of ~1,700 bp. With the obtained amplicon, ligation into a plasmid vector was performed (TOPO TA Cloning Kit for Sequencing, Invitrogen, Groningen, Netherlands). After ligation, *Escherichia coli* competent cells were transformed as instructed by the manufacturer. Transformed colonies were selected on LB agar plates containing 50 μl/ml ampicillin. Plasmid DNA was purified from overnight cultures of selected colonies (Wizard Plus Minipreps DNA Purification System, Promega, Madison, Wisconsin) and analyzed for inserts by restriction analysis with *Eco*R1 (Promega). The complete sequence of the babesial 18S rRNA gene was obtained from a single clone. Sequencing on both strands was carried out in an ABI PRISM Model 310 automated sequencer with BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California). Sequences were further analyzed with computer programs of the Lasergene 1999 software package (Dnastar, Madison, Wisconsin). The alignment of the complete babesial 18S rRNA gene sequences showed 99.9% identity of the unknown babesiae from *I. ricinus* from Slovenia with EU1, differing at only 1 nucleotide position (nt 1715: G-A). Phylogenetic relationships of babesiae used for alignment with the ClustalW algorithm are shown in Figure 1. The unique sequence determined in this study was deposited in GenBank (AY553915).

In a recent study, we used molecular methods to screen *I. ricinus* ticks collected in Slovenia for the presence of babesiae (Duh et al., 2001). We expected to detect *B. divergens*, a parasite that is transmitted by this species of tick in Europe (Gray et al., 1990). The results raised our interest because the detected babesial parasites were only closely related to, but clearly distinct from, *B. divergens*. The inability to detect *B. divergens* in ticks could be explained by the collection of ticks in woodland and forest areas. It is well known that *I. ricinus* ticks feed on many different hosts, including mammals, reptiles, and birds (Milne, 1948; L'Hostis and Seegers, 2002). In woodlands, cervids and small mammals are the main hosts for these ticks. Although they play an important role in maintaining the ticks in nature, they are not reservoirs of *B. divergens* (L'Hostis and Seegers, 2002). With the exception of a single report of naturally acquired babesiosis caused by *B. divergens* in reindeer (*Rangifer tarandus tarandus*), no other data regarding *B. divergens* infection in cervids exist (Langton et al., 2003). Recently, Herwaldt et. al. (2003) published a paper reporting 2 human cases of babesiosis from Italy and Austria caused by an etiological agent related to, but again distinct from, *B. divergens*. They referred to it as EU1, a zoonotic pathogen with previously unreported molecular characteristics. These authors compared the partial sequence of the babesial 18S rRNA gene from Slovenian ticks obtained in our screening study with EU1 and established that they are identical in the analyzed part of the gene. However, Caccio et al. (2002) illustrated the limited value of phylogenetic analysis based on the partial sequences of 18S rRNA gene by showing a discrepancy in the phylogenetic position of *Babesia canis vogeli*. Hence, for determination of relatedness of the EU1 and babesial parasites from ticks, the complete 18S rRNA gene sequence for the organism from ticks was needed. By cloning and sequencing the complete 18S rRNA gene of the unknown babesiae from *I. ricinus* ticks from Slovenia, we have provided strong molecular evidence that the unknown parasite is indeed *Babesia* EU1. The complete sequences of both parasites were 99.9% identical, differing at only 1 nucleotide position. Two important facts can be drawn from the newly acquired information on EU1. First, *I. ricinus* ticks could be responsible for transmitting yet another human pathogen, and second, it is apparent that other species of babesiae can cause disease in humans.

The authors thank Franc Gubensek and Vera Zupunski from the Institute Jozef Stefan, Slovenia, for guidance and technical assistance with cloning reactions.

LITERATURE CITED

CACCIO, S. M., B. ANTUNOVIC, A. MORETTI, V. MANGILI, A. MARINCULIC, R. R. BARIC, S. B. SLEMENDA, AND N. J. PIENIAZEK. 2002. Molecular characterisation of *Babesia canis canis* and *Babesia canis vogeli* from naturally infected European dogs. Veterinary Parasitology **10:** 285–292.

CENTENO-LIMA, S., V. DO ROSARIO, R. PARREIRA, A. J. MAIA, A. M. FREUDENTHAL, A. M., NIJHOF, AND F. JONGEJAN. 2003. A fatal case of human babesiosis in Portugal: Molecular and phylogenetic analysis. Tropical Medicine and International Health **8:** 760–764.

DUH, D., M. PETROVEC, AND T. AVSIC-ZUPANC. 2001. Diversity of babesia infecting European sheep ticks (*Ixodes ricinus*). Journal of Clinical Microbiology **39:** 3395–3397.

GRAY, J. S. 1980. Studies on the activity of *Ixodes ricinus* in relation to the epidemiology of babesiosis in Co. Meath, Ireland. British Veterinary Journal **136:** 427–436.

———. 1985. Bovine babesiosis in Ireland. Irish Veterinary News **October:** 9–14.

———, T. M. MURPHY, S. M. TAYLOR, D. A. BLEWETT, AND R. HARRINGTON. 1990. Comparative morphological and cross transmission studies with bovine and deer babesias in Ireland. Preventive Veterinary Medicine **9:** 185–193.

HERWALDT, B. L., S. CACCIO, H. GHERLINZONI, H. ASPOCK, S. B. SLEMENDA, P. PICCALUGA, G. MARTINELLI, R. EDELHOFER, U. HOLLENSTEIN, G. POLETTI, S. PAMPIGLIONE, K. LOSCHENBERGER, S. TURA, AND N. J. PIENIAZEK. 2003. Molecular characterization of a non–*Babesia divergens* organism causing zoonotic babesiosis in Europe. Emerging Infectious Diseases **9:** 942–948.

HOMER, M. J., I. AGUILAR-DELFIN, S. R. TELFORD 3RD, P. J. KRAUSE, AND D. H. PERSING. 2000. Babesiosis. Clinical Microbiological Reviews **13:** 451–469.

KJEMTRUP, A. M., AND P. A. CONRAD. 2000. Human babesiosis: An emerging tick-borne disease. International Journal for Parasitology **30:** 1323–1337.

LANGTON, C., J. S. GRAY, P. F. WATERS, AND P. J. HOLMAN. 2003. Naturally acquired babesiosis in a reindeer (*Rangifer tarandus tarandus*) herd in Great Britain. Parasitology Research **89:** 194–198.

LEWIS, D., AND E. R. YOUNG. 1980. The transmission of a human strain of *Babesia divergens* by *Ixodes ricinus* ticks. Journal of Parasitology **66:** 359–360.

L'HOSTIS, M., AND A. CHAUVIN. 1999. *Babesia divergens* in France: Descriptive and analytical epidemiology. Parasitologia **41**(Suppl. 1): 59–62.

———, AND H. SEEGERS. 2002. Tick-borne parasitic diseases in cattle: Current knowledge and prospective risk analysis related to the ongoing evolution in French cattle farming systems. Veternary Research **33:** 599–611.

MILNE, M. A. 1948. The ecology of the sheep tick, *Ixodes ricinus*. Observations on hill and moorland grazing in Northern England. Parasitology **39:** 173–197.

PERSING, D. H., D. MATHIESEN, W. F. MARSHALL, S. R. TELFORD, A. SPIELMAN, J. W. THOMFORD, AND P. A. CONRAD. 1992. Detection of *Babesia microti* by polymerase chain reaction. Journal of Clinical Microbiology **30:** 2097–2103.

J. Parasitol., 91(?), 2005, pp. 465–467

Hesperoctenes fumarius (Hemiptera: Polyctenidae) Infesting *Molossus rufus* (Chiroptera: Molossidae) in Southeastern Brazil

Carlos E. L. Esbérard*, Andrea C. Jesus*, Adarene G. Motta*, Helena G. Bergallo†, and Donald Gettinger‡, Programa de Pós-Graduação, Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier 524, 20559-900, Rio de Janeiro, RJ, Brazil; *Projeto Morcegos Urbanos, Fundação RIOZOO, Quinta da Boa Vista s.no., 20940-040, Rio de Janeiro, RJ, Brazil; †Departamento de Ecologia, Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier 524, 20559-900, Rio de Janeiro, RJ, Brazil; ‡Manter Laboratory of Parasitology, University of Nebraska, Lincoln, Nebraska 68588-0514. *e-mail: cesberard@terra.com.br*

ABSTRACT: We analyzed the prevalence, intensity, and medium density of parasitism of *Hesperoctenes fumarius* infesting *Molossus rufus* in natural (hollow trees) and anthropogenic roosts (attics) in southeastern Brazil. The prevalence and intensity of infestations were higher in the hollow trees than in the attic roosts. We also noted a relationship between the amount of space available within the roost and the infestation levels of *H. fumarius*. One advantage of roosting in larger, often man-made, refuges may be the reduction in ectoparasite infestations.

Polyctenid bugs, or ''batbugs'' (Hemiptera: Polyctenidae), are blood-sucking ectoparasites of bats, commonly associated with the Molossidae in the Neotropics (Marshall, 1991). In southeastern Brazil, these ectoparasites are commonly collected from the pelage of *Molossus rufus* (E. Geoffroy, 1805), a medium-sized (33 g), insectivorous bat that frequently uses roofs and attics of houses and buildings as roosts (Marques, 1986; Fenton et al., 1998; Esbérard, 2002). This bat species prefers roosts where the temperature is elevated, usually by a combination of factors including the general insolation of the roost, the number and density of bats, and the decomposition of feces and urine. The colonies of *M. rufus* are large, often exceeding 500 individuals (Marques, 1986), but numbers vary through time, depending on reproduction, immigration, and emigration (Esbérard, 2002). In natural conditions, these bats often roost in hollow trees, and Esbérard et al. (2003) observed that individuals commonly returned to the same roost through 4 reproductive seasons. However, roost fidelity may have costs, i.e., long-term use of the same roosts may increase the probability of heavy parasite burdens (Lewis, 1995). In this article, we examine the parasitism of a polyctenid bug, *Hesperoctenes fumarius*, associated with the molossid bat, *M. rufus*, by comparing the infestation levels of natural and anthropogenic roosts in southeastern Brazil.

We sampled populations of polyctenid bugs infesting *M. rufus* in 3 roosts in southeastern Brazil, Rio de Janeiro State. Two were natural roosts, and the third was anthropogenic. Roost no. 1 was in a hollow ''pau-d'alho'' tree (*Agonandra brasiliensis*, Olacaeae), extending from a point 0.4 m from ground level to a height of 3.2 m, with roost space estimated at 3 m^3 and access from above; this roost was located in the Fazenda Ventania, Municipality of Casimiro de Abreu (22°33′13.98″S, 42°00′35.82″W). Roost no. 2 was also in a hollow pau-d'alho tree, extending from the level of the ground to a height of 2.2 m, with a roost

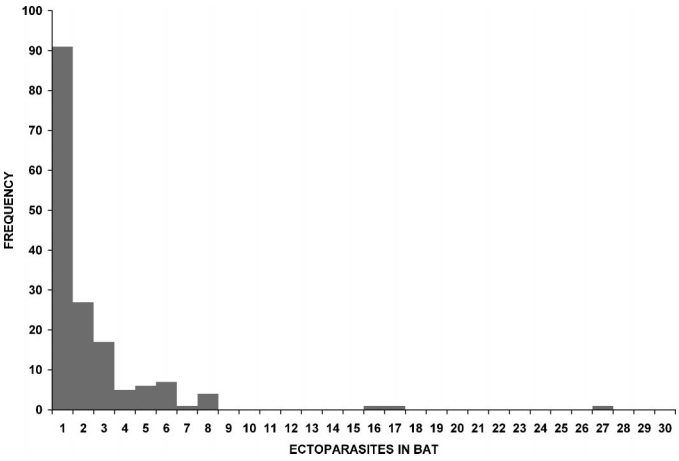


FIGURE 1. Frequency of *Hesperoctenes fumarius* on *Molossus rufus* in 3 refuges sampled in southeastern Brazil.

TABLE I. Data of the collections, number of bats, number of positive bats, and total ectoparasites (*Hesperoctenes fumarius*) collected in the 3 roosts in the State of Rio de Janeiro, southeastern Brazil.

Roost nos.	Date	Size of colony	Positive bats	Number of ectoparasites
1	24 May 2003	34	26	98
1	06 September 2003	60	32	84
2	11 November 2000	29	10	10
2	24 March 2001	59	19	35
2	28 April 2001	26	11	11
2	21 July 2001	37	32	47
2	29 September 2001	14	2	4
2	02 November 2001	39	19	42
3	13 January 2001	68	7	7
3	17 March 2001	92	5	6
3	21 April 2001	64	4	4
3	02 June 2001	57	3	3
3	25 July 2001	23	2	2
3	20 October 2001	57	9	15
3	02 November 2001	113	11	19

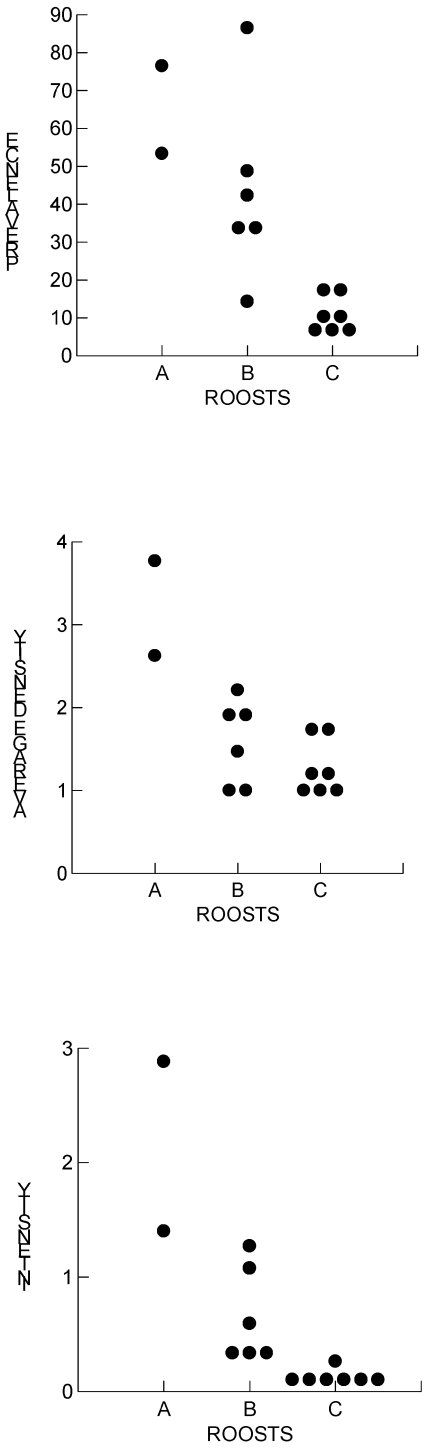


FIGURE 2. Parasitism of *Hesperoctenes fumarius* on *Molossus rufus* in southeastern Brazil. **A.** Refuge in a hollow tree of 4 m^3. **B.** Refuge in a hollow tree with 3 m^3. **C.** Attic of house.

space estimated at 4 m^3, with access from above; it was located in the Fazenda da Barra, Municipality of Casimiro de Abreu (22°33′03.3″S, 42°03′02″W). Roost no. 3 was an enclosed space under the slate-roof of a building, with a roost area estimated at 30 m^3; there were large areas of empty space and 3 entrances by holes on the wall, located in the Biological Reserve of Poço das Antas, Municipality of Silva Jardim (22°33′39.2″ S, 42°16′19.3″W). In all 3 roosts, *M. rufus* shared space

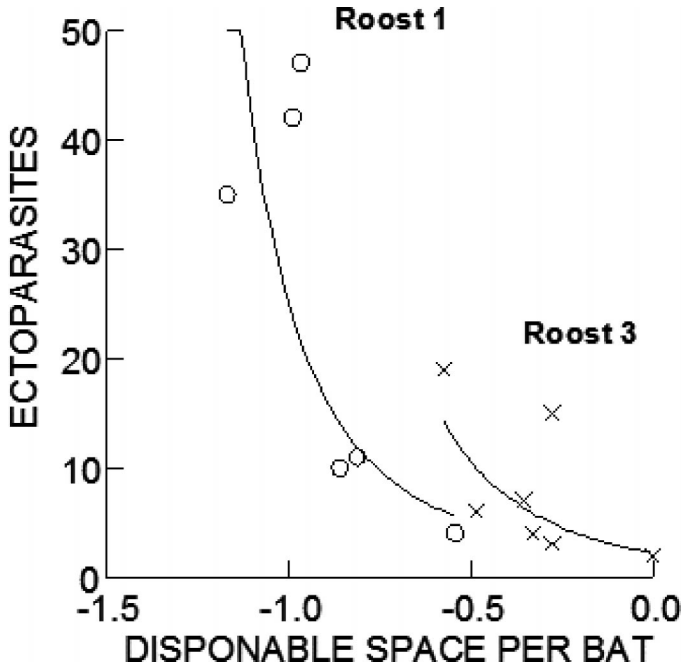


FIGURE 3. Variation of available space per bat (m^3) and the number of bats and the number of ectoparasites collected in 2 of the roosts sampled in southeastern Brazil. The circles represent the sample from the hollow tree (roost no. 1) and the crosses the collection from the attic of a house (roost no. 3).

with another bat, the large phyllostomid, *Phyllostomus hastatus*; parasitism with *H. fumarius* was never observed in these cohabiting bats.

