ELSEVIER

International Journal for Parasitology 28 (1998) 719–725

INTERNATIONAL Journal for PARASITOLOGY

Determinants of transmission success of individual clones from mixed-clone infections of the rodent malaria parasite, *Plasmodium chabaudi*

L.H. Taylor*, A.F. Read

Institute of Cell, Animal and Population Biology, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JT, U.K

Received 7 October 1997; received in revised form 10 February 1998; accepted 10 February 1998

Abstract

Interactions between malaria parasite clones within mixed infections can have a profound effect on transmission and therefore the epidemiology of the disease. However, factors which determine the relative transmission success of individual clones from mixed infections are unknown. We have used two clones of the rodent malaria *Plasmodium chabaudi* to investigate changes in the clonal composition of asexual parasites over the course of mixed-clone infections in mice and how these relate to the clonal composition of transmission (oocyst) populations in mosquitoes. Clonal composition was determined using monoclonal antibody analyses for the asexual blood stage populations and PCR analysis of single oocysts for the transmission populations in mosquitoes. The relative frequency of the two clones changed dramatically during the course of the infection in mice, depending on their ratio in the inoculum. The clonal composition of parasites within mosquitoes most closely resembled that in the asexual infection at the time of transmission rather than that at any point earlier in the infection. These results provide no evidence that clones increase rates of gametocytogenesis in response to competitive suppression. Most likely, transmission success follows from asexual success in the later parts of the infection. The clone which dominated the earlier part of the infection, when most parasites are produced, did not necessarily dominate the transmission from the infection. The two clones differed in competitive ability and the data suggest that interactions with the host immune system may be a major factor in determining transmission success from mixed-clone infections. © 1998 Australian Society for Parasitology. Published by Elsevier Science Ltd.

Key words: Malaria; *Plasmodium chabaudi*; Transmission: Mixed-clone; Competition; Oocysts; Infectivity

1. Introduction

Mixed infections of human malaria parasites are common in many, if not most, endemic areas [1]. The relative transmission success of co-infecting clones will therefore be a major component of natural selection on parasite genes expressed in vertebrate hosts. Any within-host interactions between clones which affect transmission may also affect levels of infectiousness, outcrossing rates, and population-wide gene frequencies. Despite the theoretical and practical importance of these issues, only recently has it become technically feasible to examine the dynamics of competing clones within

*Corresponding author (new address): Centre for Tropical and Veterinary Medicine, University of Edinburgh, Easter Bush, Roslin, Midlothian EH25 9RG, U.K. Fax: (0131) 445 5099; email: ltaylor@lab0.vet.ed.ac.uk.

S0020-7519/98 $19.00+0.00
PII: S0020-7519(98)000320

the vertebrate host and the consequences of these dynamics on the transmission of individual clones.

To that end, we have been studying the performance of two clones (denoted ER and CR) of the rodent malaria *Plasmodium chabaudi* in laboratory mice, using mAb labelling of asexual parasites and PCR analysis of single oocysts to distinguish the clones of parasites in mice and mosquitoes respectively [2, 3]. Interactions between clones within mixed infections were complex and had a significant effect on both overall infectiousness and the transmission success of individual clones. Total transmission from mixed infections was higher than from single-clone infections [2]. Competing clones could substantially suppress asexual replication rates and hence the population size of individual clones within mice. The relative frequencies of clones within asexual parasite populations often altered dramatically over a few days and the clonal composition of the transmission stages was often different from that of the inoculum or that of the asexual population only four days before the feed. Often, when a clone was initially rare, and suppressed during the bulk of the asexual infection, it was nonetheless the majority parasite in mosquitoes [3].

There are two alternative explanations for the high transmission success of clones which were relatively rare during most of the infection [3]. First, gametocyte densities (and hence transmission) of each clone might be a simple consequence of their asexual densities at the time of gametocyte production. If so, marked alterations in the clonal composition of the asexual infection must have occurred just prior to the feed. Second, in mixed-clone infections, there might be facultative alterations in the rate at which clones produce gametocytes. Environmental modulation of gametocytogenesis occurs in *Plasmodium* [4] and in some cases it is possible to interpret this as an adaptive response to local conditions [5]. Theoretically, a clone which has its asexual replication suppressed by a competitor should increase investment into transmission stage production [6].

