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S. Chatterjee · E. Ngonseu · P. Druilhe · E. Van Marck

Improved quantification of *Plasmodium* exoerythrocytic forms in rodents

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Abstract The result of a *Plasmodium* sporozoite challenge is currently evaluated either by detecting the emergence or not of parasites in the blood, or by estimating the 'prepatent period', which is the time between sporozoite inoculation and the appearance of parasites in the blood. This type of measurement is relatively rough and has given way to another method of measuring sporozoite infectivity, which is to enumerate the exoerythrocytic forms (EEF) by microscopic examination of liver sections. Up until now, two different methods have been proposed to calculate and estimate the number of *Plasmodium* EEF forms in the livers of infected rodents, both of which are unfortunately biased to some extent. Here, we propose a different method of calculation, which more faithfully reflects the EEF number in the liver. This method is based on the calculated mean number of consecutive liver sections in which a schizont appears, and is host related.

Introduction

The development from partially to fully effective anti-sporozoite vaccines could be enhanced if their assessment could be improved by the use of quantitative instead of qualitative assays. Malaria transmission to vertebrates occurs when a feeding female *Anopheles* mosquito injects sporozoites into the host. Exoerythrocytic forms (EEF), or liver stages, of the parasite follow the sporozoites' entry into hepatocytes. The number of EEF influences the duration of the prepatent period of the parasitic infection, the duration and severity of subsequent clinical attacks, and depends on the effect of subunit vaccines in immunised hosts. The irradiated sporozoite vaccine, which induces full protection, has been influencing the read-out of further subunit vaccines, which were not fully protective. The read-out remains qualitative, i.e. only the emergence or not of blood forms is determined. In this case, even a strongly effective vaccine, reducing the liver form load by 95–99%, is considered non-effective, thereby preventing any improvement in subunit vaccines, which cannot be expected to be 100% effective. For these reasons, many studies now use methods aimed at estimating the number of EEF in the challenged liver. These estimations generally rely on two methods of calculation: the first developed by Garnham and Bray (as given in Wéry 1966; Vanderberg 1977; Khan and Vanderberg 1991), and the second developed by Scheller et al. 1994.

In the first method, the number of EEF in the whole liver was estimated as follows: density of EEF in the liver = (mean number of EEF per section)/(mean EEF diameter)×(mean section surface area).

The total number of EEF per liver is calculated by multiplying the density of EEF so obtained by the total liver volume previously estimated by volumetric displacement. According to Garnham and Bray, working with 4-μm-thick liver sections, a schizont measuring for instance 30 μm in diameter would appear in seven or eight consecutive sections. This approach holds true on the sole condition that we accept that all of the liver stage schizonts have a spherical shape. This is, however, not the case, as observed in EEF slice pictures (Yoeli and Most 1965). By calculating "(mean EEF diameter)×(mean surface area section)" in the calculation of the EEF density, these authors disregard the fact that EEF are non-homogeneous 3-dimensional structures. The measures done on EEF slices (diameter, length or width) under the microscope are in two dimensions

S. Chatterjee (✉) · E. Van Marck
Pathology Unit, Department of Medicine, University of Antwerp, Campus Drie Eiken, Antwerp, Belgium
E-mail: shyama.Chatterjee@ua.ac.be
Tel.: +32-3-8202546
Fax: +32-3-8202532

E. Ngonseu
Department of Parasitology, Institute of Tropical Medicine, Nationalestraat-155, 2000 Antwerp, Belgium

P. Druilhe
Biomedical Parasitology, Institut Pasteur, 25 Rue du Dr. Roux, 75015 Paris, France

which are different from the third one involving the EEF slice thickness. In their pioneer work on EEF of *Plasmodium berghei*, Yoeli and Most (1965) observed 65 EEF in 304 consecutive sections, and observed that two schizonts were rarely found in the same section. Taking this together, we conclude that the average number of sections in which a schizont appears is approximately 4.5. Moreover, the average size of 18 of these schizonts, chosen randomly, was 39–43 μm. Based on the Garnham and Bray method, the average number of sections in which a schizont appears should be at least ten. This value is not the observed result (4.5 after the work of Yoeli and Most and 4.47 according to our own calculations). Based on this, Garnham and Bray's method leads to an underestimate of EEF in the liver.