Each roost was sampled for 2–7 nights using mist nets opened all night next to the entrances or in the case of the anthropogenic roost, modified Davis traps (Esbérard, 2002, 2003). Captured bats were individually marked with plastic collars provided with colored beads following an established code (see Esbérard and Daemon, 1999). The bats were examined carefully; all batbugs were removed with forceps and preserved in vials of 70% ethanol. After marking and sampling for ectoparasites, the bats were released at the point of capture.

We analyzed the prevalence (proportion of bat individuals infested), the intensity of infestation (mean number of batbugs sampled per host sample or the total colony), and medium density of parasitism (mean number of batbugs per infested host sample) (Margolis et al., 1982). The prevalence, intensity, and medium density of the 3 roosts were compared with an analysis of variance realized through SYSTAT 7.0. The aggregation of parasites was calculated by the formula $k = m^2/(s^2 - m)$, where s = variance and m = mean of parasites per host. This index indicates whether the distribution of the parasite tends to be random ($k > 20$) or aggregated ($k = 1$) (Wilson et al., 2002).

Molossus rufus was captured 762 times and found positive for *H. fumarius* on 161 occasions (21%), with a total of 387 batbug ectoparasites collected (Table I). The number of ectoparasites per bat varied from 1 to 27, with a medium of 2.22 ± 2.86 and mode of 1 ectoparasite per bat (52.5%). Three samples displayed elevated infestations (more than 15 ectoparasites per bat), corresponding to 16.2% of the ectoparasites collected ($k = 0.0068$), demonstrating that this parasite displays an aggregated distribution (Fig. 1).

The prevalence showed variation among the 3 roosts analyzed, the 2 hollow trees (roosts nos. 1 and 2) were represented by mean prevalences (48.54 ± 23.67%) higher than the attic (roost no. 3) (8.78% ± 3.70) ($F_{2,12} = 12.07$, $P = 0.001$). The prevalence differed between roost nos. 1 and 3 ($P = 0.003$) and roost no. 2 with no. 3 ($P = 0.008$) (Fig. 2). The intensity presented a similar pattern, with the hollow trees presenting values higher (1.035 ± 0.863) than the attic (0.115 ± 0.076) ($F_{2,12} = 19.48$, $P < 0.001$). Only roost nos. 2 and 3 did not differ significantly ($P = 0.074$) (Fig. 2). The medium density varied ($F_{2,12} = 13.80$, $P = 0.001$) among the 3 roosts considered, although roost nos.

2 and 3 did not differ significantly ($P = 0.382$) (Fig. 2). Of the 2 most sampled roosts, a clear relationship was observed between the space available for the bats (roost space) and the number of ectoparasitic batbugs collected (Fig. 3).

The size of the bat colony varies seasonally, with largest concentrations observed during the reproductive season (Esbérard, 2002). Less roost space imposes higher contact between the bats and, consequently, increases the probability of parasitism. Larger spaces may permit, and even maintain, more plentiful colonies and can result in dilution of the number of ectoparasites infesting the colony. The use of anthropogenic roosts, generally with larger amounts of available roost space, is shown to have advantages in avoiding parasitism. Also, living in an urban area often provides an elevated availability of prey for insectivorous bats because insects are attracted to artificial illumination. *Molossus rufus* is a common bat in urban areas of southeastern Brazil and frequently roosts in roofs and attics of houses and buildings (Esbérard et al., 1999). Various species change localities to avoid parasites (Lewis, 1995). This bat uses the refuge for short periods of time, being sited 3 mo of each year (Esbérard, 2002), and this can be a strategy to limit the chance of elevated infestation by parasites.

We thank Gustavo Gracioli for the identification of the voucher material. We thank the Secretaria de Agricultura e Pesca de Casimiro de Abreu that paid for part of the resources necessary for this work. We thank the Reserva Biológica de Poço das Antas (Processo 02045.000233/00-68) and the Fazenda da Barra for permission to collect. We thank the Associação Mico Leão for the use of their installations and João Branco (SMAP—Casimiro Abreu) e Carlos (Fazenda da Barra) for their support in the realization of this project. We thank Adriene Areas and Débora Moraes for help with the fieldwork. Ludmilla Aguiar and Cristina Nassar edited a version of this work. During these proceedings H.G.B. received a research grant from CNPq.

LITERATURE CITED

ESBÉRARD, C. 2002. Composição de colônia e reprodução de *Molossus rufus* (E. Geoffroy) (Chiroptera, Molossidae) em um refúgio no sudeste do Brasil. Revista Brasileira de Zoologia **19:** 1153–1160.

———. 2003. Armadilha para retirada de morcegos abrigados em telhado. Chiroptera Neotropical **9(1-2):** 164–166.

———, A. S. CHAGAS, AND E. M. LUZ. 1999. Uso de residências por morcegos no Estado do Rio de Janeiro (Mammalia: Chiroptera). Revista Brasileira de Medicina Veterinaria **21:** 17–20.

———, AND C. DAEMON. 1999. Novo método para marcação de morcegos. Chiroptera Neotropical **5:** 116–117.

———, A. G. MOTTA, D. M. OLIVEIRA, A. F. ÁREAS, R. T. V. RODRIGUES, AND H. G. BERGALLO. 2003. Observações de fidelidade ao refúgio em *Molossus rufus* no Estado do Rio de Janeiro, sudeste do Brasil. Chiroptera Neotropical **9(1-2):** 175–178.

FENTON, M. B., I. L. RAUTENBACH, J. RYDELL, H. T. ARITA, J. ORTEGA, S. BOUCHARD, M. D. HOVORKA, B. LIM, E. ODGREN, C. V. PORTFORS, W. M. SCULLY, D. M. SYME, AND M. J. VONHOF. 1998. Emergence, echolocation, diet and foraging behavior of *Molossus ater* (Chiroptera: Molossidae). Biotropica **30:** 314–320.

LEWIS, S. E. 1995. Roost fidelity of bats: A review. Journal of Mammalogy **76:** 481–496.

MARGOLIS, L., G. W. ESCH, J. C. HOLMES, A. M. KURIS, AND G. A. SCHAD. 1982. The use of ecological terms in parasitology (report of an ad hoc committee of the American Society of Parasitologists). Journal of Parasitology **68:** 131–133.

MARQUES, S. A. 1986. Activity cycle, feeding and reproduction of *Molossus ater* (Chiroptera: Molossidae) in Brazil. Boletim do Museu Paraense Emilio Goeldi **2:** 159–179.

MARSHALL, A. G. 1991. The ecology of ectoparasitic insects. Academic Press, New York, 445 p.

WILSON, K., O. N. BJØRNSTAD, A. P. DOBSON, S. MERLER, G. POGLAYEN, S. E. RANDOLPH, A. F. READ, AND A. SKORPING. 2002. Heterogeneities in macroparasite infections: Patterns and process. *In* Ecology of wildlife diseases, P. J. Hudson, A. Rizzoli, B. T. Grenfell, H. M. Heesterbeek, and A. P. Dobson (eds.). Oxford University Press, Oxford, U.K., p. 6–44.

J. Parasitol., 91(2), 2005, pp. 467–470

Suppressed Cytokine and Immunoglobulin Secretions by Murine Splenic Lymphocytes Infected In Vitro with *Toxoplasma gondii* Tachyzoites

S.-M. Guk, J. Kook*, Y.-H. Jeon†, J.-H. Choi‡, E.-T. Han§, E.-H. Shin, and J.-Y. Chai¶, Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine, and Institute of Endemic Diseases, Seoul National University Medical Research Center, Seoul 110-799, Korea; *Department of Biochemical Engineering, Yanbian University of Science and Technology, Yanji City, Jilin Province 133000, P.R. China; †Department of Pediatrics, Inha University College of Medicine, Inchon 400-711, Korea; ‡Department of Pediatrics, Seoul National University Children's Hospital, Seoul 110-744, Korea; §Department of Parasitology, Kangwon National University College of Medicine, Chunchon 200-701, Korea; ¶To whom correspondence should be addressed. *e-mail: cjy@snu.ac.kr*

ABSTRACT: Mechanisms of host immunosuppression after infection with *Toxoplasma gondii* are unclear. This study was performed to observe cytokine and immunoglobulin secretions by murine splenic lymphocytes infected in vitro with live, nonreplicating (irradiated) RH tachyzoites on stimulation with concanavalin A (Con A) or lipopolysaccharide (LPS). For lymphocyte cultivation, 3 groups were prepared: coculture with live nonirradiated tachyzoites separated by a transwell (group T), live irradiated tachyzoites without a transwell (group R), and no tachyzoites (group C). Compared with group T, groups R and C, on stimulation with Con A, revealed significantly ($P < 0.05$) lower levels of interleukin-2 (IL-2) and IFN-γ, but not IL-10. The levels of IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM were also significantly ($P < 0.05$) lower in groups R and C than in group T after stimulation with LPS. The results suggest that intracellular infection of murine splenic lymphocytes with *T. gondii* tachyzoites could impair their capacity to produce cytokine and immunoglobulin secretions.

Lymphocyte functions have been reported to be suppressed in humans (Channon and Kasper, 1996, 1999) and rodents (Suzuki et al., 1981; Suzuki and Kobayashi, 1983; Candolfi et al., 1994, 1995; Haque et al., 1995; Khan et al., 1995; Haque et al., 1998; Neyer et al., 1998) during acute *Toxoplasma gondii* infection. In mice, both primary antibody responses and the initiation of memory cells were suppressed after *T. gondii* infection (Suzuki and Kobayashi, 1983). It has been suggested that some soluble mediators inducing immunosuppression are released by the parasites (Channon and Kasper, 1996). Subsequently, microneme (MIC-1 and MIC-2) and surface proteins (SAG-1 and SAG-2) were shown to be candidate immunosuppression mediators (Channon et al., 1999). In addition, an increase in interleukin-10 (IL-10) and a reduction in IL-2 were associated with the down-regulation of host immunity by *T. gondii* (Haque et al., 1994, 1995). Yet, the precise mechanisms of immunosuppression are still unknown.

Our previous studies showed that *T. gondii* can invade murine splenic T and B lymphocytes and proliferate in these cells (Chai et al., 1997; Shin et al., 2004); B cells are slightly more susceptible than T cells (Shin et al., 2004). According to transmission electron microscope (TEM) observations, the rough endoplasmic reticulum of tachyzoite-

infected B lymphocytes, where immunoglobulins are produced, is significantly reduced, and it was suggested that immunoglobulin production by these cells would be seriously affected (Chai et al., 1997).

With regard to *Trypanosoma cruzi*, another species of intracellular protozoan, human dendritic cells infected in vitro with this protozoan were shown to be functionally impaired as their capacity to produce cytokines and other molecules was reduced (Van Overtvelt et al., 1999). However, in the case of *T. gondii*, no reports are available that demonstrate functional impairments of parasite-infected T or B lymphocytes. Thus, this study was performed to examine the functional capacity of murine splenic lymphocytes infected in vitro with irradiated *T. gondii* tachyzoites, in terms of cytokine and immunoglobulin secretions.

Virulent RH tachyzoites of *T. gondii* were serially passaged in ICR mice every 5 days. Peritoneal exudates of the mice were harvested, suspended in phosphate-buffered saline (PBS), and centrifuged at 1,500 rpm for 5 min. Tachyzoites were isolated from the interlayer between 40 and 50% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradients and resuspended in PBS. After washing with PBS, the isolated tachyzoites were suspended in RPMI 1640 (Gibco, Grand Island, New York) containing 10% inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES buffer (pH 7.4) at a parasite density of 1×10^6 tachyzoites per well in 96-well plates (Costar, Cambridge, Massachusetts).

Splenic lymphocytes were obtained from a total of 30 naïve 8-wk-old male BALB/c mice. Briefly, spleen cells from 5 mice were pooled, and erythrocytes were lysed by hypotonic shock with 0.2% ammonium chloride. Lymphocytes were purified by density gradient centrifugation with Histopaque 1.077 (Sigma, St. Louis, Missouri), washed in PBS, and suspended in RPMI 1640 containing 10% FBS at a density of 1×10^6 cells per well in 96-well plates. Lymphocyte viability as determined by trypan blue exclusion testing was >99%. Lymphocytes were stimulated by coculture with mitogens, namely, concanavalin A (Con A, 10 μg/ml; Sigma) for 2 days or lipopolysaccharide (LPS; Sigma) for 7 days at 37 C in a 95% air–5% CO_2 atmosphere.

Three groups were prepared: a coculture of lymphocytes with live nonirradiated tachyzoites separated by a transwell system (group T), a coculture of lymphocytes in close contact with live irradiated tachyzoites (group R) to allow parasites to invade lymphocytes, and a culture of naïve lymphocytes only (group C). Each group was divided into 2 subgroups, with or without Con A (to stimulate T lymphocytes) and with or without LPS (to stimulate B lymphocytes). In each subgroup, triplicate wells were prepared to a final volume of 200 μl per well. Experiments were repeated 3 times with similar results.

For group T, the dual-chamber transwell culture system (Costar) was used. Each transwell measured 24.5 mm in diameter and consisted of 2 compartments separated by a semipermeable polycarbonate membrane (pore size 0.4 μm). The distance between the 2 chambers was 1 mm, allowing cells to be cocultured in close proximity, but without contacting the tachyzoites. In group R, tachyzoites in PBS were gamma irradiated with a Cs-137 irradiator (JL Sheperd and Associates Co., San Fernando, California) at a rate of 2.74 Gy/min from 40 cm. The irradiation dose was determined as 300 Gy, which inhibited intracellular proliferation of *T. gondii* tachyzoites (Kook et al., 1995); this took 79 min.

The ultrastructure of the splenic lymphocytes infected with irradiated tachyzoites was observed by TEM. Samples were washed with cacodylate buffer (pH 7.4) and fixed with 2.5% glutaraldehyde at 4 C for 12 hr. After washing, they were postfixed with 1% osmium tetroxide for 2 hr and then dehydrated in a graded ethanol series. To prepare ultrathin sections, semithin sections were further processed and stained with uranyl acetate and lead citrate. Sections were then observed by TEM (1200 EX-II, JEOL, Tokyo, Japan) at 80 kV.

After 2 (for cytokines) or 7 days (for immunoglobulins) of incubation, the culture supernatant of each well was collected by centrifugation and stored at −70 C. The concentrations (pg/ml) of IL-2, IFN-γ, and IL-10 were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Cambridge, Massachusetts), with standard curves for recombinant murine IL-2, IFN-γ, and IL-10. The amounts of IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA secreted into the culture supernatant were measured with an ELISA kit (Pharmingen, San Diego, California) at an optical density (OD) of 410 nm, again with the use of standard curves for respective immunoglobulin isotypes. Statistical significance of differences was determined with the 2-tailed Student's *t*-test. $P < 0.05$ was considered significant for all comparisons.

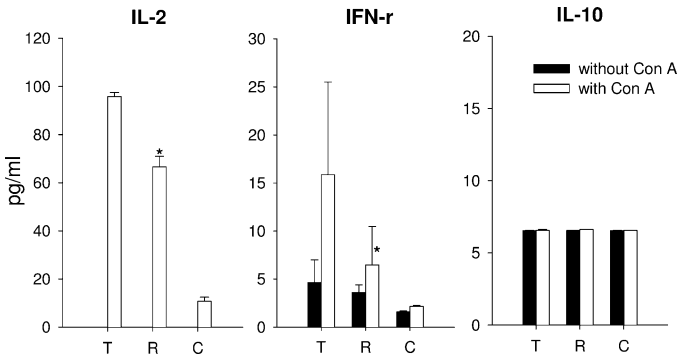


FIGURE 1. Concentrations of IL-2, IFN-γ, and IL-10 secreted by splenic lymphocytes and incubated with *Toxoplasma gondii* tachyzoites for 2 days in vitro with (open bar) or without (solid bar) concanavalin A (Con A). Group T: coculture (separately) with live tachyzoites in a transwell system. Group R: coculture with irradiated tachyzoites without a transwell system. Group C: culture of lymphocytes only. Vertical bars indicate the means ± SD. The levels of IL-2 and IFN-γ were significantly ($P < 0.01$) lower in group R than in group T.