We were unable to eliminate either of these explanations using data from our previous experiments. This was because of insufficient sampling during the period when the unexpectedly rapid changes in clonal composition would have to have occurred. Here we report experiments aimed at rectifying that. We initiated *P. chabaudi* infections in mice with the two clones used previously. All infections were initiated with equal numbers of parasites, but with the clones inoculated in ratios of 9:1, 1:9 and 5:5. The first two treatments replicate those used in the earlier experiments [2, 3]; the third provided a more direct test of competitive ability. Blood samples were taken from all mice on the day of the mosquito feed, as well as 2 and 4 days earlier.

2. Materials and methods

2.1. Infections, sampling and analysis

The methods used to infect, maintain and sample infected mice and mosquitoes together with the monoclonal and PCR techniques used to analyse these samples were described by Taylor et al. [3]. The hosts used were inbred C57Bl/6J/Ola mice (Harlan, England) which are homozygous for haplotype b at the Histocompatability-2 (H-2) locus.

2.2. Experimental design

Three treatment groups of six mice each were infected with initial ratios of 1ER:9CR, 9ER:1CR and 5ER:5CR. All mice received a total of 10^6 parasites. Samples of the asexual parasites for mAb analysis were taken on days 10, 12 and 14 p.i. from all mice. On day 14 p.i., blood smears were taken to assess asexual parasitaemia and gametocytaemia, then mosquitoes were allowed to feed on all mice in the 5ER:5CR treatment group, and four mice from each of the 1ER:9CR and 9ER:1CR treatment groups. Very little gametocyte production occurs early in *P. chabaudi* infections [7] and in those initiated with 10^6 parasites, peak gametocytaemias occur on day 14 p.i. [5]. Transmission sampled at this time point should therefore be representative of the bulk of transmission from the infections. Single oocysts were dissected from mosquito midguts 8 to 9 days after the feed. To assess the ratio of the two clones in the transmission population, these were later analysed by PCR of a poly-

morphic antigen gene MSP-1 [3]. A mean (±S.E.M.) of 10.85 (±0.99) oocysts from each infection were analysed by PCR. One infection in a mouse produced no infected mosquitoes, and was excluded from the analyses of transmission.

2.3. Data analysis

To establish 95% confidence limits for the ratios of the two clones in the asexual and oocyst populations, generalised linear models with binomial error structures were fitted to counts of the ER alleles and total alleles sampled from each mouse [8]. For asexual parasites, one allele represented one haploid asexual parasite counted. For the transmission stages it represented one of the two alleles identified by PCR in a single oocyst (i.e. the equivalent of one successful gamete).

Ordinary least squares regression analyses were used to test for correlations between gametocytaemia (number of gametocytes per 1,000 red blood cells) and asexual parasitaemia (percentage of red blood cells infected with asexual parasites). All data were arcsine transformed to give normal error structures prior to these analyses. Correlations between the proportion of parasites in asexual and oocyst populations that were derived from the ER clone were carried out using GLIM [8]. The proportion of ER parasites in the oocyst population was used as the dependent variable in models with binomial error structure, and the proportion of ER parasites in the asexual population was arc-sine transformed prior to use as the independent variable.

3. Results

3.1. Relative frequencies of the two clones in asexual populations

When the ER clone started as 10% of the inoculum, it was able to increase its representation of the asexual population dramatically between days 10 and 14 p.i. ER parasites constituted 21% of the asexual parasites on day 10 p.i., 65% on day 12 p.i. and 76% on day 14 p.i. (Fig. 1a).

When CR constituted 10% of the inoculum, it too increased its proportion of the asexual population over days 10 to 14 p.i., but not as dramatically. By day 14 p.i. CR constituted only 33% of the asexual population (Fig. 1b).

The infections initiated with equal numbers of ER and CR parasites were all dominated by the ER