In the second method proposed by Scheller et al. (1994), the number of EEF in the whole liver was calculated by dividing the volume of mouse liver by the volume of a liver section. The resultant value was multiplied by the mean number of EE stages in 100 cm^2, to give a total number of EE stages per liver: (volume of liver/0.0004 cm)×(mean EE/100 cm^2).

To avoid counting the same EEF more than once in sequential liver sections, Scheller and co-workers stained and examined one every tenth section. By doing this, the distance between two consecutively examined sections was thus 40 μm, the diameter of a mature *Plasmodium* schizont used in their study, according to the authors.

According tothe literature and also our experience, the mean size of *P. berghei* schizont slices in mice is 28.8 μm (Wéry 1966); 23.2–32.5 μm (Vanderberg et al. 1968); 30 μm (Zechini et al. 1999) and 29.0–24.6 μm (Ngonseu et al. 1998).

From these observations, it is clear that the schizont size is less than 40 μm and, thus, a schizont could not appear in ten consecutive sections. Moreover, what some authors consider as the diameter of an EEF slice (or whole EEF) cannot be considered as the schizont thickness. Since a given schizont does not appear in ten consecutive sections but only in from two to four, Scheller et al.'s calculation also leads to an underestimate of EEF numbers in the liver.

Given this background, we propose a novel calculation method to estimate the EEF number in the liver, and the subsequent re-evaluation of the percentage of infective sporozoites, following the inoculation of a known number of sporozoites.

Materials and methods

Parasites

The *P. berghei* ANKA strain, isolated by Bafort in 1965 (Vincke et al. 1966) was used in this study. *P. berghei* ANKA sporozoites were dissected from the salivary glands of *Anopheles stephensi* mosquitoes 21 days after the infective blood meal. The ANKA strain is maintained by cyclical transmission in OF1 mice and *A. stephensi* mosquitoes, in an insectarium, as previously reported (Ngonseu et al. 1998).

Invertebrate host and parasite maintenance and sporozoite production

Breeding of the malaria invertebrate host *A. stephensi* and production of sporozoites were performed as previously reported (Chatterjee et al. 1996).

Grammomys surdaster and C57BL/6 mouse hosts

G. surdaster (Chatterjee et al. 2001), also called the African tree rat, is the natural host of *P. berghei*. The animals used in this study came from a colony established in our laboratory in late 1995 from 60 animals trapped in an area of 5 km around Kaindu. This village is located 20 km north-west of Mumbwa (Zambia), near the Kafua National Park (location 14°41′S 26°35′E; altitude 1,130 m; mean annual temperature 19.1°C). Confirmation that the animals belonged to the species *G. surdaster* was based on examination of their mitochondrial DNA by Prof. Verheyen at the Department of Animal Ecology of the University of Antwerp.

Female 6-week-old C57BL6 mice (IFFA Credo, Brussels) were also used.

Liver dissection and detection of liver stages

All of the rodents (four *Grammomys* and two C57BL/6 mice) were inoculated intravenously with 500,000 sporozoites each and were killed at 48 h post-inoculation. The large sporozoite inoculum was used to facilitate tracing back the EEF slices in the liver sections. The sporozoites were from three batches: A, B, C (Table 1). After necropsy, the liver volume of each rodent was estimated by volumetric displacement, and then the liver from each *Grammomys* was cut into 9–12 pieces, while those from mice were each cut into six pieces. All of the pieces were fixed in Carnoy's solution, embedded in paraffin, and serial sections (4 μm thick) were made. The presence of *P. berghei* hepatic stages in liver sections was determined using the Giemsa collophonium staining technique as described by Wéry (1966).