TEM showed solitary tachyzoites in the parasitophorous vacuoles of splenic lymphocyte cytoplasm, usually 1 vacuole per lymphocyte, through 7 days postculture (data not shown). The conoid of the tachyzoites was intact, and rhoptries were reduced in size, but increased in number. Irradiated tachyzoites successfully invaded lymphocytes but did not undergo intracellular proliferation to cause host cell rupture.

Stimulation of naïve splenic lymphocytes with Con A for 2 days in vitro resulted in their markedly increased IL-2 and IFN-γ production (Fig. 1). Compared with group T (with Con A), however, group R (with Con A) produced significantly ($P < 0.01$) lower levels of IL-2 and IFN-γ, whereas group C showed only background levels of these 2 cytokines. In contrast, the levels of IL-10 showed no notable differences between the 3 groups (Fig. 1).

Stimulation of naïve splenic lymphocytes with LPS for 7 days in vitro resulted in increased production of immunoglobulins in the culture supernatant (Fig. 2). However, the ODs at 410 nm for IgG1, IgG2a, IgG2b, and IgG3 in groups R and C (with LPS) were significantly ($P < 0.01$) lower than those in group T (with LPS). IgG2b had the lowest OD and was the most highly suppressed member of the IgG subclasses (Fig. 2). Overall, the levels of IgG subclasses in group R were similar to those of group C. Patterns similar to IgG were observed for IgA and IgM (Fig. 2). The level of IgM was generally higher than those of the other immunoglobulin isotypes.

The results of this study show that the capacity of tachyzoite-exposed murine splenic lymphocytes to produce cytokines or immunoglobulins is enhanced or unchanged when compared with lymphocytes exposed only to Con A or LPS. However, significantly decreased synthesis in cytokines and immunoglobulins was observed when the comparison was made between lymphocytes exposed to tachyzoites and lymphocytes separated from tachyzoites in a transwell system. It is speculated, therefore, that tachyzoite-infected lymphocytes might undergo functional impairments in terms of their capacities to produce cytokines and immunoglobulins. Irradiated tachyzoites were used because they can invade mammalian cells, maintain their metabolic functions, and elicit cellular immunity similar to natural infection in mice, without multiplying in the host cells (Kook et al., 1995; Hiramoto et al., 2002). The cytokines shown to be suppressed by tachyzoite infection were IL-2 and IFN-γ, but not IL-10. All the immunoglobulin isotypes and subtypes examined (i.e., IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA) revealed suppressed secretion.

It is suggested that occupation of the cytoplasmic space of immune cells, particularly lymphocytes, by live tachyzoites might be a factor for host immunosuppression. In *T. cruzi*, parasite-infected human dendritic cells were shown to be functionally impaired, with their lowered capacity to produce cytokines and other molecules (Van Overtvelt et al., 1999). With regard to *T. gondii*, the effect of intracellular infection of

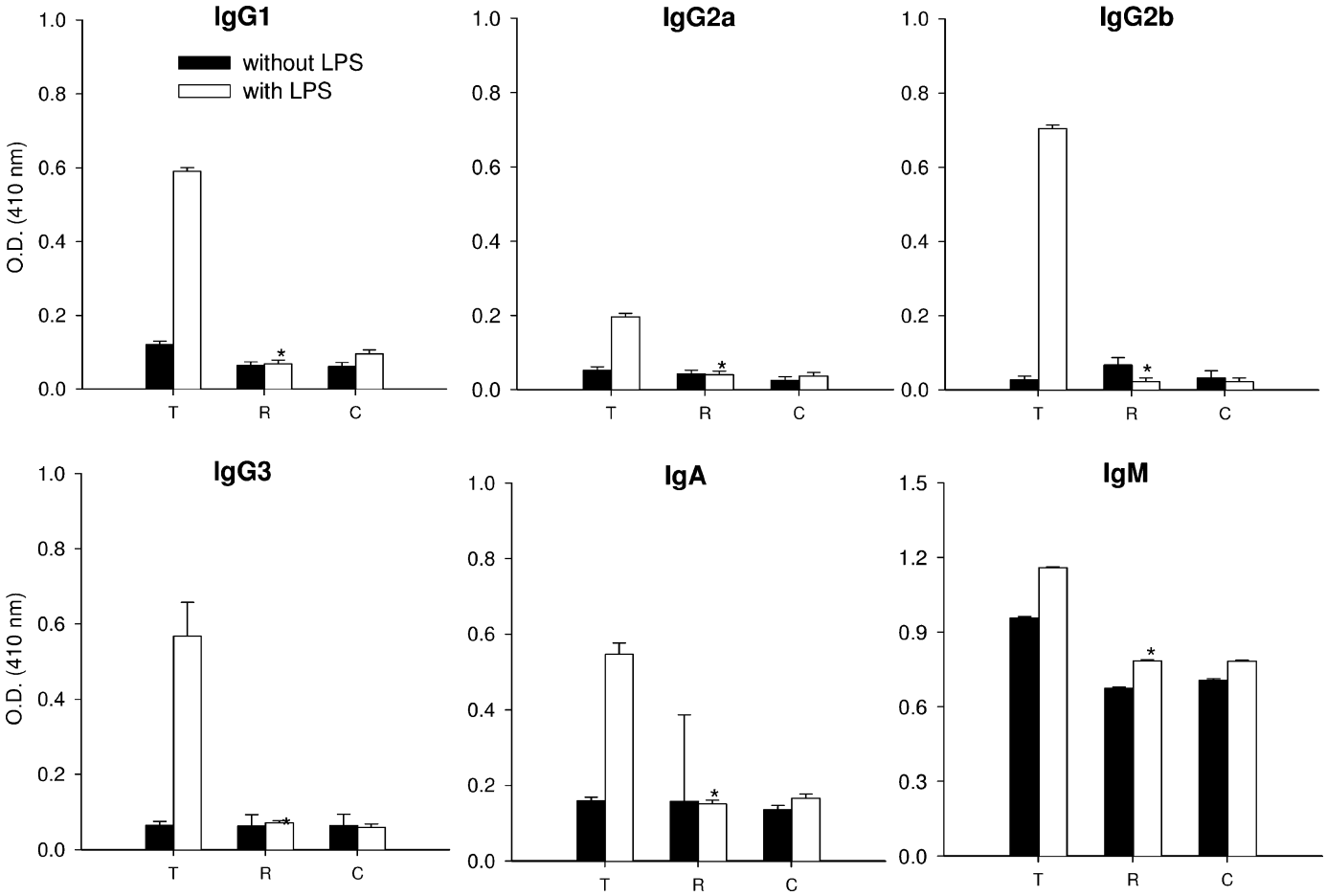


FIGURE 2. Concentrations of IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM as revealed by optical density (OD) at 410 nm, secreted by splenic lymphocytes incubated with *T. gondii* tachyzoites for 7 days in vitro with (open bar) or without (solid bar) lipopolysaccharide (LPS). Group T: coculture (separately) with live tachyzoites in a transwell system. Group R: coculture with irradiated tachyzoites without a transwell system. Group C: culture of lymphocytes only. Vertical bars indicate means ± SD. Immunoglobulin secretions were all significantly ($P < 0.01$) lower in group R than in group T.

immune cells and their functional impairments, and subsequent rupture of these cells, would be detrimental to host immunity, considering that up to one third of splenic T and B lymphocytes were found to be infected during the first 12 hr of coculture with *T. gondii* tachyzoites (Shin et al., 2004). However, in vivo experiments are required to elucidate this point.

Other factors of host immunity down-regulation include soluble mediators secreted from host immune cells or by the parasites themselves. These included IL-10, transforming growth factor β, nitric oxide, prostaglandin generated by splenocytes in response to parasite antigens (Haque et al., 1994, 1995; Khan et al., 1995; Channon and Kasper, 1996; Wei et al., 2002), and antigenic proteins liberated by the parasites (Channon et al., 1999). Even IFN-γ, the major mediator of resistance against *T. gondii* (Suzuki et al., 1988), appeared to be, paradoxically, the principal component of up to 50% soluble factor-mediated suppression of mitogen-induced lymphocyte DNA synthesis in vitro (Channon and Kasper, 1996). In this connection, immunosuppression of the host in acute toxoplasmosis is considered to be due to multiple factors.

We used the transwell system (group T) to protect host cells from infection with tachyzoites. In this group, cytokine and immunoglobulin secretions were active. This group could be used as a positive control for group R, in which the transwell system was not used and lymphocyte infection was allowed. The possible secretion of soluble immunosuppression mediators in group T can be excluded because the down-regulatory response requires contact between a small number of parasites and host cells (Channon and Kasper, 1996).

The observed attenuation of the secretion of Th1 cytokines, IL-2 and IFN-γ, by splenic lymphocytes infected in vitro with tachyzoites was consistent with other studies in mice (Candolfi et al., 1995; Khan et al., 1995; Mun et al., 2003). However, IL-10, a Th2 cytokine produced by T cells and macrophages and antagonistic to IL-2 and IFN-γ (Haque et al., 1994; Khan et al., 1995), was produced only at background levels, regardless of cell-to-parasite contact, seemingly because of the in vitro culture and, in particular, the shorter cultivation time with Con A stimulation prior to IL-10 measurement (2 days) compared with the 7–14 days required until maximum immunosuppression in mice (Candolfi et al., 1994).

In this study during the 2 or 7 days of in vitro culture, some of the tachyzoite-infected lymphocytes might have undergone apoptosis, and this could have resulted in anomalous results. It is of interest, however, that *T. gondii*–infected cells have been reported to be resistant to multiple apoptotic inducers (Nash et al., 1998; Wei et al., 2002). In these studies, the host cells tested included murine splenic T and B lymphocytes, and apoptotic inhibition required live intracellular parasites and ongoing protein synthesis (Nash et al., 1998; Wei et al., 2002). Host cells, parasites, and culture conditions in this study were similar to those described previously. Therefore, it is presumed that lymphocytes infected with irradiated tachyzoites were resistant to apoptosis, and it appeared that our assay system worked well.

This work was supported by a grant (R01-2002-000-00422-0) from the Basic Research Program of the Korea Science and Engineering

Foundation and the BK21 Human Life Sciences, Ministry of Education, Republic of Korea.

LITERATURE CITED

CANDOLFI, E., C. A. HUNTER, AND J. S. REMINGTON. 1994. Mitogen- and antigen-specific proliferation of T cells in murine toxoplasmosis is inhibited by reactive nitrogen intermediates. Infection and Immunity **62:** 1995–2001.

———, ———, AND ———. 1995. Roles of gamma interferon and other cytokines in suppression of the spleen cell proliferative response to concanavalin A and *Toxoplasma* antigen during acute toxoplasmosis. Infection and Immunity **63:** 751–756.

CHAI, J. Y., J. KOOK, S. M. GUK, Y. P. CHANG, AND C. K. YUN. 1997. Experimental infection of murine splenic lymphocytes and granulocytes with *Toxoplasma gondii* RH tachyzoites. Korean Journal of Parasitology **35:** 79–85.

CHANNON, J. Y., AND L. H. KASPER. 1996. *Toxoplasma gondii*–induced immune suppression by human peripheral blood monocytes: Role of gamma interferon. Infection and Immunity **64:** 1181–1189.

———, E. I. SUH, R. M. SEGUIN, AND L. H. KASPER. 1999. Attachment ligands of viable *Toxoplasma gondii* induce soluble immunosuppressive factors in human monocytes. Infection and Immunity **67:** 2547–2551.

HAQUE, S., H. DUMON, A. HAQUE, AND L. H. KASPER. 1998. Alteration of intracellular calcium flux and impairment of nuclear factor-AT translocation in T cells during acute *Toxoplasma gondii* infection in mice. Journal of Immunology **161:** 6812–6818.

———, A. HAQUE, AND L. H. KASPER. 1995. A *Toxoplasma gondii*–derived factor(s) stimulates immune downregulation: An in vitro model. Infection and Immunity **63:** 3442–3447.

———, I. KHAN, A. HAQUE, AND L. KASPER. 1994. Impairment of the cellular immune response in acute murine toxoplasmosis: Regulation of interleukin 2 production and macrophage-mediated inhibitory effects. Infection and Immunity **62:** 2908–2916.

HIRAMOTO, R. M., A. J. GALISTEO, JR., N. D. NASCIMENTO, AND H. F. DE ANDRADE, JR. 2002. 200 Gy sterilised *Toxoplasma gondii* tachyzoites maintain metabolic functions and mammalian cell invasion, eliciting cellular immunity and cytokine response similar to natural infection in mice. Vaccine **20:** 2072–2081.

KHAN, I. A., T. MATSUURA, AND L. H. KASPER. 1995. IL-10 mediates immunosuppression following primary infection with *Toxoplasma gondii* in mice. Parasite Immunology **17:** 185–195.

KOOK, J., Y. J. KIM, M. SEO, AND J. Y. CHAI. 1995. TEM ultrastructure of gamma-irradiated *Toxoplasma gondii* RH tachyzoites. Seoul Journal of Medicine **36:** 159–165.

MUN, H. S., F. AOSAI, M. CHEN, L. X. PIAO, K. NOROSE, Y. IWAKURA, AND A. YANO. 2003. Pathogenicity of *Toxoplasma gondii* through B-2 cell-mediated downregulation of host defense responses. Microbiology and Immunology **47:** 533–542.

NASH, P. B., M. B. PURNER, R. P. LEON, P. CLARKE, R. C. DUKE, AND T. J. CURIEL. 1998. *Toxoplasma gondii*–infected cells are resistant to multiple inducers of apoptosis. Journal of Immunology **160:** 1824–1830.

NEYER, L. E., H. KANG, J. S. REMINGTON, AND Y. SUZUKI. 1998. Mesenteric lymph node T cells but not splenic T cells maintain their proliferative response to concanavalin A following peroral infection with *Toxoplasma gondii*. Parasite Immunology **20:** 573–581.

SHIN, E. H., S. B. KIM, H. W. NAM, E. T. HAN, J. H. PARK, H. J. AHN, AND J. Y. CHAI. 2004. Use of monoclonal antibodies for flow cytometric detection of intracellular *Toxoplasma gondii* in murine splenic lymphocytes. Journal of Parasitology **90:** 161–166.

SUZUKI, Y., AND A. KOBAYASHI. 1983. Suppression of unprimed T and B cells in antibody responses by irradiation-resistant and plastic-adherent suppressor cells in *Toxoplasma gondii*-infected mice. Infection and Immunity **40:** 1–7.

———, M. A. ORELLANA, R. D. SCHREIBER, AND J. S. REMINGTON. 1988. Interferon-γ: The major mediator of resistance against *Toxoplasma gondii*. Science **240:** 516–518.

———, N. WATANABE, AND A. KOBAYASHI. 1981. Nonspecific suppression of primary antibody responses and presence of plastic-adherent suppressor cells in *Toxoplasma gondii*–infected mice. Infection and Immunity **34:** 30–35.

VAN OVERTVELT, L., N. VANDERHEYDE, V. VERHASSELT, J. ISMAILI, L. DE VOS, M. GOLDMAN, F. WILLEMS, AND B. VRAY. 1999. *Trypanosoma cruzi* infects human dendritic cells and prevents their maturation: Inhibition of cytokines, HLA-DR, and costimulatory molecules. Infection and Immunity **67:** 4033–4040.

WEI, S., F. MARCHES, J. BORVAK, W. ZOU, J. CHANNON, M. WHITE, J. RADKE, M. F. CESBRON-DELAUW, AND T. J. CURIEL. 2002. *Toxoplasma gondii*–infected human myeloid dendritic cells induce T-lymphocyte dysfunction and contact-dependent apoptosis. Infection and Immunity **70:** 1750–1760.

J. Parasitol., 91(2), 2005, pp. 470–472

Prevalence of Antibodies to *Trypanosoma cruzi* in Raccoons (*Procyon lotor*) From an Urban Area of Northern Virginia

Katie Hancock, Anne M. Zajac, Oscar J. Pung*, Francois Elvinger†, Alexa C. Rosypal, and David S. Lindsay‡, Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, Virginia 24061-0342; *Department of Biology, Georgia Southern University, Statesboro, Georgia 30460-8042; †Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, Virginia 24061-0342; ‡To whom correspondence should be addressed. *e-mail: lindsayd@vt.edu*

ABSTRACT: Raccoons (*Procyon lotor*) are reservoir hosts for *Trypanosoma cruzi*. A 3-yr-long serological survey was conducted to determine the prevalence of antibodies to this zoonotic parasite in raccoons collected from Fairfax County, Virginia, a suburban/urban area outside Washington, D.C. Serum samples from 464 raccoons were examined for *T. cruzi* antibodies at a 1:40 dilution with an indirect fluorescent antibody test (IFAT) and Brazil strain *T. cruzi* amastigotes and trypomastigotes as antigen. A positive IFAT test was found in 154 (33%) of the 464 samples. The yearly prevalence was 49 of 132 (37%) in 2000; 19 of 120 (16%) in 2001; and 86 of 212 (41%) in 2002. Our study indicates that raccoons in this area of Virginia are frequently exposed to *T. cruzi*.