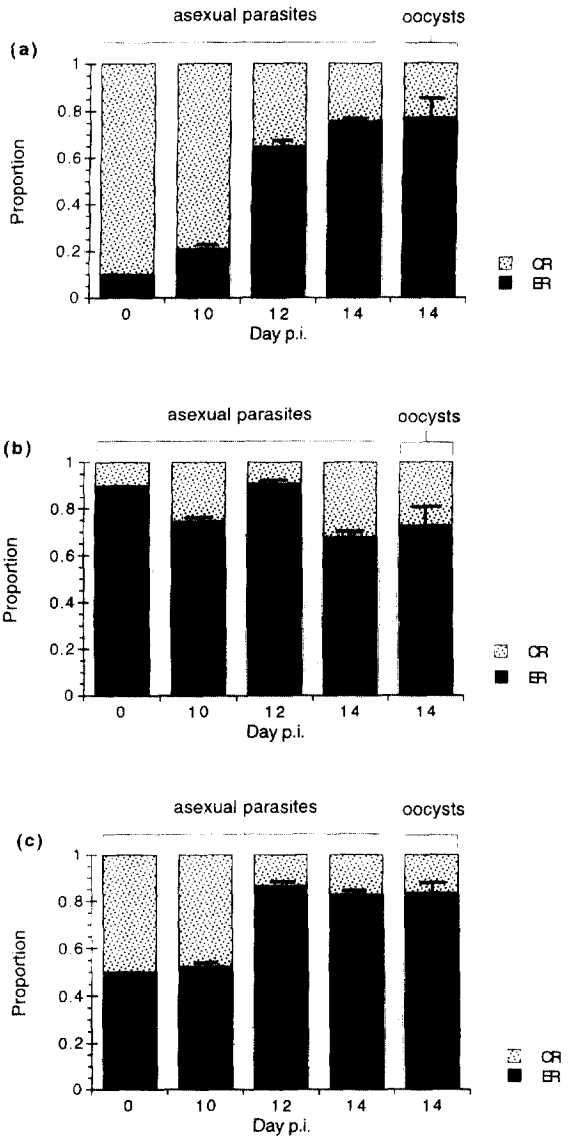

Fig. 1. Clonal composition of asexual and oocyst parasite populations from infections initiated with inocula of 1ER:9CR (a), 9ER:1CR (b) and 5ER:5CR (c). Error bars are 95% confidence limits from models with binomial errors.

clone in the later stages of infection. ER parasites formed 52% of the asexual parasites on day 10 p.i., but this rose to 87% on day 12 p.i. and 83% on day 14 p.i. (Fig. 1c).

3.2. Relative frequencies of the two clones in the oocyst populations

The oocyst populations were dominated by the ER clone for all three treatment groups of mice. Mice given an inocula with 10%, 50% and 90% ER produced transmission populations in which ER contributed 77, 84 and 73% of the oocysts respectively (Fig. 1a, 1b and 1c). These ratios differed only marginally ($\chi^2_1 = 3.52$, $P \approx 0.07$).

In all three treatment groups the clonal composition of the oocyst population conformed to the Hardy–Weinberg equilibrium, consistent with random mating between the two clones ((χ^2_2 3.29, $p > 0.1$ for 10% ER inoculum, χ^2_2 0.09, $P > 0.5$ for 90% ER inoculum, χ^2_2 0.69, $P > 0.5$ for 50% ER inoculum).

3.3. Determinants of the transmission populations

3.3.1. *Gametocytaemia.* Gametocytaemia on day 14 p.i. was highly correlated with the asexual parasitaemia on both day 14 p.i. ($F_{1,17} = 10.45$, $P < 0.01$, Fig. 2), and day 12 p.i. ($F_{1,17} = 6.60$, $P < 0.05$). Day 14 asexual parasitaemia explained significantly more variance in gametocytaemia when added to a model containing day 12 asexual parasitaemia ($F_{1,17} = 7.07$, $P < 0.05$), but day 12 asexual parasitaemia did not explain significantly more variance in gametocytaemia when added to a model containing day 14 asexual parasitaemia ($F_{1,16} = 1.81$, $P > 0.05$).

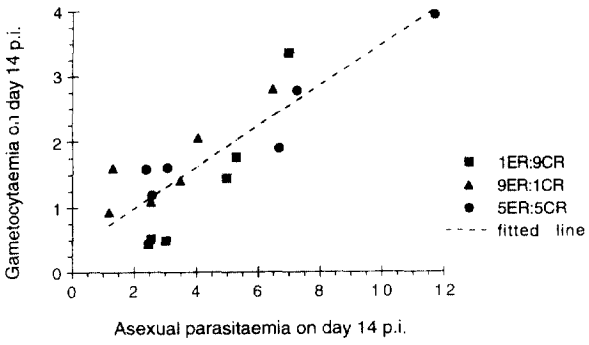

Fig. 2. The relationship between gametocytaemia and asexual parasitaemia on day 14 p.i. of all infections.

