J. Parasitol., 95(2), 2009, pp. 472–473

Positive Correlation Between Hemosporidian Parasitemia and Likelihood of PCR Detection in Co-Infected Birds

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ABSTRACT: As shown previously, the nested PCR method of detection of avian blood parasites, commonly referred to as Waldenström's method, sometimes amplifies only 1 parasite species of the several that may be present in the same bird, and not always the one with the highest parasitemia. This result raises questions regarding the use of the molecular method for the identification of bird parasites. Additionally, it is unclear whether the amplified parasite, among the several present in the same host, reflects the intensity of infection. However, I performed statistical analyses on a dataset in which there were multiply infected birds and showed that the parasites with the highest parasitemia are the most likely to be amplified. Such a positive correlation between the likelihood of amplification and the parasitemias of the different blood parasites supports the use of Waldenström's method for the comparison of the parasite content of groups of birds exposed to the same parasites.

Hemosporidian parasites of birds can be identified by a nested PCR method described by Waldenström et al. (2004) and broadly used by avian parasitologists (Westerdahl et al., 2005; Bonneaud et al., 2006; Križanauskienė et al., 2006; Bensch et al., 2007; Ortego et al., 2007; Loiseau et al., 2008). However, Valkiūnas et al. (2006) compared results of avian hemosporidian identifications by microscopic examination of stained blood films with the molecular method. In their study, the PCR failed to identify as co-infected 36 birds of 83 examined. This happened, for instance, in simultaneous infection of spotted flycatchers, *Muscicapa striata*, by the parasites *Haemoproteus* sp., *Haemoproteus pallidus*, and *Haemoproteus balmorali*. In 4 birds of the 5 cases reported by Valkiūnas et al. (2006), *Haemoproteus* sp. was the only parasite amplified by the PCR method. Microscopic examination is thus necessary to detect the different parasite species infecting individual birds. Furthermore, Valkiūnas et al. (2006) found that in 9 of the co-infection cases, the parasite detected by PCR was not the one that produced the highest parasitemia. Although it did not have the greatest parasitemia, *Haemoproteus* sp. was amplified in 3 of the 4 spotted flycatchers that were infected by *Haemoproteus* sp., *H. pallidus*, and *H. balmorali*.

These results question the use of Waldenström's method for identifying the several parasites that can simultaneously infect the same birds and, thus, for measuring their resistance to these parasites (here defined as the ability of the hosts to stop or limit infection). However, a positive association between the parasitemia of the co-infecting parasites and the likelihood of detection by PCR would show that, in most cases, the method reveals the parasite that causes the strongest infection. This would consequently support the use of nested PCRs in empirical studies of hemosporidian infections, even though the method provides a somewhat noisy estimate of the most common parasites of a group of birds.

Although in the co-infection cases reported by Valkiūnas et al. (2006) the majority of amplified hemosporidian parasites were those with greater intensity of parasitemia, the authors did not carry out statistical analyses on their data. It thus remains unknown whether the parasite with the highest parasitemia was amplified more often than would have been expected by chance, i.e., whether or not the selection of the amplified parasite is a random process. To answer that question, I performed 2 statistical analyses on the data reported by Valkiūnas et al. (2006) and found a significant positive association between parasitemia and likelihood of PCR detection.

To analyze the data, I first created a nominal variable that described for each co-infected bird whether or not the amplified parasite was also the one with the highest parasitemia. I had to exclude the birds where the 2 parasites that infected the greatest number of red blood cells were in equal numbers (7 cases from 36 co-infections) because I could not predict which parasite should have been detected. I then performed a 1-tailed binomial test to determine whether more than 50% of the amplified parasites were dominant. Note that since 14 hosts were infected by more than 2 parasites, the random amplification of 1 of them would happen on average less than 50% of the time, e.g., 33.3% of the time when there are 3 parasites. Comparing the frequency of amplification to 50% is more stringent than to any lower value and is thus conservative. I found that the amplified parasite had the highest parasitemia in more than half of the cases; indeed, it happened 20 times out of 29 ($P = 0.0307$).

I also tested whether there was a quantitative relationship between the intensity of the parasitemia of the different parasites and the likelihood of PCR detection. I created the variable log (parasitemia of the amplified parasite/parasitemia of the non-amplified parasite that had the greatest parasitemia). This variable was positive when the parasite with the highest parasitemia had been amplified, equal to zero when 2 parasites had the same parasitemia and that 1 of them was amplified, negative when the parasite amplified was not the one with the highest parasitemia (Fig. 1). I then tested whether the mean of this (normally distributed) variable was greater than 0. The mean of the variable was 0.96 (SE 0.54), and a *t*-test significantly rejected the null hypothesis ($t = 1.79$, $P = 0.041$).