This study was conducted to determine the serological prevalence of antibodies to *Trypanosoma cruzi* in a common reservoir host, the raccoon (*Procyon lotor*), from Virginia. Our interest was in part a result of the report of clinical *T. cruzi* infection (Barr et al., 1995) in dogs (Walker hounds) from Virginia. Schaffer et al. (1978) indicated that none of 10 raccoons from Virginia were blood culture–positive for *T. cruzi*. Raccoons used in the present study originated in various locations in Fairfax County, Virginia, a suburban/urban area outside Washington, D.C. Raccoons used for this study were live-trapped as part of a larger study on rabies. Blood samples were collected from all trapped raccoons. Raccoons were released immediately after sampling was completed. The serum was collected, placed in a tube, and frozen at −70

TABLE I. Prevalence of *Trypanosoma cruzi* in raccoons from the United States.

Location	No. examined/ No. positive (% positive)	Test used*	Reference
Florida	184/2 (1%)	BS	Telford and Forrester, 1991
Florida	33/4 (12%)	Culture	Schaffer et al., 1978
Florida/Georgia	608/9 (1.5%)	Culture	McKeever et al., 1958
Georgia	10/5 (50%)	Culture	Schaffer et al., 1978
Georgia	54/12 (22%)	Culture	Pung et al., 1995
Georgia	30/13 (43%)	Culture	Pietrzak and Pung, 1998
Georgia	87/51 (59%)	IFAT	Yabsley and Noblet, 2002
Maryland	400/5 (1%)	Culture	Walton et al., 1958
North Carolina	20/3 (15%)	Culture	Karsten et al., 1992
Oklahoma	8/5 (63%)	Culture	John and Hoppe, 1986
South Carolina	134/53 (40%)	IFAT	Yabsley and Noblet, 2002
Tennessee	6/0 (0%)	Culture	Schaffer et al., 1978
Tennessee	3/2 (67%)	Culture	Herwaldt et al., 2000
Texas	25/6 (24%)	Culture	Schaffer et al., 1978
Virginia	10/0 (0%)	Culture	Schaffer et al., 1978
Virginia	464/154 (33%)	IFAT	Present study
West Virginia	10/0 (0%)	Culture	Schaffer et al., 1978

* BS = blood smear, Culture = blood culture; IFAT = indirect fluorescent antibody test.

C. Frozen sera were sent to the Center for Molecular Medicine and Infectious Diseases (Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia) for testing.

The indirect fluorescent antibody test (IFAT) was used because it has been shown to be more sensitive than blood culture in detecting *T. cruzi* infections in raccoons (Yabsley et al., 2001). Brazil strain *T. cruzi* amastigotes and trypomastigotes were collected from bovine monocyte cell cultures. Parasites were washed in phosphate-buffered saline (PBS) by centrifugation, air-dried onto 12-well microscope slides, fixed in 100% methanol for 30 sec, and stored at −20 C until used. Sera were diluted to 1:40 in PBS and incubated with *T. cruzi* antigens for 30 min at room temperature. The slides were then washed 2 times in PBS for 5 min per washing. Bound antibodies were detected by incubation with goat anti-raccoon IgG(H&L) (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) at a 1:40 dilution in PBS for 30 min at room temperature. The slides were then washed 2 times in PBS for 5 min per washing. All incubations were done in a humidified box. The IFAT slides were mounted in fluoromount-G (Southern Biotechnology Associates, Birmingham, Alabama) and covered with cover slips (24 × 60 mm) and viewed with an Olympus BX60 fluorescent microscope. Sera from *T. cruzi* blood culture–negative and blood culture–positive raccoons (Pung et al., 1995) were used as controls on each IFAT slide.

Serological examination by IFAT found that 154 of the 464 (33%) raccoons sampled during the 3-yr period were positive. The yearly prevalence was 49 of 132 (37%) in 2000; 19 of 120 (16%) in 2001; and 86 of 212 (41%) in 2002.

Trypanosoma cruzi has been isolated from raccoons from as far north as Maryland (Walton et al., 1958). Infectivity of these isolates from Maryland for mammals (Diamond and Rubin, 1958; Walton et al., 1958) and triatomid insects (Walton et al., 1958) has been demonstrated. The prevalence of infection in raccoons from Maryland was 1% (5 of 400), which is lower than the 33% (154 of 464) for raccoons from Virginia in this study. Our prevalence results are similar to those reported by others for raccoons in the southern United States (Table I) when IFAT or culture are used to conduct examinations. Schaffer et al. (1978) did not isolate *T. cruzi* from the 10 raccoons they examined from Virginia.

Clinical canine *T. cruzi* infections have been reported in dogs from Virginia (Barr et al., 1995). We are not aware of autochthonous human infections acquired in Virginia. There are apparently only 5 cases of autochthonous vectorborne human *T. cruzi* infections reported in the United States (see Herwaldt et al., 2000). Three cases were in children <10 mo old from Texas, 1 case was in an 18-mo-old child from Tennessee, and 1 case was in a 56-yr-old adult from California (see Herwaldt et al., 2000).

This research was supported in part by a Clinical Research grant from the Virginia-Maryland Regional College of Veterinary Medicine to D.S.L. We thank Cheryl Davis for providing the stock culture of *T. cruzi* and John Dame for the bovine monocyte cell culture.

LITERATURE CITED

BARR, S. C., O. VAN BEEK, M. S. CARLISLE-NOWAK, J. W. LOPEZ, L. V. KIRCHHOFF, N. ALLISON, A. M. ZAJAC, A. DE LAHUNTA, D. H. SCHLAFER, AND W. T. CRANDALL. 1995. *Trypanosoma cruzi* infection in Walker hounds from Virginia. American Journal of Veterinary Research **56:** 1037–1044.

DIAMOND, L. S., AND R. RUBIN. 1958. Experimental infection of certain farm mammals with a North American strain of *Trypanosoma cruzi* from the raccoon. Experimental Parasitology **7:** 383–390.

HERWALDT, B. L., M. J. GRIJALVA, A. L. NEWSOME, C. R. MCGHEE, M. R. POWELL, D. G. NEMEC, F. J. STEURER, AND M. L. EBERHARD. 2000. Use of polymerase chain reaction to diagnose the fifth reported U.S. case of autochthonous transmission of *Trypanosoma cruzi*, in Tennessee, 1998. The Journal of Infectious Diseases **181:** 395–399.

JOHN, D. T., AND K. L. HOPPE. 1986. *Trypanosoma cruzi* from wild raccoons in Oklahoma. American Journal of Veterinary Research **47:** 1056–1059.

KARSTEN, V., C. DAVIS, AND R. KUHN. 1992. *Trypanosoma cruzi* in wild raccoons and opossums in North Carolina. Journal of Parasitology **78:** 547–549.

MCKEEVER, S., G. W. GORMAN, AND L. NORMAN. 1958. Occurrence of a *Trypanosoma cruzi*–like organism in some mammals from southwestern Georgia and northwestern Florida. Journal of Parasitology **44:** 583–587.

PIETRZAK, S. M., AND O. J. PUNG. 1998. Trypanosomiasis in raccoons from Georgia. Journal of Wildlife Diseases **34:** 132–136.

PUNG, O. J., C. W. BANKS, D. N. JONES, AND M. W. KRISSINGER. 1995. *Trypanosoma cruzi* in wild raccoons, opossums, and triatomine bugs in southeast Georgia, U.S.A. Journal of Parasitology **81:** 324–326.

SCHAFFER, G. D., W. L. HANSON, W. R. DAVIDSON, AND V. F. NETTLES. 1978. Hematotropic parasites of translocated raccoons in the southeast. Journal of the American Veterinary Medical Association **173:** 1148–1151.

TELFORD, S. R., JR., AND D. J. FORRESTER. 1991. Hemoparasites of raccoons (*Procyon lotor*) in Florida. Journal of Wildlife Diseases **27:** 486–490.

WALTON, M. J., P. M. BAUMAN, L. S. DIAMOND, AND C. M. HERMAN. 1958. The isolation and identification of *Trypanosoma cruzi* from raccoons in Maryland. American Journal of Tropical Medicine and Hygiene **7:** 603–610.

YABSLEY, M. J., AND G. P. NOBLET. 2002. Seroprevalence of *Trypanosoma cruzi* in raccoons from South Carolina and Georgia. Journal of Wildlife Diseases **38:** 75–83.

———, ———, AND O. J. PUNG. 2001. Comparison of serological methods and blood culture for detection of *Trypanosoma cruzi* infection in raccoons (*Procyon lotor*). Journal of Parasitology **87:** 1155–1159.

J. Parasitol., 91(2), 2005, pp. 472–474

Vertebrate Host Specificity of Two Avian Malaria Parasites of the Subgenus *Novyella*: *Plasmodium nucleophilum* and *Plasmodium vaughani*

Tatjana A. Iezhova, Gediminas Valkiūnas, and Franz Bairlein*, Institute of Ecology, Vilnius University, Akademijos 2, LT-2600 Vilnius, Lithuania; *Institute of Avian Research 'Vogelwarte Helgoland,' An der Vogelwarte 21, 26386 Wilhelmshaven, Germany. *e-mail: tatjana@ekoi.lt*

ABSTRACT: The susceptibility of wild-caught European passeriform birds to naturally isolated malaria parasites, *Plasmodium* (*Novyella*) *nucleophilum* and *Plasmodium* (*Novyella*) *vaughani*, was studied by means of intramuscular subinoculation of infected citrated blood. *Plasmodium nucleophilum* of the great tit, *Parus major*, was transmitted to 3 great tits, but 3 blackcaps (*Sylvia atricapilla*) were not susceptible. *Plasmodium vaughani* of the robin, *Erithacus rubecula*, was transmitted to 1 robin and 1 blackcap, but 1 dunnock, *Prunella modularis*, was not susceptible. The prepatent period was between 8 and 10 days in all experimental infections. Maximum experimental parasitemia (3.4% of red cells) was detected in great tits infected with *P. nucleophilum* 23 days postexposure. A light (<0.01%) transient parasitemia of *P. vaughani* developed in the robin and blackcap. This study is in accord with former experimental observations on host specificity of *P. nucleophilum* and *P. vaughani*, which are characterized by a wide, but selective, range of avian hosts. Two new host–parasite associations were recorded.

Avian malaria parasites (Plasmodiidae, *Plasmodium*) of the subgenus *Novyella* Corradetti, Garnham and Laird, 1963 are some of the most common hematozoa, found particularly in passeriform birds. They are characterized by small meronts (less than the size of the erythrocyte nucleus), scanty cytoplasm, and elongated gametocytes (Corradetti et al., 1963). Ten species in the subgenus have been described, but there is limited information about their life cycles, pathogenicity, and vertebrate host specificity (Telford et al., 1997; Valkiūnas, 1997). Although numerous surveys report *Novyella* species in passeriform birds from all over the world, frequently with a high prevalence of infection (Bishop and Bennett, 1992; Valkiūnas et al., 2003), there are few experimental studies that detail the host specificity of these parasites (Corradetti and Scanga, 1973; Christensen et al., 1983; Telford et al., 1997; Valkiūnas, 1997). In the present paper, we present results that detail the experimental transmission of 2 malaria parasites of the subgenus *Novyella*, i.e., *Plasmodium nucleophilum* Manwell, 1935 and *Plasmodium vaughani* Novy and MacNeal, 1904, which have frequently been reported in wild birds from different families, particularly passeriforms, worldwide (Garnham, 1966; Bishop and Bennett, 1992; Valkiūnas, 1997).

The work was carried out at the Institute of Avian Research 'Vogelwarte Helgoland,' Wilhelmshaven, Germany. In all, 220 passeriform birds belonging to 15 species were caught with mist nets in the environs of the institute in June 2002. Blood was taken by puncturing the brachial vein. Three slides were prepared from each bird. Blood films were air dried, fixed in methanol, and stained with Giemsa. The slides were examined for 10–15 min at low magnification (×400), and then at least 100 fields were studied at high magnification (×1,000). Intensity of infection was estimated as a percentage by actually counting the number of parasites per 1,000 erythrocytes examined and per 10,000 erythrocytes at low parasitemias, i.e., <0.1%, as recommended by Godfrey et al. (1987). Malaria parasites were identified according to Valkiūnas (1997). One great tit, *Parus major*, and 1 robin, *Erithacus rubecula*, that were naturally infected with *P. nucleophilum* and *P. vaughani*, respectively, and 9 uninfected birds of 4 species were held in cages during this study. The infected birds with active parasitemia were used as donors of malaria parasites to infect the recipient birds, which were 3 great tits, 1 robin, 4 blackcaps (*Sylvia atricapilla*), and 1 dunnock (*Prunella modularis*). Blood from the recipient birds was taken once per day for 5 days before subinoculation. The blood smears from each recipient bird were examined for hematozoa at high magnification for 3 hr. At least 1,000,000 red blood cells were examined during this time. Malaria parasites were not seen in them.

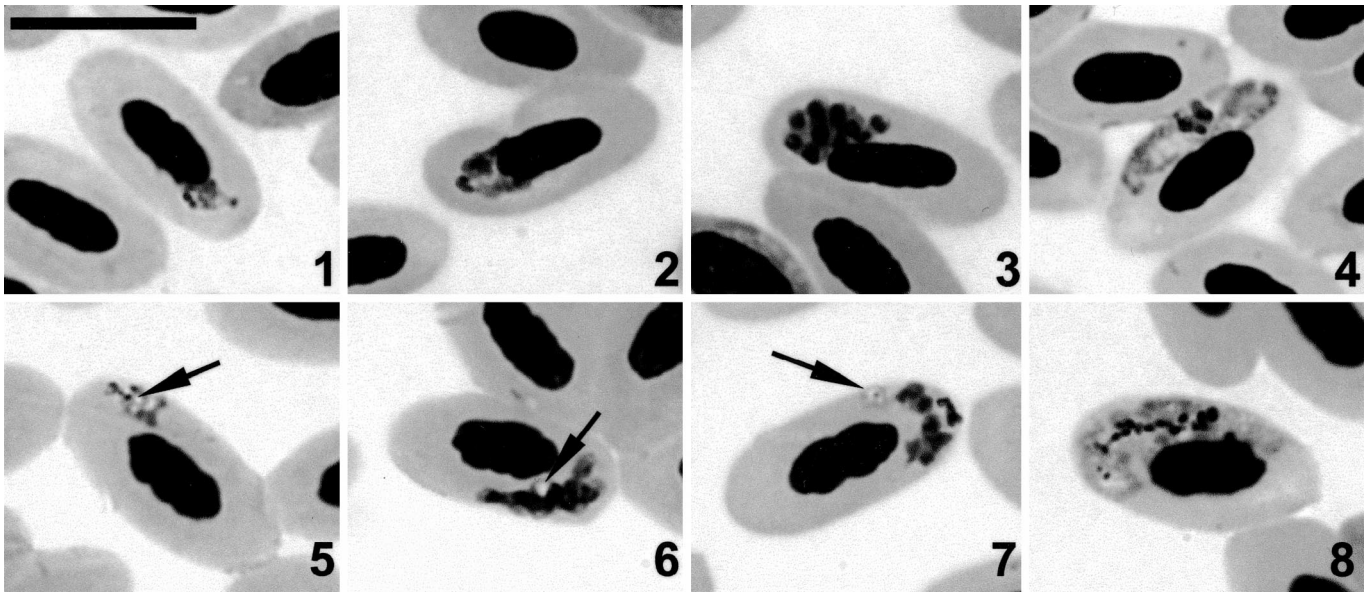
All birds were taken under license from the wild and kept indoors under controlled conditions (20 ± 1 C; 50–60% relative humidity; a constant light/dark period of 12/12 hr), given a standard diet (Bairlein, 1986), and protected from vectors.

Uninfected birds were exposed to the parasites by subinoculation of 50 μl of a freshly prepared mixture of infected blood in a 3.7% solution of sodium citrate (1 part of the solution to 4 parts of the blood) into their pectoral muscle as described by Valkiūnas (1997). Parasitemia of *P. nucleophilum* and *P. vaughani* was 6.8 and 1.9%, respectively, in the donor birds on the day of the exposure. The blood was taken as described above once per 2 days during the first 10 days postinfection (PI) and then once per wk for 53 days. The blood films were examined as described above for nonrecipient birds.

