Conserved Polymerase Chain Reaction Primers Fail in Diagnosis of Parasitic Infections

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ABSTRACT: We demonstrate that a set of previously described polymerase chain reaction primers used for detection of hemogregarines in reptiles will also amplify the same region of the 18S rRNA gene of reptiles, amphibians, mammals, and insects and thus should not be used for molecular diagnosis. These same primers have also been used to differentiate 2 species of *Plasmodium* that infect lizards. We provide evidence that the observed variance may have been dependent on parasitemia and not representative of actual molecular differences between the 2 parasite species.

The advent of modern molecular techniques has allowed parasitologists and epidemiologists to develop species-specific polymerase chain reaction (PCR) primers for the identification of various pathogens of animals and humans (e.g., Roos and Grant, 1993; Snounou et al., 1993; Li et al., 1995; Tsuji et al., 1997). These assays can provide quick, reliable diagnoses of infectious diseases both in field and clinical samples, including instances where infection by more than 1 species of parasite is possible. Great care must be taken when designing primers that will be useful to detect parasites, however, that they will not also amplify host DNA and thus give false-positive results. For example, as we demonstrate below, the primers used by Wozniak et al. (1996) to differentiate 2 species of saurian *Plasmodium* species are extremely conservative and will amplify the 18S rRNA gene in vertebrates (hosts), insects (vectors), as well as apicomplexans (parasites). These conservative primers were used by the same authors in an earlier study (1994) to survey captive reptiles for hemogregarine infections; thus, it is possible that many of their infections detected by PCR were, in fact, false positives.

Blood samples from western fence lizards (*Sceloporus occidentalis,* California), an anole (*Anolis evermanni,* Puerto Rico), a ctenosaur (*Ctenosaurus similis,* Costa Rica), and a wood frog (*Rana sylvatica,* Vermont) were obtained by clipping toes. Thin blood smears were made for each lizard and these were fixed in absolute methanol and Giemsa stained. All slides were scanned at 1,000× under oil immersion for 6 min (this allows inspection of approximately 10,000 host blood cells) and the presence of hemoparasites (*Plasmodium,* hemogregarines) was noted for each. In addition to thin blood smears, dried blood spots on Whatman filter paper were also made for the lizards and the frog. DNA was extracted from these dried blood spots using sodium dodecyl sulfate lysis followed by either phenol–chloroform extraction and ethanol precipitation/washing or ammonium acetate (7.5 M) precipitation of proteins with subsequent isopropanol precipitation/ethanol washing. Lizards designated as uninfected were either hatched in the laboratory (*S. occidentalis*) or of a species in which infection with *Plasmodium* is rare (*A. evermanni,* Schall and Vogt, 1993). Screening with nested PCR primers specific to *Plasmodium* species (Li et al., 1995) showed that the western fence lizard, anole, and ctenosaur were free of malaria parasites. These *Plasmodium*-specific primers are capable of detecting infections of less than 1 parasite per 10,000 host cells (Perkins et al., 1998). The ctenosaur was infected with an unidentified hemogregarine.

PCRs were set up in 25-μl reactions with Ready-to-Go™ PCR beads (Pharmacia Biotech, Piscataway, New Jersey) using 2.5 mM MgCl$_2$, 2.5 μM of each primer, and approximately 250 ng of genomic DNA. In addition to the lizard and frog samples, we also amplified DNA from an uninfected human, a fruitfly (*Drosophila melanogaster*), a mosquito (*Anopheles gambiae*), and clonal *P. falciparum.* Samples were amplified using the same conditions used by Wozniak et al. (1996): 94 C (1 min), 56 C (1 min), and 72 C (1 min) for 10 cycles followed by 25 cycles with the annealing temperature lowered to 54 C and a final polymerization step of 5 min at 72 C. Products were separated on a 2% agarose gel containing ethidium bromide and visualized over UV light. PCR products from samples to be sequenced were purified by incubation with polyethylene glycol and washed with ethanol. These samples were subjected to cycle sequencing using primer 18AP853.F and run in an ABI Prism automated sequencer (Vermont Cancer Center, University of Vermont).

Amplification of all samples, with the exception of the cloned *P. falciparum,* resulted in an approximately 590-bp fragment. The cloned *P. falciparum* produced a fragment of approximately 610 bp. Amplification of DNA from lizards infected with *Plasmodium mexicanum* resulted in 2 bands, 1 of each of these sizes (Fig. 1). The sequence of the single PCR fragment from the wood frog was 99.71% identical to the published sequence for this region of the 18S rRNA gene in *Xenopus laevis* (GenBank accession no. X04025). The sequence of the PCR fragment from the uninfected western fence lizard was 99.41% identical to the published sequence for this region of the 18S rRNA gene in *Sceloporus undulatus* (GenBank accession nos. M59400/M36359.) A BLAST search in GenBank of the primer sequences showed that these regions are highly conserved in the rRNA genes of a wide range of organisms, from green algae to vertebrates. Thus, these data demonstrate that the primers designated as 18AP853.F and 18AP1488.R by Wozniak et al. (1994, 1996) will amplify the rRNA genes from most samples, including those of potential hosts and vectors.

