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Author for correspondence:
Fabian H. Leendertz
e-mail: leendertzf@rki.de

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Pathogen biology

Age-related effects on malaria parasite infection in wild chimpanzees

Hélène M. De Nys[1,2], Sébastien Calvignac-Spencer[1], Ursula Thiesen[1], Christophe Boesch[2], Roman M. Wittig[2], Roger Mundry[2] and Fabian H. Leendertz[1]

[1]Project Group Epidemiology of Highly Pathogenic Microorganisms, Robert Koch-Institut, Nordufer 20, 13353 Berlin, Germany
[2]Department of Primatology, Max Planck Institute for Evolutionary Anthropology, Deutscher Platz 6, 04103 Leipzig, Germany

Wild great apes are widely infected with a number of malaria parasites (*Plasmodium* spp.). Yet, nothing is known about the biology of these infections in the wild. Using faecal samples collected from wild chimpanzees, we investigated the effect of age on *Plasmodium* spp. detection rates. The data show a strong association between age and malaria parasite positivity, with significantly lower detection rates in adults. This suggests that, as in humans, individuals reaching adulthood have mounted an effective protective immunity against malaria parasites.

1. Introduction

The development of molecular diagnostic tools and the use of non-invasive faecal samples have recently prompted broad-scale investigations of malaria parasite (*Plasmodium* spp.) infections in wild apes [1,2]. This has notably led to the discovery of at least six distinct parasite species infecting wild chimpanzees (*Pan troglodytes*). Three of them, *Plasmodium reichenowi* (C1 in [2]), *Plasmodium gaboni* (C2) and *Plasmodium billcollinsi* (C3) are part of the *Laverania* clade, to which *P. falciparum*, the species responsible for malignant malaria in humans also belongs [3,4]; they are by far the most frequently encountered [2]. The three other species are, respectively, related to the human-infecting *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* parasites and occur more rarely [1,2].

While the genetic diversity of chimpanzee malaria parasites has now been extensively studied in the wild, substantially less is understood about the epidemiological drivers of infection. Important in this context are the virulence of the parasite—i.e. the added mortality owing to infection—and age-related variation in prevalence. In human populations, for example, parasite prevalence and malaria-related morbidity/mortality decrease with age, which reflects the progressive mounting of a protective immunity [5,6].

Here, we present a cross-sectional study which addresses the age-distribution of malaria parasite infection in a group of wild chimpanzees.

2. Material and methods

(a) Sample collection

One hundred forty-one faecal samples were collected in the Taï National Park, Côte d'Ivoire, from seven female and 12 male wild chimpanzees (*Pan troglodytes verus*) belonging to an habituated community of 25 individuals [7], in which malaria parasites are known to circulate [1,8]. Faecal samples were collected within 5 min after defaecation using single-use plastic bags, transported to camp in a cool box

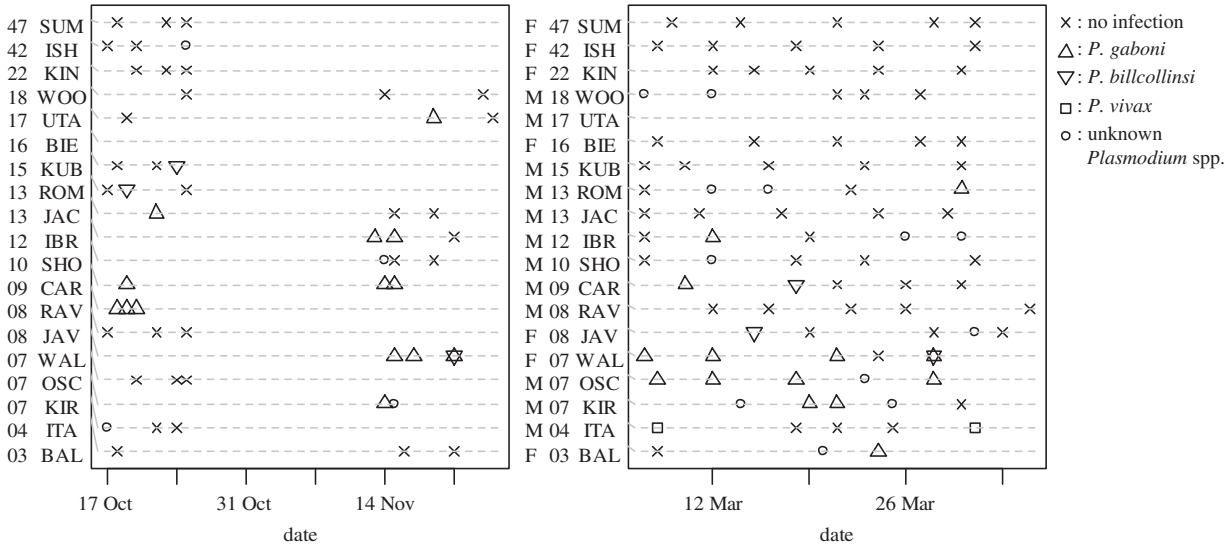

Figure 1. Malaria parasite infection through time. Each line represents an individual (identity is given by the three letter code). Individuals are ordered according to their age, which is shown next to individual short names. The sex of each individual is indicated by F for females and M for males.

within 12 h and then stored in liquid nitrogen until shipment to Germany. On average, 7.42 samples per individual were collected over two time periods (October–November 2011, average 2.94; March–April 2012, average 4.88). Ages at time of sampling ranged from 3 to 47 years (figure 1).