Samples of blood from naturally and experimentally infected birds were cryopreserved according to Garnham (1966) for future experimental work. Representative preparations of the blood stages of *P. nucleophilum* (accession numbers 8007–8020, NS) and *P. vaughani* (8021–8029, NS) were stored at the Institute of Ecology, Vilnius University, Lithuania.

It may be asked whether it is justifiable to make conclusions about experimental evaluation of a host range on the basis of the microscopic examination of blood smears from a small number of wild-caught recipient birds. Microscopic examination of blood films is of low sensitivity for diagnosing malaria infections (Forrester et al., 1974). Evidence must be provided that the recipient birds did not have a chronic or subpatent natural infection. Lines of evidence that support our reasoning include the low prevalence of infection in the wild population from which the birds were removed and the examination of multiple smears from multiple dates. Overall prevalence of malaria parasites of the subgenus *Novyella* was 2.3% at the study site, so the probability that the recipient birds had natural malaria infections was low. Extended microscopy (between 2 and 3 hr) has been shown to be effective in detecting low-intensity chronic malaria infections (Demina and Pavlova, 1962; Valkiūnas, 1997). Because intensive microscopy of blood smears was carried out on each of the 5 days before subinoculation, it seems likely that the recipient birds were not infected before their experimental exposure.

Plasmodium nucleophilum of the great tit was transmitted to 3 great tits. Three blackcaps were not susceptible. The prepatent period was 10 days. The maximum parasitemia was detected 23 days PI, when up to 3.4% of erythrocytes were parasitized. Light, but rather constant para-



FIGURES 1–8. Blood stages of *Plasmodium* (*Novyella*) *nucleophilum* (**1–4**) from the great tit and *Plasmodium* (*Novyella*) *vaughani* (**5–8**) from the robin. (**1, 5**) Trophozoites. (**2, 6**) Growing erythrocytic meronts. (**3, 7**) Mature erythrocytic meronts. (**4, 8**) Mature macrogametocytes. Methanol-fixed and Giemsa-stained thin films. Arrow indicates a refractive globule. Bar = 10 μm.

sitemia (on average, 0.4%) was maintained to the end of this study in all infected birds. Advanced erythrocytic trophozoites, erythrocytic meronts, and gametocytes were nucleophilic, which is an important diagnostic feature for this species (Figs. 1–4). Mature erythrocytic meronts (n = 33) contained 5–9 (on average, 6.9 ± 1.2) merozoites.

Plasmodium vaughani of the robin was transmitted to 1 robin and 1 blackcap, with a prepatent period of 8 and 10 days, respectively. Only light transient parasitemia (<0.01%) was detected in both infected birds on the days they were bled. Parasites were not seen in 1 subinoculated dunnock. In the robin, a clear refractive globule was seen in erythrocytic trophozoites and meronts (Figs. 5–7). Mature meronts (n = 33) contained 4–8 (on average, 6.8 ± 1.1) merozoites. Gametocytes were elongated (Fig. 8). Trophozoites, meronts, and most gametocytes did not touch the nuclei of infected erythrocytes (Figs. 5–7), but some gametocytes were attached to the nuclei (Fig. 8). A few parasites were seen in the blackcap. They were morphologically similar to the blood stages seen in the robin.

It is worth noting that the prepatent period (8–10 days) in all experimental infections in this study was not more than half of the period that has formerly been recorded in intramuscularly blood-induced infections of *P. nucleophilum* and *P. vaughani* (Corradetti and Scanga, 1973; Valkiūnas, 1997). Parallels may be difficult to draw because of the different strains and doses of subinoculated parasites and the different recipient hosts used in different studies. In the present study, the short prepatent period may be due to the high intensity of parasitemia in donor birds and, thus, a large number of parasites that were subinoculated in susceptible avian hosts.

No obvious clinical signs or mortality occurred in any avian host during this study, which agrees with the low virulence formerly recorded for the majority of *Novyella* species during their development in wild birds (Garnham, 1966; Valkiūnas, 1997).

According to Bennett et al. (1982), Bishop and Bennett (1992), and Valkiūnas (1997), natural infection of *P. vaughani* and *P. nucleophilum* has been recorded in >150 and 40 species of birds belonging to different families, respectively. The great tit and the robin are new hosts for *P. nucleophilum* and *P. vaughani*, respectively.

The present communication indicates that both *P. nucleophilum* and *P. vaughani* are host restricted. *Plasmodium nucleophilum* of the great tit (Paridae) did not develop in the blackcap (Sylviidae). A low transient parasitemia of *P. vaughani* developed in the robin (Turdidae) and in the blackcap, but the dunnock (Prunellidae) was not susceptible. The obstacles that preclude development of *Plasmodium* spp. in different avian hosts remain unclear. It seems that the insusceptibility observed for some of the birds in this study is not due to their previous natural infection and acquired immunity. In avian malaria, the acquired immunity is premunition, i.e., only parasitized birds are resistant to reinfection (Garnham, 1966; Valkiūnas, 1997). Because malaria parasites were not seen in any recipient bird before experimental infection and parasites were not observed in uninfected birds for 63 days PI, it is possible that the insusceptibility is a natural innate resistance.

It is probable that there is a marked intraspecific divergence of specificity of avian malaria parasites. On the basis of host restriction, 3 subspecies of *P. vaughani* have been described (Corradetti and Scanga, 1973; Valkiūnas, 1997). These are: *P. vaughani vaughani* Novy and MacNeal, 1904, *P. vaughani tenuis* (Laveran and Marullaz, 1914), and *P. vaughani merulae* Corradetti and Scanga, 1972. The type vertebrate host of *P. vaughani vaughani* is the American robin *Turdus migratorius* belonging to the family Turdidae. The average number of merozoites in its erythrocytic meronts is around 4. This parasite develops in canaries, but it does not complete sporogony in the mosquito *Culex pipiens*. The type vertebrate host of *P. vaughani tenuis* is the Pekin robin *Leiothrix lutea* belonging to the family Timaliidae. The average number of merozoites in its erythrocytic meronts is 5.2. This parasite can be adapted to the canary, but it does not infect the blackbird *Turdus merula* belonging to the family Turdidae; the susceptibility of its vectors is unknown. The type vertebrate host of *P. vaughani merulae* is the blackbird. The average number of merozoites in its erythrocytic meronts is 6. This parasite does not develop in the canary or Pekin robin, but its sporogony is completed in *C. pipiens*.

According to the present study, *P. vaughani* of the robin will infect the blackcap, but it will not infect the dunnock in northern Germany. The huge geographic and vertebrate host ranges, the variability of the average number of merozoites in erythrocytic meronts, and the different specificity of strains and subspecies with respect to vertebrate hosts and vectors (summarized by Garnham, 1966; Corradetti and Scanga, 1973; Valkiūnas, 1997) suggest that *P. vaughani* is the evolutionary stage of active differentiation into subspecies or even distinct species.

The same is probably true for the *P. nucleophilum* subspecies, of which *P. nucleophilum nucleophilum* Manwell, 1935 and *P. nucleophilum toucani* Manwell and Sessler, 1971 are characterized by their different abilities to develop in the canary and produce different levels of virulence in this bird (Manwell and Sessler, 1971; Valkiūnas, 1997). According to our data, *P. nucleophilum* of the great tit will not infect the blackcap. It is worth noting that, in spite of numerous surveys,

natural infection with *P. nucleophilum* has not been recorded in the blackcap (Bennett et al., 1982; Bishop and Bennett, 1992; Valkiūnas, 1997).

The data presented in our study are in accordance with information about the huge genetic divergence that is documented for avian malaria parasites and other hemosporidians recorded using polymerase chain reaction–based techniques (Ricklefs and Fallon, 2002; Bensch and Åkesson, 2003). More field and experimental transmission investigations, combined with a molecular approach, are needed to gain sufficient knowledge regarding the speciation of avian malaria parasites and the mechanism that accounts for their host restriction, particularly because of the large ranges of their vertebrate hosts and their immense geographic ranges in all parts of the world.

The authors are grateful to Eva Nivinski and Olga Dolnik for help with the caring for and bleeding of birds. This article benefited from comments made by Richard W. Ashford and 2 anonymous reviewers. The present study was generously supported by the European Science Foundation, the Institute of Avian Research 'Vogelwarte Helgoland,' Germany, the Lithuanian State Science and Studies Foundation, and the Wellcome Trust.

LITERATURE CITED

BAIRLEIN, F. 1986. Ein standardisiertes Futter für Ernährungsuntersuchungen an omnivoren Kleinvögeln. Journal fuer Ornithologie **127:** 338–340.

BENNETT, G. F., M. WHITEWAY, AND C. WOODWORTH-LYNAS. 1982. A host-parasite catalogue of the avian haematozoa. Occasional Papers in Biology, Memorial University of Newfoundland **5:** 1–243.

BENSCH, S., AND S. ÅKESSON. 2003. Temporal and spatial variation of hematozoans in Scandinavian willow warblers. Journal of Parasitology **89:** 388–391.

BISHOP, M. A., AND G. F. BENNETT. 1992. Host-parasite catalogue of the avian haematozoa, supplement 1, and bibliography of the avian blood-inhabiting haematozoa, supplement 2. Occasional Papers in Biology, Memorial University of Newfoundland **15:** 1–244.

CHRISTENSEN, B. M., H. J. BAMES, AND W. A. ROWLEY. 1983. Vertebrate host specificity and experimental vectors of *Plasmodium* (*Novyella*) *kempi* sp. n. from the eastern wild turkey in Iowa. Journal of Wildlife Diseases **19:** 204–213.

CORRADETTI, A., P. C. C. GARNHAM, AND M. LAIRD. 1963. New classification of the avian malaria parasites. Parassitologia **5:** 1–4.

———, AND M. SCANGA. 1973. The *Plasmodium vaughani*–complex. Experimental Parasitology **34:** 344–349.

DEMINA, N. A., AND E. A. PAVLOVA. 1962. The duration of *Plasmodium gallinaceum* infection. Medicinskaya Parazitologiya i Parazitarniye Bolezni (Moscow) **31:** 648–651. [In Russian.]

FORRESTER, D. J., L. T. HOT, L. E. WILLIAMS, AND D. H. AUSTIN. 1974. Blood protozoa of wild turkeys in Florida. Journal of Protozoology **21:** 494–497.

GARNHAM, P. C. C. 1966. Malaria parasites and other Haemosporidia. Blackwell Scientific Publications, Oxford, U.K., 1114 p.

GODFREY, R. D., A. M. FEDYNICH, AND D. B. PENCE. 1987. Quantification of hematozoa in blood smears. Journal of Wildlife Diseases **23:** 558–565.

MANWELL, R. D., AND G. J. SESSLER. 1971. Malaria parasites of toucans. Journal of Protozoology **18:** 570–574.

RICKLEFS, R. E., AND S. M. FALLON. 2002. Diversification and host switching in avian malaria parasites. Proceedings of the Royal Society of London B Biological Sciences **269:** 885–892.

TELFORD, S. R., J. K. NAYAR, G. W. FOSTER, AND J. W. KNIGHT. 1997. *Plasmodium forresteri* n. sp., from raptors in Florida and southern Georgia: Its distinction from *Plasmodium elongatum* morphologically within and among host species and by vector susceptibility. Journal of Parasitology **83:** 932–937.

VALKIŪNAS, G. 1997. Bird Haemosporida. Acta Zoologica Lituanica **3–5:** 1–607. [In Russian.]

———, T. A. IEZHOVA, AND A. P. SHAPOVAL. 2003. High prevalence of blood parasites in hawfinch *Coccothraustes coccothraustes*. Journal of Natural History **37:** 2647–2652.

J. Parasitol., 91(2), 2005, pp. 474–476

Trematodes in Snails near Raccoon Latrines Suggest a Final Host Role for this Mammal in California Salt Marshes

K. D. Lafferty and E. J. Dunham*, Western Ecological Research Center, United States Geological Survey, c/o Marine Science Institute, University of California, Santa Barbara, California 93106, *Penn State Biology Department, 513A Mueller Lab, University Park, Pennsylvania 16802-5301. *e-mail: lafferty@lifesci.UCSB.edu*

ABSTRACT: Of the 18 trematode species that use the horn snail, *Cerithidea californica*, as a first intermediate host, 6 have the potential to use raccoons as a final host. The presence of raccoon latrines in Carpinteria Salt Marsh, California, allowed us to investigate associations between raccoons and trematodes in snails. Two trematode species, *Probolocoryphe uca* and *Stictodora hancocki*, occurred at higher prevalences in snails near raccoon latrines than in snails away from latrines, suggesting that raccoons may serve as final hosts for these species. Fecal remains indicated that raccoons fed on shore crabs, the second intermediate host for *P. uca*, and fish, the second intermediate host for *S. hancocki*. The increase in raccoon populations in the suburban areas surrounding west coast salt marshes could increase their importance as final hosts for trematodes in this system.

Upstream host communities should strongly affect the composition of parasite communities in downstream hosts. As a consequence, parasite communities in downstream hosts should indicate the composition of upstream-host communities. We used this logic to indirectly determine whether raccoons serve as final hosts for trematodes in west coast salt marshes by studying associations between raccoon latrines and the prevalence of trematodes in snails at the Carpinteria Salt Marsh, California.

Trematodes in west coast salt marshes use *Cerithidea californica* (and closely related snail species in more southern latitudes) as first intermediate hosts. Birds are common predators on second intermediate hosts, e.g., polychaetes, snails, clams, crabs, and fishes, and all 18 trematode species that infect *C. californica* use birds as final hosts; none is known from wild mammals. Mammals are less common in salt marshes than birds, a potential explanation for why they have not been considered as final hosts. However, some trematodes that use *C. californica* have the potential to infect mammals.

Tracks of the raccoon, *Procyon lotor*, are common on mudflats and tidal channels, where their broad diet would expose them to several species of second intermediate hosts. The tendency of raccoons to defecate in communal latrines aided our investigation of the importance of raccoons as hosts. Some latrines occur on logs that are adjacent to habitat that supports populations of *C. californica*. Trematodes that regularly complete their life cycle within raccoons should be more common in first intermediate host snails occurring near latrines than in snails occurring away from latrines.

To avoid making this comparison for all 18 trematode species (which would increase the probability of a type 1 statistical error), we examined the life cycle of each trematode species to assess which ones might be viable in mammals. For example, *Probolocoryphe lanceolata* (*glandu-*

losa) in Florida parasitizes raccoons (Heard and Sikora, 1969), rice rats (Kinsella, 1988), and a wide variety of birds (Heard and Sikora, 1969), suggesting that *Probolocoryphe* (*Maritrema*) *uca* in California, might parasitize raccoons. *Probolocoryphe uca* uses crabs as a second intermediate host. Other trematodes from *C. californica* believed to primarily use birds as final hosts might also infect mammals, as demonstrated in experiments or from observations of closely related species. Adults of *Euhaplorchis californiensis* (which encyst on the brain of the second intermediate host, the killifish *Fundulus parvipinnis*) and *Phocitremoides ovale* (which encyst under the scales of several salt marsh fish species) can parasitize mammals in experimental infections (Martin, 1972). Although *Stictodora hancocki* (which encyst in the tissues of several salt marsh fish species) is only known to use bird as final hosts (Martin, 1972), adults of a similar species, *Stictodora cursitans*, from Florida, infect a wide range of mammals, including raccoons (Kinsella and Heard, 1974). *Mesostephanus appendiculatus* (that encysts in the musculature of several salt marsh fish species) occurs in shorebirds (Martin, 1972). However, Hutton and Sogandares-Bernal (1964) successfully infected a raccoon from Florida with *Mesostephanus appendiculatoides* by feeding it infected mullet. Finally, *Acanthoparyphium spinulosum*, a trematode that uses mollusks as second intermediate hosts, will develop to maturity in rats (T. Huspeni, pers. obs.).

In mid-November, we collected 2 snail populations near substantial raccoon latrines in the Carpinteria Salt Marsh. From the first, we collected 306 snails from a pan that contained a large latrine on a stump. From the second, we collected 30 snails from a small tidal channel adjacent to a stump with a medium-size latrine. We found 3 other latrines, but their small size or lack of adjacent snail populations (or both) precluded their use in this study. Snails from 14 other sites distributed through the marsh were also sampled for trematodes during the first 2 wk of August 2004. Of these, 7 were from tidal channels (a habitat similar to the location of the snails adjacent to the medium latrine) and 7 were from pans or flats (habitats similar to the location of the snails adjacent to the large latrine).

We collected 10 fecal samples at the larger latrine between 13 and 20 March 2004. We chose samples so that each appeared to be from a separate defecation event, although it was impossible to determine how many raccoons contributed to the samples. Samples were mixed thoroughly with 15 ml of zinc sulfate solution by rotating the fecal insert inside an OVASSAY Plus fecal device. Cover slips (25 × 25 mm) were placed on the meniscus of the fecal device for 10 min, and the floating material adhering to the cover slips was inspected for the presence of trematode eggs under a compound microscope.