I thus show for data from wild birds that Waldenström's nested PCR method preferentially amplified the hemosporidian parasite with the highest parasitemia when co-infection occurred. These results are in agreement with the experimental study by Pérez-Tris and Bensch (2005) in which they mixed *Plasmodium* sp. and *Haemoproteus* sp., parasites to various concentrations and found a positive relationship between concentration and the result of a PCR. I conclude that when co-infection occurs, the selection of the parasite amplified by Waldenström's method is not a random process but relates to intensity of infection. It is, however, important to underline that the nested PCR method may exhibit marked selective amplification of some parasites, which could be due to the selectivity of the primers. For instance, such selective amplification might have happened in the cases of infection by *Haemoproteus* sp., *H. pallidus*, and *H. balmorali* reported by Valkiūnas et al. (2006). Indeed, the former parasite was amplified in 3 of the 4 cases where it did not have the greatest parasitemia. Consequently, Waldenström's method should only be used to compare the parasite that produces the heaviest infection among groups of birds exposed to the same parasites and, thus, when the artifacts due to PCR amplification biases are likely to be similar among these groups. Furthermore, since parasitemia varies over time (Zehtindjiev et al., 2008), it may be necessary to study a large number of birds within each group to identify accurately their most important parasites.

Although many investigations report only the variation in prevalence and distribution of avian malaria infection, numerous studies deal with the association between individual genotype or experimental treatments and resistance to blood parasites (Bonneaud et al., 2006; Loiseau et al., 2008). In particular, when comparing groups of birds exposed to similar conditions, the Waldenström method would identify in each of them the parasite species that infects the greatest number of red blood cells, hence the one for which development is least inhibited by the host. It is important to note that, although this method does not accurately recognize qualitative resistance, i.e., whether or not a host is infected by 1, or 2, particular parasites, it does correlate with quantitative resistance to different parasites. In other words, it is related to the intensity of parasitic infections. Indeed, one could hypothesize that, on average, the amplified parasite is the one for which the bird host has the least quantitative resistance. Such a difference between qualitative and quantitative resistance has important implications for the epidemiology and evolution (Gandon and Michalakis, 2000) of avian malaria.

I thank Staffan Bensch, Aurélie Coulon, Antoinette 'Tania' Jenkins, Mari Kimura, Claire Loiseau, Gabriele Sorci, François Urvoy, 2 anonymous referees, and Gerald W. Esch for critical comments on the manuscript.

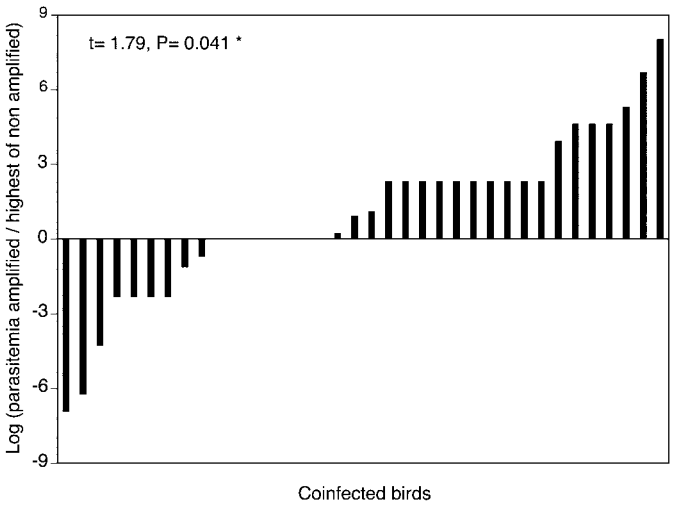

FIGURE 1. Log-transformed ratio of the percentage of blood cells infected by the amplified parasite to the percentage of blood cells infected by the non-amplified parasite that had the greatest parasitemia. Positive values indicate the amplification of the parasite that infected the greatest number of red blood cells; null values that 2 parasites had the same parasitemia and that 1 of them was amplified; negative values show that the parasite amplified did not have the greatest parasitemia. Data for naturally co-infected wild birds from Valkiūnas et al. (2006).

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