(b) Molecular biology

DNA was extracted from pea-sized faecal samples using EURx Gene MATRIX stool DNA purification kit (Roboklon, Berlin, Germany). Total DNA was measured by fluorometry, using a Qubit (Invitrogen, Carlsbad, CA). Content in mammal mitochondrial DNA (mtDNA) was assessed using a quantitative PCR assay targeting a 200-bp-long fragment of the 16S gene (16Smam1/2; primers and conditions described in Calvignac-Spencer *et al*. [9]).

For malaria parasite detection, 2 µl of DNA extract were used to seed a first PCR reaction targeting a 170-bp-long fragment of malaria parasite mtDNA, followed by a nested qPCR targeting a 90-bp-long fragment.

Positive samples were then subjected to a semi-nested PCR targeting a longer malaria parasite mtDNA fragment (350 bp; see the electronic supplementary material, methods).

PCR products were sequenced on both strands according to Sanger's methodology. Sequences were analysed using Geneious v. 5.4 [10] and compared with publicly available sequences using BLAST [11]. All sequences identified in this study exhibited 99–100% sequence identity to published sequences, which allowed for unambiguous species assignment. Sequences were deposited in EMBL under accession numbers: HF952925–HF952956.

Of note, the PCR systems described here were newly designed and were therefore first validated on a broad range of malaria parasite-positive samples (data not shown).

(c) Statistical analyses

To investigate what influenced the probability of a positive PCR test for *Plasmodium* spp., we used a generalized linear-mixed model (GLMM; [12]) with binomial error structure and logit link function. Into this, we included age and sex, defaecation time and defaecation time-squared as fixed effects and the individual identity as a random effect. The rationale for including defaecation time-squared into the model was that it seemed possible that the response showed a nonlinear diurnal pattern. We *z*-transformed defaecation time and age to a mean of zero and a standard deviation of one. To control for differences in the amount of material analysed, we included it as an offset term into the model. However, we had two measures of the amount of material analysed (total_DNA and mammal_mt_DNA) and, hence, ran two models, one with each of the two included as an offset term (after log-transforming).

The probability of a positive result seemed likely to be temporally autocorrelated beyond what could be explained by the model. To control for autocorrelation, we explicitly incorporated it into the model, using an approach identical to that described in Furtbauer *et al*. [13]. To determine whether the period of sampling (October–November 2011 or March–April 2012) had any effect on the models, two alternative models that included it as a random effect were also tested.

To determine the significance of age and sex, the two predictors we were mainly interested in, we compared the fit of the full model with that of a null model lacking these two fixed effects, but comprising all other terms present in the full model [14] using a likelihood ratio test [15].

To rule out the possibility that the results were largely dependent on certain individuals, we excluded each individual once from the data and repeated the analysis. The estimated coefficients were similar to the ones derived from all data. Hence, the results were not unduly driven by certain individuals. The model was fitted in R (v. 2.14.1; R Development Core Team 2012) using the function lmer of the package lme4 [16], and the autocorrelation term was derived using an R-script written by R.M. and using the R-function optimize. Likelihood ratio tests were conducted using the R-function ANOVA with the argument 'test' set to 'Chisq'.

3. Results

From a nested qPCR assay, *Plasmodium* spp. sequences were detected in 35 per cent of the samples. All individuals but three (84%) were found to be positive at least once over the course of this sampling scheme (*ca* two months; figure 1). Malaria parasite presence was detected in at least one member of the group on 31 out of the 43 (72%) days for which samples were acquired. Days for which no malaria parasite could be detected were also those less intensively sampled (for 'positive' days: mean four samples and standard deviation 1.9; for 'negative' days: mean 1.25 sample and standard deviation 0.4).

Table 1. Results of GLMM model with total_DNA as the offset term. There was no overall effect of day time (likelihood ratio test comparing the full model with a reduced model lacking age and age^2: $\chi^2 = 1.52$, d.f. = 2, $p = 0.468$). Age, day time and the autocorrelation term were *z*-transformed.

term	estimate	s.e.	*z*	*p*
intercept	−0.923	0.575	−1.606	0.108
age	−1.317	0.515	−2.557	0.011
sex (0 = F, 1 = M)	0.463	0.572	0.808	0.419
day time	0.238	0.217	1.098	0.272
day time^2	−0.120	0.218	−0.550	0.582
autocorrelation term	0.523	0.192	2.726	0.006

Table 2. Results of GLMM model with mammal_mt_DNA as the offset term. There was no overall effect of day time (likelihood ratio test comparing the full model with a reduced model lacking age and age^2: $\chi^2 = 0.91$, d.f. = 2, $p = 0.634$). Age, day time and the autocorrelation term were *z*-transformed.