Where possible, 200 snails were collected from each of the sites and distributed evenly between 20–25 and 25–30 mm size classes. Snails were dissected and trematode infections were identified to species (Martin, 1972). The prevalence (Bush et al., 1997) of each trematode species was calculated. For species with the potential to use mammals and having a prevalence high enough to analyze (>1%), we investigated the effects of snail size class, habitat type, and the presence of a latrine on trematode prevalence.

Of the 11 collected fecal samples, 8 were sufficiently fresh to assess diet. Two of the samples contained egg-like items with filaments, but because there was no clear operculum, this prevented confirmation as trematode eggs. Five samples contained remains of large numbers of shore crabs, probably *Pachygrapsus crassipes*. One fecal sample included only fish bones, and 2 samples contained fish bones and crab carapaces. No mollusk remains was found in the fecal samples. These results suggest that raccoons ingest metacercariae of trematodes that use crabs and fishes as second intermediate hosts. Four fecal samples had larval nematodes of *Baylisascaris procyonis*, the common raccoon roundworm. Larvae of *Enterobius* sp. (nematode) and *Entamoeba muris* (protozoan) also occurred in the feces.

We collected 336 snails at latrine sites and 2,698 snails at nonlatrine sites. *Mesostephanus appendiculatus* and *P. ovale* were too rare (<1% prevalence) for an analysis to be completed, and it seems unlikely that raccoons play an important role in their transmission. The angular transformation (arcsine of the square root of the proportion infected) was used to help normalize the distribution of prevalence for statistical comparisons (to ease interpretation, we present the actual values of prevalence instead of the transformed values). A separate linear model was determined for each species by determining whether snail size class, habitat, or latrine explained a significant proportion of the variance in transformed prevalence.

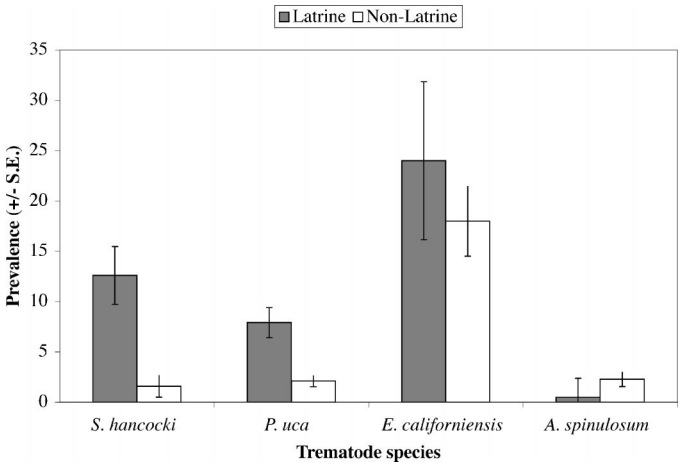


FIGURE 1. Comparison of prevalence of trematodes, by species, at sites near and faraway from raccoon latrines in Carpinteria Salt Marsh, California. Error bars are SEs. *Probolocoryphe uca* and *Stictodora hancocki* were significantly more prevalent near raccoon latrines.

Size and habitat, but not latrine, affected the prevalence of *E. californiensis*. Latrine sites (24%) did not have significantly different prevalence than nonlatrine sites (18%) (df = 1, *F*-ratio = 0.20, *P* = 0.67). Size did not affect the remaining 3 species, and data from the 2 size classes were combined. The prevalence of *A. spinulosum* did not differ among habitat or significantly between latrines (average = 0.5%) and nonlatrine sites (average = 2.3%) (N = 16, df = 1, *F*-ratio = 0.79, *P* = 0.39). The prevalence of *P. uca*, however, was significantly higher at latrine sites (7.1%) than at the nonlatrine sites (1.8%) (N = 16, df = 1, *F*-ratio = 8.37, *P* = 0.0126). This pattern is consistent with the prediction that raccoon latrines serve as sites of focal transmission for *P. uca* (Fig. 1). There was a marginally independent effect of habitat type on the prevalence of *P. uca*. Channel sites (1.7%) had a lower prevalence than flat and pan sites (3.4%) (N = 16, df = 1, *F*-ratio = 4.66, *P* = 0.050). The prevalence of *S. hancocki* was significantly higher in the latrine sites (14.6%) than at the nonlatrine sites (1.6%) (N = 16, df = 1, *F*-ratio = 14.08, *P* = 0.0024), an effect due greatly to a high prevalence of *S. hancocki* at the smaller latrine site (Fig. 1). There was an independent effect of habitat type on the prevalence of *S. hancocki*. Channel sites (5.4%) had a higher prevalence than flat and pan sites (1.0%) (N = 16, df = 1, *F*-ratio = 4.95, *P* = 0.0445).

Multiple statistical comparisons, such as for the 4 species cited above, require an adjustment of the critical *P*-value to assess significance. Application of the Bonferroni correction indicated that the equivalent of a 0.05 critical *P*-value for a set of 4 similar tests is 0.0127. The *P*-values for *P. uca* and *S. hancocki* were both below this adjusted level and, therefore, indicated statistically significant differences between latrine and nonlatrine sites for these species.

Raccoons forage on shore crabs and fishes in Carpinteria Salt Marsh. These prey serve as second intermediate hosts for some trematodes that use the California horn snail, *C. californica*, as a first intermediate host and birds as final hosts. Some of these species are able to use mammals as final hosts and, therefore, could infect raccoons that eat infected crabs or fish. Comparisons between trematode communities in snails at latrine and nonlatrine sites indicated a 4-fold higher prevalence of *P. uca* (a species using shore crabs as second intermediate hosts) and an 8-fold higher prevalence of *S. hancocki* (a species using fishes as second intermediate hosts) adjacent to raccoon latrines. These correlative data are consistent with the hypothesis that raccoons are likely to be hosts for *P. uca* and *S. hancocki* in California Salt Marshes. Direct support for this hypothesis was not found from the examination of raccoon feces. However, fecal examination for eggs is often an insensitive means of assessing parasitism. Necropsy of raccoons that forage in salt marshes would be necessary to conclude that raccoons serve as final hosts for *P. uca* and *S. hancocki*.

A possible alternative explanation for our results is that birds use

stumps for perches and, therefore, transmit trematode eggs at raccoon latrines more than at sites without stumps. Another possibility is that trematode prevalence was higher in November (when collected from the latrine sites) than in August (when collected from the control sites). Either alternative would predict that the entire trematode community (not just those suspected of using raccoons) would be more prevalent at the latrine sites. However, when averaged or summed, the other 12 species of trematodes were not associated with latrines. The summed prevalence of nonraccoon trematodes found in 20- to 25-mm snails was the same (34% vs. 33%) in latrine and nonlatrine sites, and the prevalence of nonraccoon trematodes found in 25- to 30-mm snails was lower (46 vs. 81%) at latrine than at nonlatrine sites. Data on the entire community of trematodes will be analyzed for future publications.

Other trematode species with the potential to use raccoons as final hosts were either too rare to evaluate or did not have a positive association with latrines. Of the relatively common trematode species with the potential to infect mammals that were not associated with latrines, one, *A. spinulosum*, uses mollusks as a second intermediate host that, according to the limited diet information available, may not be important prey for raccoons in this system. The other, *E. californiensis*, is specific to 1 species of fish (*F. parvipinnis*) that might not be commonly preyed on by raccoons.

Latrines result in a high degree of aggregation of raccoon feces, and this should lead to spatial structure in transmission of the trematode eggs/miracidia in these feces. Although the prevalence of *P. uca* and *S. hancocki* were much higher near latrine than at nonlatrine sites, latrines were relatively rare within the marsh, suggesting that, at the scale of the salt marsh, transmission of trematodes by raccoons contributed to a relatively small proportion of the total population of these 2 species in the salt marsh, relative to transmission by birds.

Urbanization may be changing the abundances of shorebirds and raccoons in this system. A decrease in the area of salt marsh habitat due to development could crowd shorebirds into remaining areas, whereas degradation of some habitats might reduce bird use. Either pattern could alter parasitism (Holmes, 1996). Urbanization favors raccoons by providing a convenient source of food and a dearth of top predators (DeLap and Knight, 2004). These factors may have changed and will continue to change the relative importance of raccoons and birds as hosts for trematodes in California Salt Marshes.

T. Huspeni assisted with assessment of the fecal samples. Many individuals assisted by dissecting snails from the control sites. R. Hechinger and K. Phillips provided comments on an early draft. This manuscript has also benefited from support received from the National Science Foundation through the NIH/NSF Ecology of Infectious Disease Program (DEB-0224565) and a grant from the U.S. Environmental Protection Agency's (EPA) Science to Achieve Results (STAR) Estuarine and Great Lakes (EaGLe) program through funding to the Pacific Estuarine Ecosystem Indicator Research (PEEIR) Consortium, U.S. EPA Agreement R-882867601. However, it has not been subjected to any EPA review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The University of California Natural Reserve System provided access to Carpinteria Salt Marsh.

LITERATURE CITED

BUSH, A. O., K. D. LAFFERTY, J. M. FONT, AND A. W. SHOSTAK. 1997. Parasitology meets ecology: Definitions, clarifications, examples and Margolis et al. revisited. Journal of Parasitology **83:** 575–583.

DELAP, J. H., AND R. L. KNIGHT. 2004. Wildlife response to anthropogenic food. Natural Areas Journal **24:** 112–118.

HEARD, R. W., AND W. B. SIKORA. 1969. *Probolocoryphe otagaki*, 1958 (Trematoda: Microphallidae), a senior synonym of *Mecynophallus* Cable, Conner, and Balling, 1960, with notes on the genus. Journal of Parasitology **55:** 674–675.

HOLMES, J. C. 1996. Parasites as threats to biodiversity in shrinking ecosystems. Biodiversity and Conservation **5:** 975–983.

HUTTON, R. F., AND F. SOGANDARES-BERNAL. 1964. A list of parasites from marine and coastal animals of Florida. Transactions of the American Microscopical Society **79:** 287–292.

KINSELLA, J. M. 1988. Comparison of helminthes of rice rats, *Oryzomys palustris*, from freshwater and saltwater marshes. Proceedings of the Helminthological Society of Washington **55:** 275–280.

———, AND R. W. I. HEARD. 1974. Morphology and life cycle of *Stictodora cursitans* N. Comb. (Trematoda: Heterophyidae) from mammals in Florida salt marshes. Transactions of the American Microscopical Society **93:** 408–412.

MARTIN, W. E. 1972. An annotated key to the cercariae that develop in the snail *Cerithidea californica*. Bulletin of the Southern California Academy of Sciences **71:** 39–43.

J. Parasitol., 91(2), 2005, pp. 476–479

Expression of P23 of *Cryptosporidium parvum* in *Toxoplasma gondii* and Evaluation of its Protective Effects

Hiroaki Shirafuji, Xuenan Xuan*, Isao Kimata†, Yasuhiro Takashima‡, Shinya Fukumoto, Haruki Otsuka, Hideyuki Nagasawa, and Hiroshi Suzuki, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan; *To whom correspondence should be addressed; †Department of Medical Zoology, Faculty of Medicine, Osaka City University, Abeno-ku, Osaka 545-8585, Japan; ‡Department of Global Agricultural Science, Graduate School of Agriculture and Life Science, The University of Tokyo, Bunkyo-ku, Tokyo, Japan. *e-mail: gen@obihiro.ac.jp*

ABSTRACT: In this study, P23 of *Cryptosporidium parvum* sporozoites, an immunodominant surface protein, was stably expressed in *Toxoplasma gondii* (Tg/P23) and its protective effects were evaluated in a mouse model. The molecular weight and antigenic property of P23 expressed by Tg/P23 were similar to those of the native P23. Mice immunized with lysed Tg/P23 tachyzoites produced specific neutralizing antibodies against *C. parvum*. These findings indicate that the *T. gondii* vector may provide a new tool for the production of a recombinant vaccine against cryptosporidiosis in animals.

Cryptosporidium parvum is a coccidian parasite that infects the intestinal epithelium in humans and animals (Fayer et al., 1997). Exposure of immunocompetent individuals to *C. parvum* results in a transient infection that may be asymptomatic or can result in self-limited diarrhea. In contrast, diarrhea persists and is life-threatening in immunocompromised patients, especially individuals with human immunodeficiency virus–acquired immune deficiency syndrome (Brasseur, 1997; Okhuysen and Chappell, 2002). The contamination of watersheds by cattle feces has been indicated as a primary source of outbreaks in human populations (Smith and Rose, 1990). Therefore, controlling *C. parvum* infections in cattle is important to resolve public health concerns. However, to date, there are no effective vaccines for cryptosporidiosis (de Graaf et al., 1999).

A 23-kDa glycoprotein (P23) of *C. parvum* was identified as an antigen with neutralization-sensitive epitopes, and, therefore, it is considered to be a good candidate for the development of an effective vaccine against cryptosporidiosis (Perryman et al., 1996, 1999). It was reported that immunization with a recombinant protein containing the amino acid

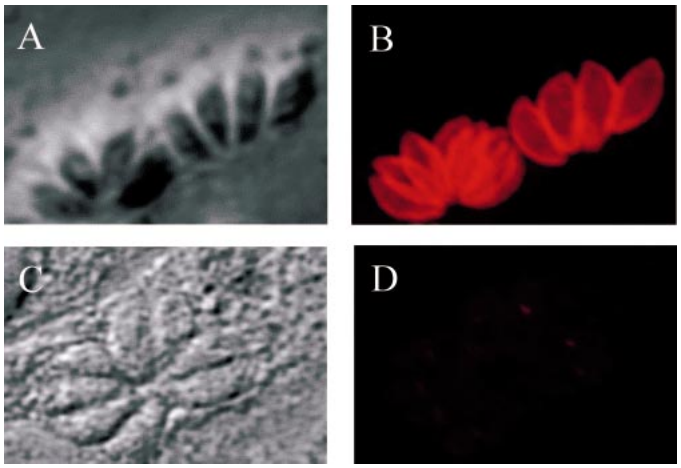


FIGURE 1. IFAT of recombinant P23 expressed in *Toxoplasma gondii*. Tg/P23 (**A**, **B**) or Tg/wt (**C**, **D**) tachyzoites were allowed to infect Vero cells, and IFAT was performed 24 hr after infection, with anti-P23 serum followed by Alexa Flour-594–conjugated secondary antibodies (Molecular Probes, Eugene, Oregon). Alexa Flour-594–stained proteins are observed in red. Panels **A** and **C** show differential interference contrast fields, and panels **B** and **D** show fluorescence images of the same fields.

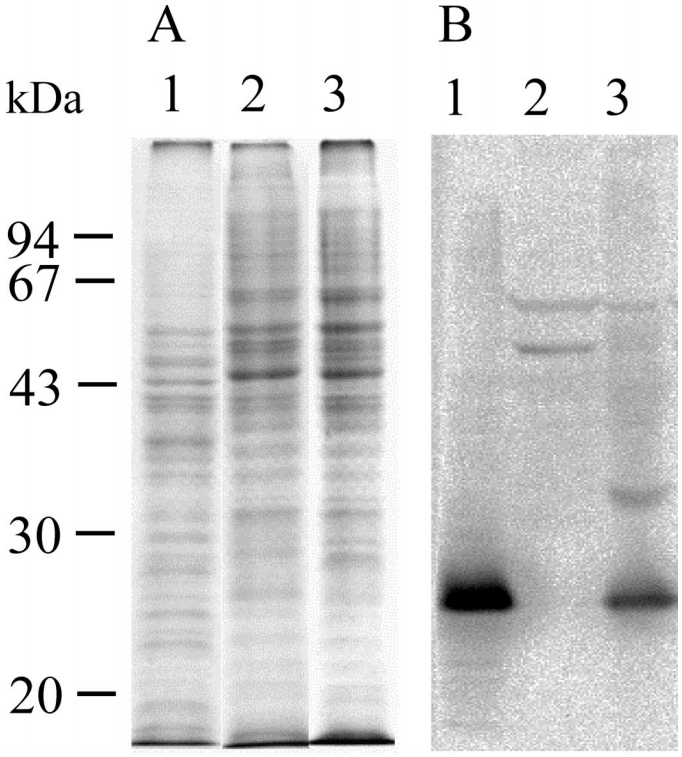


FIGURE 2. Western blot analysis of recombinant P23 expressed in *Toxoplasma gondii*. Oocysts of *Cryptosporidium parvum* (lane 1), Tg/wt tachyzoites (lane 2), and Tg/P23 tachyzoites (lane 3) were lysed and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (panel A) after immunoblotting using anti-P23 serum as the primary antibody (panel B).

sequence of the major part of P23 induced immune bovine colostrum that protected calves against cryptosporidiosis (Perryman et al., 1996, 1999). Recently, Takashima et al. (2003) reported that a recombinant bovine herpes virus–1 expressing P23 induced neutralizing antibodies in rabbits.