Wozniak et al. (1996) conclude that 2 species of lizard malaria could be differentiated electrophoretically because *Plasmodium floridense* consistently produced 2 bands, whereas *Plasmodium chiricahuae* only produced 1. Although these authors do not provide data on parasitemias of the infected lizards, it is possible that their results were affected by lower parasitemias of *P. chiricahuae* in its host, *Sceloporus jarrovi,* than those of *P. floridense* in the 2 anole hosts. Amplification of *S. occidentalis* with very low parasitemias of *P. mexicanum* (<6 parasites/10,000 host blood cells) produced a single PCR frag-
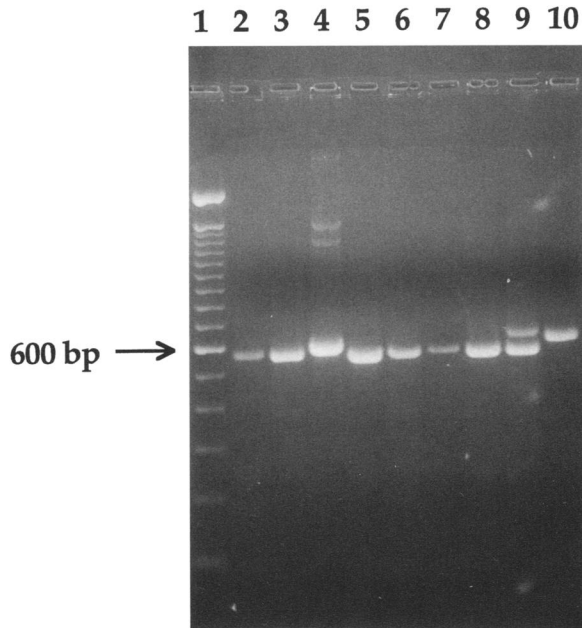
FIGURE 1. Agarose gel containing PCR products from a range of organisms amplified with primers 18AP853.F and 18AP1488. Lane 1, 100-bp molecular standard; lane 2, human; lane 3, *Drosophila melanogaster*; Lane 4, *Anopheles gambiae*; lane 5, *Rana sylvatica*; lane 6, *Anolis evermanni*; lane 7, uninfected *Sceloporus occidentalis*; lane 8, *Ctenosaura similis* infected with unidentified hemogregarine; lane 9, *Sceloporus occidentalis* infected with *Plasmodium mexicanum*; lane 10, *Plasmodium falciparum*.
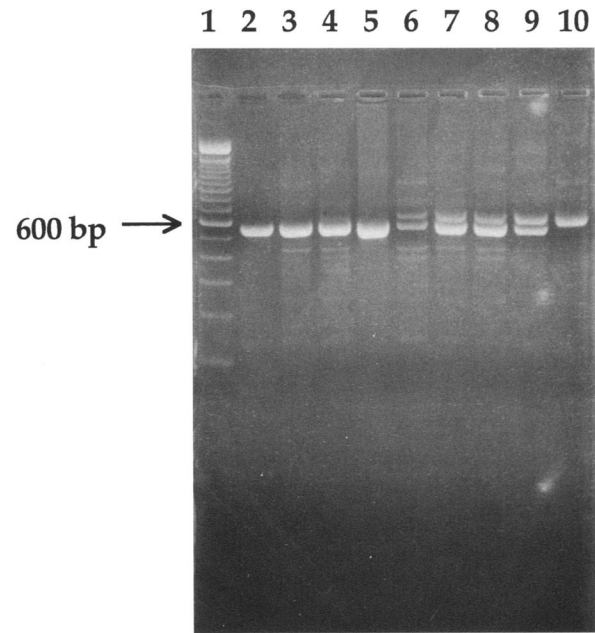
FIGURE 2. Agarose gel containing PCR products from *Sceloporus occidentalis* with varying parasitemias (expressed as number of cells per 1,000 host erythrocytes) of *Plasmodium mexicanum*. Lane 1, 100-bp molecular marker; lane 2, uninfected; lane 3, <1; lanes 4 and 5, 2; lane 6, 21; lane 7, 58; lane 8, 180; lane 9, 202; lane 10, *Plasmodium falciparum* control. Lizards with weak infections (lanes 3–5) show only a single host band, whereas those with higher parasitemias (lanes 6–9) show both an approximately 590-bp band from the host and an approximately 630-bp band from the parasite.

ment, that of the lizard host, whereas heavier infections produced 2 fragments of different sizes, 1 of the host and 1 of the malaria parasite (Fig. 2).