term	estimate	s.e.	*z*	*p*
intercept	−1.193	0.543	−2.197	0.028
age	−1.103	0.453	−2.433	0.015
sex (0 = F, 1 = M)	0.760	0.545	1.395	0.163
day time	0.212	0.222	0.956	0.339
day time^2	0.030	0.223	0.135	0.893
autocorrelation term	0.703	0.202	3.482	0

Overall, a full model including an effect of age and sex on the probability of a positive result was preferred to a null model not accounting for the effect of these variables (likelihood ratio test comparing full and null model, model with total_DNA as offset term: $\chi^2 = 11.44$, d.f. = 2, $p = 0.003$; model with mammal_mt_DNA: $\chi^2 = 12.2$, d.f. = 2, $p = 0.002$). However, detailed inspection of individual results revealed that only age and the autocorrelation term were significant (sex of animal and time of day were not; tables 1 and 2). A clear decrease of the detection probability with age was observed for both models (model with total_DNA as offset term: estimate + s.e. = −1.32 + 0.51, $z = -2.56$, $p = 0.011$; model with mammal_mt_DNA: estimate + s.e. = −1.10 + 0.45, $z = -2.43$, $p = 0.015$; figure 2; electronic supplementary material, figure S1). Models including sampling time as a random effect yielded essentially the same results (see the electronic supplementary material, tables S1 and S2).

For most positive samples, it was also possible to determine informative sequences. This revealed a clear predominance of *P. gaboni*, which was identified in 82 per cent of the positive samples. *Plasmodium billcollinsi* and *P. vivax* were, however, also detected in 18 per cent and 6 per cent of the positive samples, respectively (two cases of co-infection with *P. gaboni* and *P. billcollinsi* were detected). Although most series of positive samples involved a single species, two cases of an apparent switch from one parasite species to another were observed (CAR in March and WAL in November; figure 1).

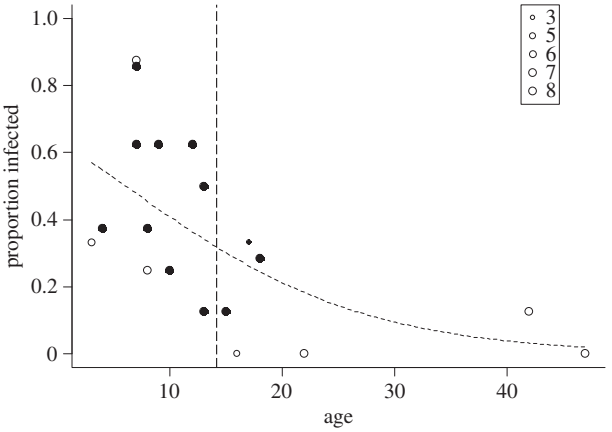

Figure 2. Malaria parasite infection frequency as a function of age. Circles represent individuals. Circle areas are proportional to the number of samples analysed for a given individual; females are shown using open circles, males using filled circles. The vertical dashed line represents the age of maturity in female chimpanzees in Taï (14 years; [7]). Offset term, mammal_mt_DNA.

4. Discussion

The general detection rate was 35 per cent overall, which is well in line with previous estimates in wild chimpanzees [1,2]. However, almost every individual chimpanzee was found positive at least once in the course of this short study. Taking into account sampling intensity, our data also suggest that at every point in time at least one individual of this group of 25 wild chimpanzees is infected. Given the low chimpanzee density in the region [7], it appears likely that circulation of malaria parasites is mainly supported by transmission within groups. Because these groups are, in general, quite small, it can be hypothesized that malaria parasites are locally (within groups) exposed to intense genetic drift. This could end up with group-specific infection with particular parasite species and/or shift of locally dominant parasite species through time, particularly when chimpanzee group size decreases.

Although negative samples do not necessarily mean the absence of infection, but possibly low parasitaemia and shedding in the intestinal lumen, the age-related decrease of the detection probability of *Plasmodium* spp. most probably reflects a decline in parasite prevalence and/or parasite density with age, as has been observed in human populations from endemic areas [5,6]. To our knowledge, this is the first indication that epidemiological characteristics of malaria parasite infection in wild chimpanzee populations might be comparable to those in human populations.

As in humans [5], the development of acquired immunity probably plays an important role in explaining the observed patterns. Throughout this process, malaria parasites might also contribute to directly decimating young chimpanzees. Histopathological findings and molecular analyses performed on more than 30 dead adult chimpanzees from the same area and community do not support a marked pathogenicity of malaria

parasites in adults, because malaria was thus far never identified as a possible cause of death [1]. For young chimpanzees, however, the question remains completely open. While it is known that mortality in young chimpanzees is high [17], their bodies are rarely accessible, either because they have less chances to be found opportunistically or because their carcasses are carried for several days by their mothers (data not shown). This makes the determination of young chimpanzees' cause of death a tricky issue and the involvement of malaria parasites currently cannot be ruled out. Even though at this stage, we cannot pinpoint pathogenicity of malaria parasites found in wild chimpanzees, our results suggest a continuous exposure of this population, leading to the development of a resistance to infection and/or high parasitaemia.

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