3.3.2. *Clonal composition.* Clone ER was dominant in the oocyst populations from all three treatments, even though the clonal composition of the infections in the three treatments was very different four days earlier. There was no evidence that the clonal composition of oocysts in mosquitoes was inversely proportional to the clonal composition in mice (Fig. 3a–c): CR did not transmit at higher frequencies when it was in the minority during the bulk of the infection (Fig. 1b), or in the minority in the days just prior to transmission (Fig. 1a–c). Thus, there was no consistent evidence that suppressed clones increase their relative frequency in transmission populations by increasing rates of gametocytogenesis. In contrast, there is evidence supporting the hypothesis that asexual dynamics alone account for the relative frequency of clones in the oocyst population: the numerical dominance of ER in the oocyst populations best matches its numerical dominance when the mosquitoes were allowed to feed (Fig. 3a–c).

Analysing the data from each infection confirms this qualitative picture. The clonal composition of oocysts (analysed as the proportion of alleles contributed by the ER clone) was not correlated with the clonal composition of the asexual population on four or two days before the feed ($\chi^2_1 = 0.35$, $P > 0.5$, slope = 0.38; $\chi^2_1 = 1.94$, $P > 0.1$, slope = 1.10 respectively). The correlation between the clonal composition of oocysts and the clonal composition of the asexual population on the day of the feed was stronger, though still short of two-tailed significance ($\chi^2_1 = 2.55$, $P \approx 0.1$, slope of correlation = 1.31). This relationship may be weak because of the limited variation in the proportion of ER in the oocysts (no more than expected by chance alone; comparison with binomial expectation, $\chi^2_{12} = 12.71$, $P > 0.2$). At all time points the correlations were positive, so there was no evidence that clones compensated for competitive suppression by increasing the rate of gametocytogenesis.

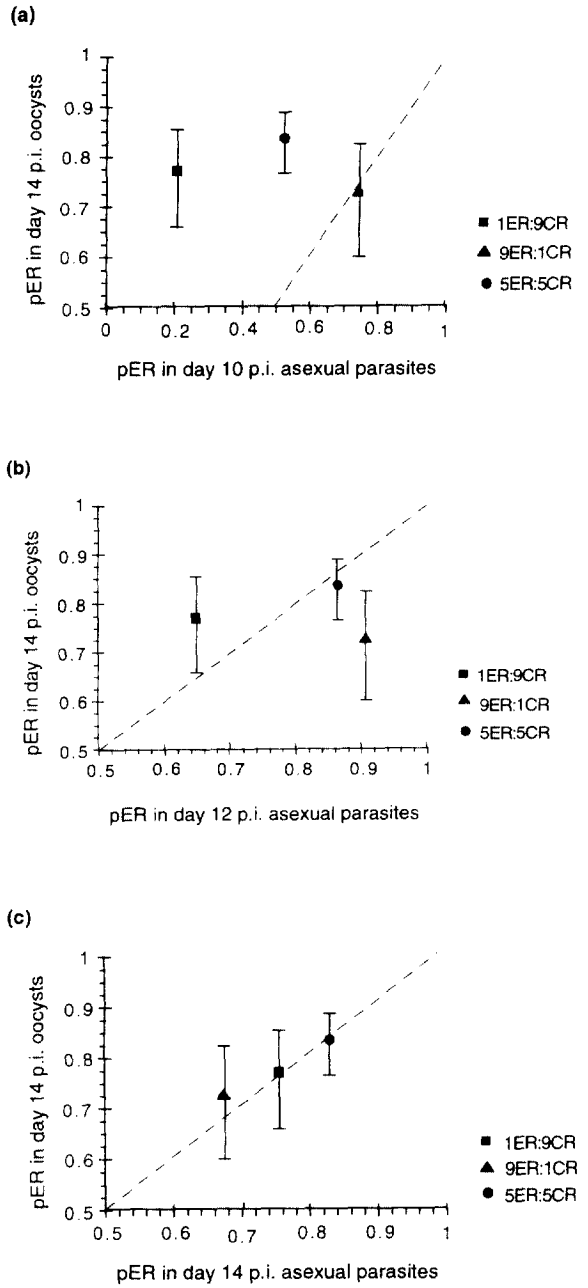

Fig. 3. The relationship between the proportion of oocyst population contributed by the ER clone, and the clonal composition of the asexual infection on day 10 p.i. (a), day 12 p.i. (b) and 14 p.i. (c). Error bars are 95% confidence limits from models with binomial errors. 95% confidence limits for the x-axis are around ± 0.025. The dotted line represents the expected proportion of oocysts contributed by the ER clone if it were determined entirely by the frequency of ER parasites in the asexual infection.