Evaluation of the number of liver forms

The evaluation of EEF number in the liver was estimated by the methods of Garnham and Bray (see Wéry 1966; Vanderberg 1977; Khan and Vanderberg 1991), Scheller (1994) and by a modification described below:

1. Approximately 200 consecutive sections (4 μm thick) for each *Grammomys* and 120 for each C57BL6 mouse liver were examined. An EEF slice count was then made from each section. The result of this count was expressed as number of EE slices/cm^2.
2. A number of schizonts (34 for the *Grammomys* and 27 for a C57BL6 mouse) were followed in consecutive sections, the number of sections in which each schizont appeared was recorded and the mean number of sections in which a schizont appeared was calculated (n). From this number, the thickness of a schizont (ϵ) can be obtained by multiplying n by the section thickness: 4 μm or 0.0004 cm ($\epsilon = n \times 0.0004$ cm).
3. From the results obtained in step 1, it is easy to estimate the number of 4 μm thick EEF slices in the whole liver (N): N = (EEF slices/cm^2)×(liver volume cm^3/0.0004 cm).
4. The number of schizonts in the whole liver (EEF number) was estimated by dividing the number of schizont slices in the whole liver (N) by the mean number of consecutive sections in which a schizont appears (n): EEF number = N/n.
5. From the result obtain in step 4, it is easy to estimate the percentage of injected sporozoites transformed into schizonts (P): P = (EEF number×100)/5.10^5.

Table 1 The percentage of injected *Plasmodium berghei* sporozoites that transformed into schizonts 48 h after sporozoite injection in *Grammomys surdaster* and C57BL/6 mice. Each rodent was inoculated with 5×10^5 sporozoites and killed 48 h post inoculation. Exoerythrocytic forms (EEF) slices in whole liver indicates the (EEF slices/area)×(volume of liver/0.0004). EEF in the whole liver indicates (EEF slices in whole liver/mean number of consecutive sections in which a schizont appears). % of sporozoites transformed to EEF indicates (EEF in whole liver×100)/number on injected sporozoites. The mean number of consecutive sections in which a schizont appears in *G. surdaster* and C57BL/6 mouse liver sections was 4.47 and 3.43, respectively

Animal	Sporozoite batch	Section screened			EEF slices in whole liver	EEF in the whole liver	% of sporozoites transformed into EEF
		Area (cm^2)	EEF slices	EEF slices/cm^2			
Grammomys 1	A	14.03	2,737	195.08	1,463,115	327,275	65
Grammomys 2	A	18.62	564	30.29	212,030	47,428	9.5
Grammomys 3	B	18.42	1,016	55.16	413,681	95,534	18.5
Grammomys 4	C	21.79	331	15.19	113,929	25,484	5.1
C57BL/6 1	B	11.44	111	9.70	26,683	7,783	1.6
C57BL/6 2	C	11.03	74	6.71	16,772	4,892	1

To summarise, the number of EEF in the liver following sporozoite inoculation can be obtained from the formula: EEF number $= N/n = [(\text{EEF slices/cm}^2)\times(\text{liver volume cm}^3/0.0004\text{ cm})]/n$.

The schizont dimensions were obtained by following each schizont in consecutive sections in which the size of the largest schizont slice was measured.

Results

The estimated number of schizont slices and schizonts in the whole liver, and the percentage of injected sporozoites that transform into mature schizonts, are presented in Table 1. Since sporozoite infectivity varies from one batch to another, we have indicated the batch of sporozoites used to infect each rodent. The liver volume, estimated by volumetric displacement, was as follows: *Grammomys* numbers 1, 3 and 4: 3.0 cm^3 each; *Grammomys* number 2: 2.8 cm^3; C57BL/6 mice 1 and 2: 1.1 and 1.0 cm^3, respectively. The mean number of consecutive sections in which a schizont appears in *G. surdaster* and C57BL6 mouse liver sections was 4.47 and 3.43, respectively.

The numbers of EEF in the whole liver estimated following the method described by Garnham and Bray, and by that of Scheller et al. as well as by our method, and the resulting percentage of sporozoites transformed into EEF are given in Table 2. Following the Garnham and Bray method, we considered the mean of the largest dimension as the diameter. Following the Scheller et al. method, only schizont slices in every tenth section were taken into account.