The primers designed by Wozniak et al. (1994, 1996) do have utility for researchers of *Plasmodium* parasites, however. As shown in Figure 2, they may be used as a molecular diagnostic for infections of moderate parasitemia. The presence of the host band provides a convenient internal control for PCR conditions. A similar diagnostic PCR was used by Feldman et al. (1995) to test for *Plasmodium* parasites in Hawaiian birds. These primers fail at amplifying very weak infections, however; therefore, diagnosis by microscopy would be more applicable for most systems.

Unlike most other eukaryotes, *Plasmodium* has at least 2 forms of nuclear rRNA genes. Some genes are expressed only in blood-stage parasites and others are expressed only in the sporozoites. These genes exist, not in tandemly arranged multiple copies as in most organisms but, instead, in just a few copies scattered throughout the genome (McCutchan et al., 1995). Although the primers of Wosniak et al. (1994, 1996) are general to a wide array of organisms' 18S rRNA genes, their primer 18AP853.F seems to be specific to the asexually expressed 18S rRNA gene of *Plasmodium*. These 2 types of 18S rRNA genes may vary by more than 12% overall (compare GenBank sequences M19172 and M19173 for *P. falciparum*), and fortuitously differ by 4 out of 24 nucleotides in this primer sequence. Using these primers and clonal *P. falciparum* DNA as a template in PCR and sequencing conditions as described above, we obtained a sequence that was identical to that of the published sequence for 18S rRNA in asexual parasites

(GenBank accession no. M19172). Previous phylogenetic work on *Plasmodium* has been plagued by the use of these nonhomologous rRNA genes (Corredor and Enea, 1993). It is possible then, that these primers that seem to be specific to only the asexually expressed genes may help alleviate this problem.

DNA from clonal *P. falciparum* (strain 3D7) was donated by Jane Carlton (Department of Pathobiology, University of Florida School of Veterinary Medicine), DNA from *Drosophila melanogaster* was donated by Brian Lannutti (UVM), and DNA from *Anopheles gambiae* was donated by David Onyabe (UVM). This research was supported by grants to J. J. Schall from the National Science Foundation and NSF–Vermont EPSCoR.

LITERATURE CITED

CORREDOR, V., AND V. ENEA. 1993. Plasmodial ribosomal RNA as phylogenetic probe: A cautionary note. Molecular Biology and Evolution **10**: 924–926.

FELDMAN, R. A., L. A. FREED, AND R. L. CANN. 1995. A PCR test for avian malaria in Hawaiian birds. Molecular Ecology **4**: 663–673.

LI, J., R. A. WIRTZ, G. A. MCCONKEY, J. SATTABONGKOT, A. P. WATERS, M. J. ROGERS, AND T. F. MCCUTCHAN. 1995. *Plasmodium*: Genus-conserved primers for species identification and quantitation. Experimental Parasitology **81**: 182–190.

MCCUTCHAN, T. F., J. LI, G. A. MCCONKEY, M. J. ROGERS, AND A. P. WATERS. 1995. The cytoplasmic ribosomal RNAs of *Plasmodium* spp. Parasitology Today **11**: 134–138.

PERKINS, S. L., S. M. OSGOOD, AND J. J. SCHALL. 1998. Use of PCR for detection of subpatent infections of lizard malaria: Implications for epizootiology. Molecular Ecology **7**: 1587–1590.

ROOS, M. H., AND W. N. GRANT. 1993. Species-specific PCR for the

parasitic nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis*. International Journal for Parasitology **23:** 419–421.

SCHALL, J. J., AND S. P. VOGT. 1993. Distribution of malaria in *Anolis* lizards of the Luquillo Forest, Puerto Rico: Implications for host community ecology. Biotropica **25:** 229–235.

SNOUNOU, G., S. VIRIYAKOSOL, W. JARRA, S. THAITHONG, AND K. N. BROWN. 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Molecular and Biochemical Parasitology **58:** 283–292.

TSUJI, N., S. KAWAZU, M. OHTA, T. KAMIO, T. ISOBE, K. SHIMURA, AND

K. FUJISAKI. 1997. Discrimination of eight chicken *Eimeria* species using the two-step polymerase chain reaction. Journal of Parasitology **83:** 966–970.

WOZNIAK, E. J., S. R. TELFORD, JR., AND G. L. MCLAUGHLIN. 1994. Employment of the polymerase chain reaction in the molecular differentiation of reptilian hemogregarines and its application to preventative zoological medicine. Journal of Zoo and Wildlife Medicine **25:** 538–547.