4. Discussion

Asexual parasite populations sampled on day 10 p.i. showed that the relative abundances of the two clones had not altered markedly from those in the inocula. However, between days 10 and 14 p.i. marked changes in the relative frequencies occurred. For all three treatment groups, ER parasites dominated both the asexual infection on the day of the feed and the oocyst populations which developed in mosquitoes. The clonal composition of the day 10 p.i. asexual populations and day 14 p.i. oocyst populations measured in this experiment are quantitatively very similar to those from the previous experiments [3], suggesting that these patterns are robust and repeatable.

The more intensive sampling used in this experiment allows us to distinguish between the two hypotheses raised. If the first were true, so that asexual densities are the primary determinant of clonal transmission success, then the composition of the population in mosquitoes should be predicted by the asexual population around the day of the feed. If the second were true, with competitively suppressed clones allocating more resources to gametocyte production, then the clonal composition of the asexual population around the time of the feed should be different from that of the transmitted population.

Clonal compositions of oocyst populations in mosquitoes were more closely related to those in the asexual populations on the day on which mosquitoes were fed than on days prior to it. We found no consistent evidence that when suppressed, clones compensate by producing more transmission stages. This strongly suggests that differences in the clonal composition of asexual population and the oocysts derived from feeds 4 days later came about because of marked changes in the relative frequencies of the clones towards the end of the infection. Gametocytaemia on day 14 p.i. was strongly correlated to the asexual parasitaemia on the same day. Previous experiments have shown gametocyte density on the day of a feed to be a good predictor of subsequent oocyst burden [2]. Taken together, the most parsimonious interpretation of our data is that no upregulation of gametocyte production to compensate for competitive suppression is occur-

ring in these infections. Instead, the transmission success achieved by a clone depends on its asexual parasitaemia on the day of the feed. Thus, it is most likely that mixed-clone infections are more infectious than single-clone infections because they have larger asexual densities at the point at which transmission stages are produced. This is supported by the positive correlation between transmission and asexual density on the day of the feed observed in the earlier experiments [2].

Gametocytes of *P. chabaudi* are though to take around 2 days to mature [9]. However, gametocytaemia and the clonal composition of the oocyst infection on day 14 p.i. reflect the size and composition of the asexual infection on that day rather than that of 2 days earlier. This could be because immune responses altering the clonal composition of asexual parasites over this timespan affected the newly formed (immature) gametocytes similarly. This may occur if immunity is targeted against antigenically conserved, clone-specific molecules essential for parasite growth. It could also be explained if early in the infection gametocytes were produced by the two clones in the same relative frequency as were asexual parasites, and a separate strain-specific immune response was raised against gametocyte populations in addition to that against asexual parasite populations.

Plasmodium chabaudi infections initiated with 10^6 parasites have peak asexual parasitaemias 7–8 days p.i., with between 25 and 35% of r.b.c. being infected. By day 10 p.i., the majority of parasites have been cleared from the blood stream and parasitaemias are generally less than 10% [2]. Over the first 10 days of infection, the three treatment groups in this experiment showed very different relative abundances of the clones. However, ER parasites formed the majority of the oocyst populations from all three groups, showing that dominance of the main part of the infection does not necessarily lead to greater transmission success. Instead, what matters is numerical dominance of the asexual population during the period of gametocyte production.

When ER parasites started as 10% of the inoculum, they were able to increase their representation to more than 70% of the asexual population by day 14 p.i. When CR parasites started as 10% of the inoculum, they reached only 30% of the asexual population over the same timespan. ER parasites dominated the later stages of infection whether they started as a minority, majority or half of the inoculum and thus appear the superior competitor. Nevertheless, even when ER parasites started as the majority of the inoculum they were not able to completely eliminate CR parasites from the infection.

What constitutes competitive ability in mixed infections is unclear. ER parasites may produce more copies of themselves each 24-h replication cycle, may survive better when nutrients are limiting, may stimulate a weaker immune response against themselves, or may be better able to withstand an immune response once mounted. However, as major changes in the clonal composition of the asexual infection occurred only after the main peak of infection had ended, it seems unlikely that growth advantages could account for the relative increase in ER asexual parasitaemia. Instead, differences between the clones in their interactions with the host immune system might explain the results more easily. Previous experiments with *P. chabaudi* infections in mice have shown that clones vary in the amounts of clone-specific and cross-reactive immunity that they induce [10] and immunity to different clones can lead to different alterations to the course of the infection [11]. One explanation of our results is that host immunity raised to an infection dominated by ER parasites adversely affected CR parasites, but immunity raised to an infection dominated by CR parasites affected ER parasites to a lesser extent. Experiments applying clone-specific antibodies to mixed infections may shed further light on the complex interactions between hosts and mixed parasite populations.