Toxoplasma gondii is an intracellular protozoan capable of infecting a variety of mammals and birds (Dubey and Beattie, 1988); it has been used as an expression system of heterologous proteins, i.e., circumsporozoite proteins of *Plasmodium* spp. (Di Cristina et al., 1999; Charest et al., 2000). Because *C. parvum* and *T. gondii* are closely related apicomplexans, it is expected that the antigens of *C. parvum* expressed in *T. gondii* would most likely have a similar structural conformation (O'Connor et al., 2003). In this study, we constructed the recombinant *T. gondii* expressing P23 of *C. parvum* and demonstrated that it can induce neutralizing antibodies in mice.

The plasmid containing the entire P23 gene, pUC/P23 (Takashima et al., 2003), was digested with *Bam*HI and blunt-ended using DNA polymerase I Klenow fragment, and the P23 gene was inserted into the *Eco*RV site of pDMG, a transfer vector for constructing recombinant *T. gondii* (Nishikawa et al., 2003). The P23 gene is under the control of the *T. gondii* GRA1 promoter. The resulting plasmid was designated pDMG/P23. Transfection of the *T. gondii* RH strain with pDMG/P23 and selection of recombinant *T. gondii* expressing the P23 gene were carried out as described previously (Sibley et al., 1994). In brief, freshly isolated tachyzoite cells were washed and resuspended at 2×10^7/ml to 5×10^7/ml with 50 μg of pDMG/P23 in a cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4–KH_2PO_4, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid, 2 mM ethylenediaminetetraacetic acid, 5 mM $MgCl_2$, pH 7.6) supplemented with 2 mM adenosine triphosphate and 5 mM glutathione. Tachyzoite cells were transferred to a 0.2 cm–gap cuvette and electroporated with 2 kV at 25μF and 50 Ω with BioRad Gene Pulser II (Hercules, California). After electroporation, parasites were allowed to recover for 15 min at room temperature and inoculated onto Vero cells grown in 25-cm^2 T-flasks (Nunc, Roskilde, Denmark). Recombinant parasites were selected on Vero cells in the presence of pyrimethamine at a concentration of 1 μM (Sigma, St. Louis, Missouri). After incubating for 4 days, the pyrimethamine-resistant and fluorescent parasites were isolated by a plaque assay, and a resulting recombinant *T. gondii* clone stably expressing P23 of *C. parvum* was designated Tg/P23.

The expression of P23 in the tachyzoite of Tg/P23 was examined with an indirect fluorescent antibody test (IFAT) using a mouse antibody against recombinant P23 expressed in *Escherichia coli* (Takashima et al., 2003). The specific fluorescence was mainly detected on the cell surface of recombinant Tg/P23 tachyzoites (Fig. 1). In the Western blot analysis, a specific band with a molecular mass of 23 kDa, which was identical to that of natural P23 expressed by *C. parvum*, was detected in Tg/P23 (Fig. 2). The molecular weight of the P23 expressed in Tg/P23 is clearly larger than that expected from its amino acid sequence (11 kDa). Therefore, it is expected that the P23 expressed in Tg/P23 is glycosylated as the native P23 is in *C. parvum*.

To determine whether the P23 expressed by Tg/P23 induces a specific immunity, 3 mice (female ddY mice, 11 wk old, Jla, Tokyo, Japan) were inoculated intraperitoneally with 5×10^7 lysed Tg/P23 tachyzoites, and another 3 mice were inoculated with 5×10^7 lysed Tg/wt tachyzoites. Each mouse was inoculated with Tg/P23 or Tg/wt tachyzoites 4 times at 2-wk intervals with Freund's incomplete adjuvant (Difco, Detroit, Michigan). Serum samples collected from these mice were subjected to an enzyme-linked immunosorbent assay with recombinant P23 expressed in *E. coli* to measure the antibodies against P23 (Takashima et al., 2003). Mice inoculated with lysed Tg/P23 induced a specific anti-P23 response with the production of a high level of serum IgG, and its subclass responses were IgG1 dominant (Fig. 3). This result suggested that immunization with Tg/P23 induced a type-2 immune response in the mice.

To determine whether the antibodies to P23 induced in mice immunized with Tg/P23 neutralized *C. parvum*, a parasite-neutralizing assay was used, as described previously (Takashima et al., 2003). Each serum was investigated in 4 different wells, and the tests were repeated twice. The addition of anti-Tg/P23 serum to HCT-8 cell monolayers resulted in a reduction in parasite number to 46.93% of the number counted in monolayers treated with anti-Tg/wt control serum. This difference was significant at the 0.05 level (Mann–Whitney *U*-test).

The reactivity of immunized mouse serum against *C. parvum* was also investigated by IFAT. *Cryptosporidium parvum* sporozoites were reacted with both sera from the mice immunized with Tg/P23 and Tg/

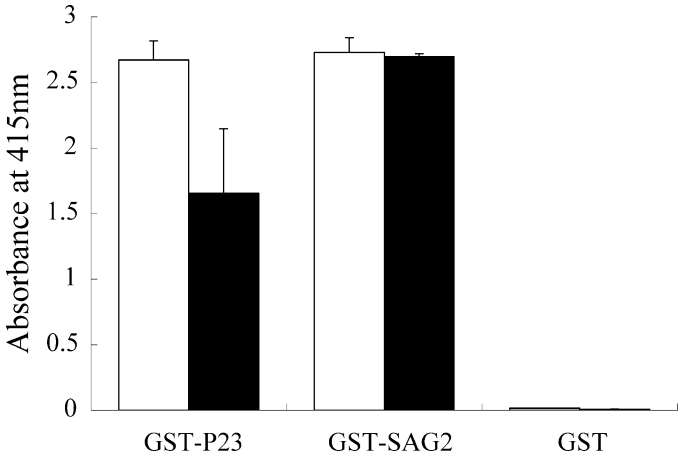


FIGURE 3. Detection of the antibody against P23 protein and serum IgG subclass profiles in Tg/P23-immunized mice. Mouse serum samples were diluted in 1:100, and duplicate wells were used for each sample. The white bars and the black bars indicate IgG1 and IgG2a, respectively. Values were expressed as mean ± SE. As the antigen, glutathione sulfotransferase (GST)-P23 protein expressed in *Escherichia coli* by pGEX-4T vector (Amersham Biosciences, Piscataway, New Jersey) was used. GST-SAG2 was used as a positive control, and GST as a negative control. SAG2 is a major surface antigen of *Toxoplasma gondii* tachyzoites.

wt (data not shown). This result indicated that there was a cross reaction between the *C. parvum* antigen and the serum of the mouse immunized with *T. gondii*. In the Western blot analysis, several cross-reactive antigens were detected in *C. parvum* with both sera from the mice immunized with Tg/P23 and Tg/wt (data not shown). However, a specific P23 was detected in *C. parvum* only with anti-Tg/P23 but not with anti-Tg/wt. This result indicated that the neutralizing antibody is induced by P23 expressed in Tg/P23 but not by cross-reactive antigens in the *T. gondii* vector.

It has been reported that the major part of immunoglobulin in the colostrum of animals is IgG1, which is derived from blood (Lascelles, 1979). In this study, we demonstrated that immunization with Tg/P23 induces a greater level of IgG1 production than IgG2a in mice. The results support the possibility of Tg/P23 as a vaccine for dams to produce colostrums containing a high level of antibodies against the P23. It is known that Th2 cells, which produce interleukin (IL)-4 and IL-5, induce the secretion of IgG1 (Stevens et al., 1988). The results indicate that the P23 expressed by Tg/P23 activates Th2 cells and induces a type-2 immune reaction, resulting in the production of IgG1 in mice.

Despite the fact that *C. parvum* has emerged as one of the most important infections carried by water (Smith and Rose, 1998), currently no vaccine is available to control cryptosporidiosis (de Graaf et al., 1999). It was reported that neutralizing monoclonal antibodies to *C. parvum* have significant efficacy in reducing *C. parvum* infection (Riggs et al., 2002). It was also reported that orally administered bovine colostrums from immunized dams prepared against recombinant P23 protected calves against cryptosporidiosis (Enriquez and Riggs, 1998). Although it was suggested that an experimental oral vaccination could protect young cattle from cryptosporidiosis, a field test of the vaccine resulted in failure (Harps and Goff, 1998), indicating the importance of providing neonatal calves with immunity against *C. parvum* within an hour of birth to protect them from cryptosporidiosis. Hence, vaccination of dams with P23 that induces the production of colostrums for the protection of calves from cryptosporidiosis could be an effective measure in controlling the disease.

The results of this study demonstrate that the immunization of mice with recombinant *T. gondii* expressing *C. parvum* P23 induced anti–P23-specific IgG that inhibited the infectivity of *C. parvum* sporozoites. In a follow-up study, it might be worthwhile to immunize dams with Tg/P23 to evaluate its effectiveness for the protection of calves from cryptosporidiosis.

This work was supported by a grant from The 21st Century COE Program (A-1) and a Grant-in-Aid for Scientific Research (14360172), both from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

LITERATURE CITED

BRASSEUR, P. 1997. Waterborne cryptosporidiosis: A major environmental risk. Journal of Eukaryotic Microbiology **44:** 67S–68S.

CHAREST, H., M. SEDEGAH, G. S. YAP, R. T. GAZZINELLI, P. CASPAR, S. L. HOFFMAN, AND A. SHER. 2000. Recombinant attenuated *Toxoplasma gondii* expressing the *Plasmodium yoelii* circumsporozoite protein provides highly effective priming for CD8+ T cell-dependent protective immunity against malaria. Journal of Immunology **165:** 2084–2092.

DE GRAAF, D. C., F. SPANO, F. PETRY, S. SAGODIRA, AND A. BONNIN. 1999. Speculation on whether a vaccine against cryptosporidiosis is a reality or fantasy. International Journal for Parasitology **29:** 1289–1306.

DI CRISTINA, M., F. GHOUZE, C. H. KOCKEN, S. NAITZA, P. CELLINI, D. SOLDATI, A. W. THOMAS, AND A. CRISANTI. 1999. Transformed *Toxoplasma gondii* tachyzoites expressing the circumsporozoite protein of *Plasmodium knowlesi* elicit a specific immune response in rhesus monkeys. Infection and Immunity **67:** 1677–1682.

DUBEY, J. P., AND C. P. BEATTIE. 1988. Toxoplasmosis of animals and man. CRC Press Inc., Boca Raton, Florida, 220 p.

ENRIQUEZ, F. J., AND M. W. RIGGS. 1998. Role of immunoglobulin A monoclonal antibodies against P23 in controlling murine *Cryptosporidium parvum* infection. Infection and Immunity **66:** 4469–4473.

FAYER, R., C. A. SPEER, AND J. P. DUBEY. 1997. The general biology of *Cryptosporidium*. In *Cryptosporidium* and cryptosporidiosis, R. Fayer (ed.). CRC Press Inc., Boca Raton, Florida, 42 p.

HARPS, J. A., AND J. P. GOFF. 1998. Strategies for the control of *Cryptosporidium parvum* infection in calves. Journal of Dairy Science **81:** 289–294.

LASCELLES, A. K. 1979. The immune system on the ruminant mammary gland and its role in the control of mastitis. Journal of Dairy Science **62:** 154–167.

NISHIKAWA, Y., X. XUAN, L. MAKALA, O. VIELEMEYER, K. A. JOINER, AND H. NAGASAWA. 2003. Characterization of *Toxoplasma gondii* engineered to express mouse interferon-gamma. International Journal for Parasitology **33:** 1525–1535.

O'CONNOR, R. M., K. KIM, F. KAHN, AND H. D. WARD. 2003. Expression of *Cpgp40/15* in *Toxoplasma gondii*: A surrogate system for the study of *Cryptosporidium* glycoprotein antigens. Infection and Immunity **71:** 6027–6034.

OKHUYSEN, P. C., AND C. L. CHAPPELL. 2002. *Cryptosporidium* virulence determinants—are we there yet? International Journal for Parasitology **32:** 517–525.

PERRYMAN, L. E., D. P. JASMER, M. W. RIGGS, S. G. BOHNET, T. C. MCGUIRE, AND M. J. ARROWOOD. 1996. A cloned gene of *Cryptosporidium parvum* encodes neutralization-sensitive epitopes. Molecular and Biochemical Parasitology **80:** 137–147.

———, S. J. KAPIL, M. L. JONES, AND E. L. HUNT. 1999. Protection of calves against cryptosporidiosis with immune bovine colostrums induced by a *Cryptosporidium parvum* recombinant protein. Vaccine **17:** 2142–2149.

RIGGS, M. W., D. A. SCHAEFER, S. J. KAPIL, L. BARLEY-MALONEY, AND L. E. PERRYMAN. 2002. Efficacy of monoclonal antibodies against defined antigens for passive immunotherapy of chronic gastrointestinal cryptosporidiosis. Antimicrobial Agents and Chemotherapy **46:** 275–282.

SIBLEY, L. D., M. MESSINA, AND I. R. NIESMAN. 1994. Stable DNA transformation in the obligate intracellular parasite *Toxoplasma gondii* by complementation of tryptophan auxotrophy. Proceedings of the National Academy of Sciences of the United States of America **91:** 5508–5512.

SMITH, H. V., AND J. B. ROSE. 1990. Waterborne cryptosporidiosis. Parasitology Today **6:** 8–12.

———, AND ———. 1998. Waterborne cryptosporidiosis: Current status. Parasitology Today **14:** 14–22.

STEVENS, T. L., A. BOSSIE, V. M. SANDERS, R. FERNANDEZ-BOTRAN, R.

L. COFFMAN, T. R. MOSMANN, AND E. S. VITETTA. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature **334:** 255–258.

TAKASHIMA, Y., X. XUAN, I. KIMATA, M. ISEKI, Y. KODAMA, N. NAGANE, H. NAGASAWA, Y. MATSUMOTO, T. MIKAMI, AND H. OTSUKA. 2003. Recombinant bovine herpesvirus-1 expressing p23 protein of *Cryptosporidium parvum* induces neutralizing antibodies in rabbits. Journal of Parasitology **89:** 276–282.

J. Parasitol., 91(2), 2005, pp. 479–481

Infection of *Saimiri boliviensis* Monkeys With *Plasmodium coatneyi*

JoAnn S. Sullivan, Amy Bounngaseng*, Ann Stewart†, James J. Sullivan, G. Gale Galland, Fleetwood Henry†, and William E. Collins‡
Division of Parasitic Diseases and Scientific Resources Program, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; *Atlanta Research & Education Foundation, Atlanta, Georgia; †Department of Immunology, Walter Reed Army Institute of Research, Silver Spring, Maryland; ‡To whom correspondence should be addressed. *e-mail: wec1@cdc.gov*

ABSTRACT: Abundant, apparently normally developing, liver-stage parasites of *Plasmodium coatneyi* were demonstrated following injection of sporozoites dissected from the salivary glands of *Anopheles dirus* mosquitoes. Erythrocytic development was not demonstrated.

Plasmodium coatneyi is a tertian, nonhuman primate malaria parasite of Southeast Asia that was initially isolated in Malaysia from an *Anopheles hackeri* mosquito and transmitted experimentally by inoculation of dissected sporozoites to a rhesus monkey, *Macaca mulatta* (Eyles et al., 1962). The parasite was later isolated from *Macaca irus* (= *fascicularis*) in Malaya and the Philippines (Eyles, 1963). In rhesus monkeys, *P. coatneyi* shows similarities to *Plasmodium falciparum* with respect to the sequestration of schizonts in the brain and the ability to produce very high parasitemia and mortality (Coatney et al., 1971; Collins et al., 2001). It has been used in macaques as a model for human cerebral malaria (Aikawa, et al., 1992; Kawai et al., 1993; Sein et al., 1993) as well as a model for malaria in pregnancy (Davison et al., 1998) and for immunologic (Udomsangpetch et al., 1993; Yang et al., 1999; Tongren et al., 2000) and drug (Maeno et al., 1993) studies.