————, ————, AND ————. 1996. Molecular differentiation of two New World saurian *Plasmodium* species in different host species using the polymerase chain reaction. Journal of Parasitology **82:** 372–375.

Helminth Communities in Audouin's Gulls, *Larus audouinii* from Chafarinas Islands (Western Mediterranean)

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ABSTRACT: A survey of intestinal helminth communities of Audouin's gulls *Larus audouinii,* from their breeding colonies in Chafarinas Islands, western Mediterranean, Spain was conducted to determine the abundance and species diversity of intestinal parasites of these birds. The sample of 58 gulls harbored intestinal helminth infracommunities composed of species that are gull generalists, including the digeneans *Cardiocephalus longicollis, Knipowitschiatrema nicolai, Condylocotyla pilodora,* and *Aporchis massiliensis,* and the cestode *Tetrabothrius cylindraceus.* Two nematodes are waterfowl generalists (*Cosmocephalus obvelatus* and *Paracuaria adunca*), whereas the digenean *Acanthotrema armata* is an Audouin's gull specialist. The relative high values of species richness and diversity of the helminth infracommunities are comparable to those of other gulls (*Larus philadelphia, Larus canus*), probably reflecting the specialized, nonselective fish diet of *L. audouinii.*

The purpose of the present study was to conduct an analysis of the intestinal helminth infracommunities of Audouin's gulls and to compare the intestinal helminth diversity parameters in this species to those found in other gull species, as *Larus philadelphia* Ord and *Larus canus* L.

Fifty eight adult specimens of *Larus audouinii* (28 males and 30 females) from the western Mediterranean (35°11′N, 2°26′W) were trapped in May of 1994 and 1995 during their breeding season on the Chafarinas Islands. Gulls were killed with an overdose of chloroform and examined for helminths. Fourteen specimens were necropsied inmediately. All other birds were frozen on dry ice within 5 min of collection. In the laboratory, the digestive tract, air sacs, heart, lungs, pancreas, kidneys, and liver were removed and placed individually in Ringer's solution for microscopical examination. Helminths from each organ were counted, washed in distilled water, fixed, mounted using standard techniques, and identified to species. Representative specimens were deposited in the Parasitology Museum of the Departamento de Biología Animal of the University of Valencia: *Cardiocephalus longicollis* (accession no. 95051710), *Knipowitschiatrema nicolai* (94051315), *Condylocotyla pilodora* (94050601), *Acanthotrema armata* (94051109), *Aporchis massiliensis* (94040524), Cyclophyllidea sp. (94051317), *Tetra-*

bothrius cylindraceus (94051521), *Cosmocephalus obvelatus* (95051404), and *Paracuaria adunca* (95051709).

The use of descriptive ecological terms follows Bush et al. (1997). Brillouin's index of diversity and evenness (Pielou, 1977; Magurran, 1988) was calculated, after logarithmic transformation (n to $\log_e$), for each helminth infracommunity using DIVERS software (Krebs, 1989). The *G*-test was employed in the analysis of the relationship between host sex and frequency of parasites (Zar, 1984), using the Bonferroni correction (Chandler, 1995).

Intestinal helminth communities of *L. audouinii* in Chafarinas Islands were composed of 9 species: 5 Digenea, 2 Cestoda (1 cestode only identified to family), and 2 Nematoda (Table I). Individual birds contained a mean of 4 species (SD = 1.21, range = 1–7) and a mean of 31.14 individual helminths (SD = 40.85, range = 2–190). There was no significant correlation between host sex and numbers of parasites ($\propto$ = 0.05).

The digeneans *C. longicollis, K. nicolai, C. pilodora,* and *A. massiliensis,* and the cestode *T. cylindraceus* are gull generalists because they have been reported only from gulls (Lafuente, 1997), whereas both nematode species (*C. obvelatus* and *P. adunca*) are waterfowl generalists. The only host specialist is the digenean *A. armata,* recorded only from Audouin's gull (Lafuente, 1997).

Larus canus and *L. philadelphia* have diversity patterns of intestinal helminth infracommunities that are similar to those of *L. audouinii* ($\bar{x}$H = 0.84; range 0–1.85). Kennedy et al. (1986) reported 0.915 as mean value of Brillouin's Index (range 0–1.702) for *L. philadelphia,* and Kennedy and Bakke (1989) reported a maximum mean value of 0.647 (range 0–1.482) for *L. canus.* Variations in abundance and diversity may be a consequence of feeding habits and diets of the gull species, because all the parasites are transmitted via food-web relationships. In *L. canus,* maximum mean species richness was 3.34 and maximum mean helminth abundance was 138.2 (Kennedy and Bakke, 1989). In *L. philadelphia,* these values were 5.5 and 50.5,