Interactions with the host immune system may therefore be an important determinant of competitive ability in mixed-clone infections. Strain-specific immunity has been experimentally demonstrated for *Plasmodium falciparum* and *Plasmodium vivax* [12–16] and recent genetic studies in the field suggest that it has an important role in the epidemiology of human malaria parasites [17]. It is possible that interactions with the host immune system are more important than more conventional indicators of competitive ability such as growth

rate or resource utilisation in shaping patterns of malaria parasite transmission and evolution.

Acknowledgements

This work was funded by a BBSRC Advanced Research Fellowship to AR. We are grateful to D. Walliker for use of his insectaries and Angus Buckling for helpful comments on earlier drafts of the manuscript.

References

[1] Day KP, Koella JC, Nee S, Gupta S, Read AF. Population genetics and dynamics of *Plasmodium falciparum*: an ecological view. Parasitology 1992;104:S35–S52.

[2] Taylor LH, Walliker D, Read AF. Mixed-genotype infections of the rodent malaria *Plasmodium chabaudi* are more infectious to mosquitoes than single-genotype infections. Parasitology 1997;115:121–132.

[3] Taylor LH, Walliker D, Read AF. Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones. Proc R Soc Lond B 1997;264:927–935.

[4] Carter R, Graves PM. Gametocytes. In: Wernsdorfer WH, McGregor I, editors. Malaria. Principles and practice of malariology. Edinburgh: Churchill Livingstone, 1988;253–305.

[5] Buckling AGL, Taylor LH, Carlton JM-R, Read AF. Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. Proc R Soc Lond B 1997;264:553–559.

[6] Stearns SC. The evolution of life history strategies. Oxford: Oxford University Press, 1992.

[7] Wery M. Studies on sporogony of rodent malaria parasites. Ann Soc Belge Med Trop 1968;48:1–138.

[8] Crawley M. GLIM for Ecologists. Oxford: Blackwell Scientific, 1993.

[9] Gautret P, Miltgen F, Gantier J-C, Chabaud AG, Landau I. Enhanced gametocyte formation by *Plasmodium chabaudi* in immature erythrocytes: patterns of production and infectivity to mosquitoes. J Parasitol 1997;82:900–906.

[10] Jarra W, Hills LA, March JC, Brown KN. Protective immunity to malaria: studies with cloned lines of *Plasmodium chabaudi chabaudi* and *P. berghei* in CBA/Ca mice. II. The effectiveness and inter- and intra-species specificity of the passive transfer of immunity with serum. Parasite Immunol 1986;8:239–254.

[11] Jarra W, Brown KN. Protective immunity to malaria: studies with cloned lines of *Plasmodium chabaudi* and *P. berghei* in CBA/Ca mice. I. The effectiveness and inter- and intra-species specificity of immunity induced by infection. Parasite Immunol 1985;7:595–606.

[12] Boyd MF. Criteria of immunity and susceptibility in naturally induced *vivax* malaria infections. Am J Trop Med Hyg 1942;22:217–226.

[13] Jeffery GM. Epidemiological significance of repeated infections with homologous and heterologous strains of *Plasmodium*. Bull WHO 1966;35:873–882.

[14] Sadun EH, Hickman RL, Wellde BT, Moon AP, Udeozo IOK. Active and passive immunisation of chimpanzees infected with West African and Southeast Asian strains of *Plasmodium falciparum*. Mil Med 1966;131:1250–1262.

[15] Cadigan FC, Chaicumpa V. *Plasmodium falciparum* in the white-handed gibbon: protection afforded by previous infection with homologous and heterologous strains obtained in Thailand. Mil Med 1969;134:1135–1139.

[16] Taliaferro WH. Immunity to the malaria infections. In: Boyd MF, editor. Malariology. Philadelphia and London: WB Saunders, 1949;935–965.

[17] Mercereau-Puijalon O. Revisiting host/parasite interactions: molecular analysis of parasites collected during longitudinal and cross-sectional surveys in humans. Parasite Immunol 1996;18:173–180.