As part of ongoing studies on the infection of New World monkeys with Old World monkey malarias, animals are injected with either sporozoites or infected erythrocytes. It was demonstrated that liver-stage parasites of *Plasmodium fieldi* (Sullivan et al., 1998) would develop in *Aotus nancymai* monkeys following sporozoite injection, but erythrocytic-stage infections would not. The same occurred when *A. nancymai* and *Saimiri boliviensis* monkeys were inoculated with sporozoites of *Plasmodium gonderi* (Sullivan et al., 2002). This contrasts with the successful sporozoite transmission and erythrocytic development of *Plasmodium knowlesi* (Collins et al., 1978; Sullivan et al., 1996), *Plasmodium cynomolgi* (Collins et al., 1975, 1985), *Plasmodium fragile* (Collins et al., 1974, 1990), and *Plasmodium inui* (Collins et al., 1981, 1988; Sullivan et al., 2003) to New World monkeys. Reported here are the results of sporozoite inoculations of *P. coatneyi* into *S. boliviensis* monkeys and a description of the exoerythrocytic stages in the liver of these monkeys. Previous attempts to infect New World monkeys by the intravenous injection of massive numbers of infected erythrocytes from macaques had failed to induce erythrocytic infections in these animals.

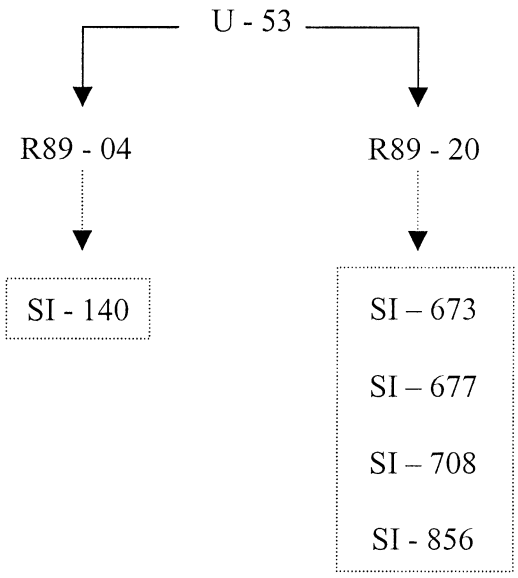


FIGURE 1. Sporozoite transmission of *Plasmodium coatneyi* from *macaca mulatta* to *Saimiri boliviensis* monkeys. R- = *Macaca mulatta*; SI = *Saimiri boliviensis*. Solid line = blood-stage inoculation; dotted line = sporozoite transmission.

Blood from rhesus U-53 was inoculated into rhesus monkeys R89-04 and R89-20 after being frozen over liquid nitrogen for approximately 141 and 139 mo, respectively. *Anopheles dirus* mosquitoes were then fed on these 2 monkeys (Fig. 1). Five *S. boliviensis* monkeys were inoculated intravenously with sporozoites dissected from the salivary glands of infected mosquitoes, each animal receiving 260,000–500,000 sporozoites (Table I). Four monkeys (SI-673, SI-677, SI-708, and SI-856) had prior malarial infections and had previously been splenectomized. One monkey (SI-140) was spleen intact and malaria naive at the time of inoculation. Liver biopsies were performed at laparotomy on day 7 or 8 after inoculation, the tissues fixed in formalin, sectioned at 5 μm, and stained with Giemsa stain. Liver sections were then examined for the presence of exoerythrocytic stages. Blood films were made daily by the method of Earle and Perez (1932) and thick films examined to detect the presence of blood-stage parasites.

Exoerythrocytic stages of *P. coatneyi* were observed in liver sections from all 5 *Saimiri* monkeys that were inoculated. They were particularly abundant in liver sections from SI-140 and SI-856 and scarce in SI-677. Seven- and 8-day liver stages were generally similar in morphology, i.e., round to oval in shape, with flocculi and/or vacuoles being prominent in some. Serial sections from SI-140 (Fig. 2A–D) and SI-856 (Fig. 2I–K) show some flocculi and vacuoles; the vacuoles do not appear in every section. An EE body from SI-140 is shown adjacent to a capillary (Fig. 2E). Liver sections from SI-673 (Fig. 2F) and SI-677 (Fig. 2G) showed few EE bodies. A liver section from SI-708 shows an oval-shaped EE body with a prominent vacuole (Fig. 2H). No completely mature liver stages were observed at days 7 or 8 and no erythrocytic stages were observed during a 60-day observation period.

Plasmodium coatneyi has been shown to develop in the liver cells of a New World monkey, *S. boliviensis*, following the intravenous inoculation of a large number of sporozoites. The morphology of the liver stages in these *S. boliviensis* monkeys closely resembles those described by Held and Contacos (1967) in rhesus monkeys. Unlike infections in the rhesus monkey, however, no blood stages were observed in any of these *S. boliviensis* monkeys. In this respect, *P. coatney* is similar to the nonhuman primate malaria parasites *P. gonderi* in *S. boliviensis* monkeys and *P. fieldi* in *Aotus nancymai* monkeys. It appears that *P. coatneyi* in splenectomized *S. boliviensis* may be useful in studies on

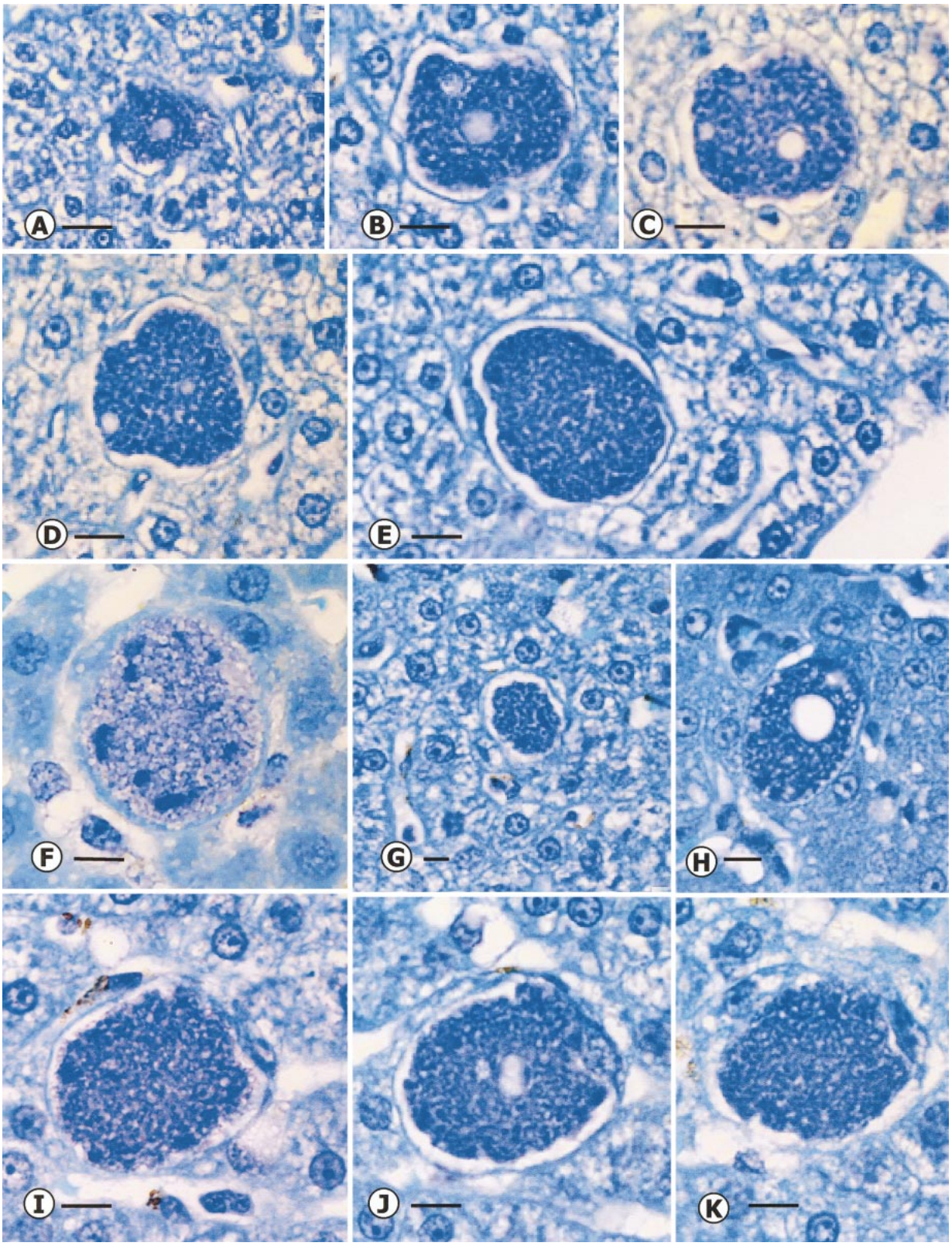


FIGURE 2. Exoerythrocytic stages of *Plasmodium coatneyi* in liver sections of *Saimiri boliviensis* monkeys. **A–D.** Serial sections fro SI-140. **E.** Liver stage in SI-140, near a capillary. **F.** Liver stage from SI-673, with flocculi. **G.** Liver stage from SI-677. **H.** Liver stage from SI-708, with vacuole. **I–K**. Serial sections from SI-856. Scale bar = 10 μm.

TABLE I. Inoculations of *Saimiri boliviensis* monkeys with sporozoites of *Plasmodium coatneyi*.

Animal ID	No. of sporozoites inoculated	Mosquito (*Anopheles*) species	Liver biopsy (days after inoculation)	Previous malaria*	EE bodies seen	Blood stages seen
SI-140	260,000	*dirus*	8	None	Yes	No
SI-673	500,000	*dirus*	8	Pf, Pv	Yes	No
SI-677	350,000	*dirus*	8	Pf, Pv	Yes	No
SI-708	320,000	*dirus*	7	Pf, Pv, Pk	Yes	No
SI-856	360,000	*dirus*	7	Pf, Pv, Pk	Yes	No

* Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pk = *P. knowlesi.*

the mechanisms that govern the invasion of liver hepatocytes or restrict the development of erythrocytic-stage infections.

This work was supported in part by an interagency agreement between the Agency for International Development, Malaria Vaccine Development Program, and the Centers for Disease Control and Prevention, Project 936-6001, and the Atlanta Research and Education Foundation of the Veterans Affairs Medical Center of Atlanta. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

LITERATURE CITED

AIKAWA, M., A. BROWN, C. D. SMITH, T. TEGOSHI, R. J. HOWARD, T. H. HASLER, Y. ITO, G. PERRY, W. E. COLLINS, AND K. WEBSTER. 1992. A primate model for human cerebral malaria: *Plasmodium coatneyi*-infected rhesus monkeys. American Journal of Tropical Medicine and Hygiene **46:** 391–397.

COATNEY, G. R., W. E. COLLINS, MCW. WARREN, AND P. G. CONTACOS. 1971. The primate malarias. U.S. Government Printing Office, Washington, D.C., p. 289–299.

COLLINS, W. E., P. G. CONTACOS,, AND W. CHIN. 1978. Infection of the squirrel monkey, *Saimiri sciureus* with *Plasmodium knowlesi*. Transactions of the Royal Society of tropical Medicine and Hygiene **72:** 662–663.

———, J. C. SKINNER, V. K. FILIPSKI, J. R. BRODERSON, P. S. STANFILL, AND C. L. MORRIS. 1990. Transmission of *Plasmodium fragile* to *Saimiri* monkeys. Journal of Parasitology **76:** 730–732.

———, ———, ———, C. WILSON, J. R. BRODERSON, AND P. S. STANFILL. 1988. Transmission of the OS strain of *Plasmodium inui* to *Saimiri sciureus boliviensis* and *Aotus azarae boliviensis* monkeys by *Anopheles* mosquitoes. Journal of Parasitology **74:** 502–503.

———, ———, AND P. MEHAFFEY. 1985. Infection of *Aotus azarae boliviensis* monkeys with the RO strain of *Plasmodium cynomolgi*. Journal of Parasitology **67:** 848–849.

———, ———, B. B. RICHARDSON, AND A. Y. HUONG. 1981. Development of *Plasmodium inui* in the *Aotus trivirgatus* monkey. Journal of Parasitology **67:** 222–225.

———, ———, ———, AND C. S. SMITH. 1974. Transmission of *Plasmodium fragile* to *Aotus trivirgatus* monkeys. Journal of Parasitology **60:** 719–720.

———, ———, ———, AND P. S. STANFILL. 1975. Transmission of *Plasmodium cynomolgi* to *Aotus trivirgatus* monkeys. Journal of Parasitology **61:** 146–148.

———, MCW. WARREN, J. S. SULLIVAN, AND G. G. GALLAND. 2001. *Plasmodium coatneyi*: Observations on periodicity, mosquito infection, and transmission to *Macaca mulatta* monkeys. American Journal of Tropical Medicine and Hygiene **64:** 101–110.

DAVISON, B. B., F. B. COGSWELL, G. B. BASKIN, K. P. FALKENSTEIN, E. W. HANSEN, A. F. TARANTAL, AND D. J. KROGSTAD. 1998. *Plasmodium coatneyi* in the rhesus monkey (*Macaca mulatta*) as a model of malaria in pregnancy. American Journal of Tropical Medicine and Hygiene **59:** 189–201.

EARLE, W. C., AND M. PEREZ. 1932. Enumeration of parasites in the blood of malarial patients. Journal of Laboratory and Clinical Medicine **17:** 1124–1130.

EYLES, D. E. 1963. The species of simian malaria: Taxonomy, morphology, life cycle, and geographical distribution of the monkey species. Journal of Parasitology **49:** 866–887.

———, L. F. YAP, MC W. WARREN, E. GUINN, A. A. SANDOSHAM, AND R. H. WHARTON. 1962. *Plasmodium coatneyi*, a new species of primate malaria from Malaya. American Journal of Tropical Medicine and Hygiene **11:** 597–604.

HELD, J. R., AND P. G. CONTACOS. 1967. Studies of the exoerythrocytic stages of simian malaria. II. *Plasmodium coatneyi*. Journal of Parasitology **53:** 910–918.

KAWAI, S., M. AIKAWA, S. KANO, AND M. SUZUKI. 1993. A primate model for severe human malaria with cerebral involvement: *Plasmodium coatneyi*-infected *Macaca fuscata*. American Journal of Tropical Medicine and Hygiene **48:** 630–636.

MAENO, Y., A. E. BROWN, C. D. SMITH, T. TEGOSHI, T. TOYOSHIMA, C. F. OCKENHOUSE, K. D. CORCORAN, M. NGAMPOCHJANA, D. E. KYLE, H. K. WEBSTER, AND M. AIKAWA. 1993. A nonhuman primate model for human cerebral malaria: Effects of artesunate (qinghaosu derivative) on rhesus monkeys experimentally infected with *Plasmodium coatneyi*. American Journal of Tropical Medicine and Hygiene **49:** 726–734.

SEIN, K. K., A. E. BROWN, Y. MAENO, C. D. SMITH, K. D. CORCORAN, P. HANSUKJARIYA, H. K. WEBSTER, AND M. AIKAWA. 1993. Sequestration pattern of parasitized erythrocytes in cerebrum, mid-brain, and cerebellum of *Plasmodium coatneyi*-infected rhesus monkeys. American Journal of Tropical Medicine and Hygiene **49:** 513–519.

SULLIVAN, J. S., V. M. JENNINGS, J. GUARNER, G. S. NOLAND, J. KENDALL, AND W. E. COLLINS. 2002. Infection of *Aotus* and *Saimiri* monkeys with *Plasodium gonderi*. Journal of Parasitology **88:** 422–425.

———, C. L. MORRIS, B. B. RICHARDSON, G. G. GALLAND, AND W. E. COLLINS. 1998. Attempts to transmit the N-3 strain of *Plasmodium fieldi* to *Aotus* monkeys. Journal of Parasitology **84:** 195–197.

———, ———, ———, ———, J. J. SULLIVAN, AND W. E. COLLINS. 1996. Sporozoite transmission of three strains of *Plasmodium knowlesi* to *Aotus* and *Saimiri* monkeys. Journal of Parasitology **82:** 268–271.

———, D. NACE, T. WILLIAMS, J. GUARNER, G. S. NOLAND, AND W. E. COLLINS. 2003. The development of exoerythrocytic stages of *Plasmodium inui shortti* in New World monkeys. Journal of Parasitology **89:** 637–639.

TONGREN, J. E., C. YANG, W. E. COLLINS, J. S. SULLIVAN, A. A. LAL, AND L. XIAO. 2000. The development of proinflammatory cytokines in four regions of the brain in *Macaca mulatta* (rhesus) monkeys infected with *Plasmodium coatneyi*. American Journal of Tropical Medicine and Hygiene **62:** 530–534.

UDOMSANGPETCH, R., A. E. BROWN, C. D. SMITH, AND H. K. WEBSTER. 1993. *Plasmodium coatneyi* ring-infected erythrocyte surface antigens. American Journal of Tropical Medicine and Hygiene **49:** 127–134.

YANG, C., L. XIAO, J. E. TONGREN, J. SULLIVAN, A. A. LAL, AND W. E. COLLINS. 1999. Cytokine production in rhesus monkeys infected with *Plasmodium coatneyi*. American Journal of Tropical medicine and Hygiene **61:** 226–229.