The mean size resulting from the compilation of the dimensions of 25 schizonts obtained in each rodent species and the mean number of liver sections in which a schizont was detected are given in Table 3. The mean sizes of the schizonts were 28.7–34.8 μm and 24.6–29.0 μm in *G. surdaster* and C57BL/6 mice, respectively. After a number of schizonts had been followed in consecutive sections (34 schizonts for the *Grammomys* and 27 for C57BL/6 mice) the mean consecutive sections in which a schizont appears were 4.47 for *G. surdaster* and 3.43 for the mice.

Knowing the mean number of consecutive sections in which a schizont appears, the thickness of EEF can be estimated to be approximately 18 μm and 14 μm in *G. surdaster* and the mice, respectively.

Discussion

The main objective of this study was to provide a more realistic method of calculation for the estimation of EEF number in the liver, since the methods used so far lead to assumptions that are debatable.

In the first method, developed by Garnham and Bray, the calculation would be correct only if the schizonts are

Table 2 Numbers of EEF in each rodent liver. According to Garnham and Bray, and Scheller et al. (1994) and our calculation methods, each rodent had been inoculated with 5×10^5 sporozoites. Garnham and Bray: The total number of EEF per liver was calculated by multiplying the density of EEF by the total liver volume. Density of EEF = mean number of EEF per square unit/(mean EEF diameter)×(square unit). Since it appears that schizont slices were not present, we considered the mean of the largest dimension as the diameter. Scheller et al.: total number of EEF per liver = (volume of liver/0.0004 cm)×mean EEF per square unit. Only schizont slices in every 10th section are taken into account

Animal	No. of EEF in each rodent liver			% of sporozoites transformed into EEF		
	Garnham and Bray	Scheller et al.	This paper	Garnham and Bray	Scheller et al.	This paper
Grammomys 1	168,130	146,310	327,275	33.62	29.26	65
Grammomys 2	24,372	21,203	47,428	4.87	4.24	9.5
Grammomys 3	47,552	41,370	95,534	9.5	8.27	18.5
Grammomys 4	13,093	11,393	25,484	2.6	2.28	5.1
C57BL/6 1	3,680	2,668	7,783	0.74	0.53	1.6
C57BL/6 2	2,314	1,678	4,892	0.46	0.34	1

Table 3 Size of 48 h old *P. berghei* schizonts in *G. surdaster* and C56BL/6 mouse liver sections, and number of consecutive sections in which a schizont appears. The mean number of consecutive sections in which a schizont appeared was 4.47 for *Grammomys* ($n = 34$ schizonts), and 3.43 for C57BL/6 ($n = 27$ schizonts)

Rodent species/strain	EEF average size (μm)	Number of liver sections	
		Mean	Range
G. surdaster	34.8/28.7	4.47	3–6
C57BL/6 mice	29.0/24.6	3.43	2–4

spherical, which is not the case as observed in EEF slice micrographs (Yoeli and Most 1965). This method, as well as the second method proposed by Scheller et al. (1994), leads to an underestimate of EEF numbers in the liver.

A summary of the problems associated with different EEF quantification methods is presented in Table 4.

Our results stress that the EEF diameter is host species related. This observation is in keeping with that reported in a previous study (Vanderberg et al. 1968). Moreover, our results also suggest that the number of consecutive sections in which a schizont appears can be host species related. The most likely explanation is that not only the diameter but also the thickness of a schizont is dependent on the host. This leads to the conclusion that the mean number of sections in which a schizont appears should be calculated for each given host, to reduce error due to approximation.

In cut sections of malaria-infected liver, the mean diameter of unpolarised parasites in the horizontal plane of the section is often different from the mean diameter in the vertical. This has been shown in EEF slice micrographs (Yoeli and Most 1965), and was also evident in our work. We should, however, not ignore the fact that the tissues have been processed and could undergo some changes in size as compared to the in vivo situation. In effect, we present a different rationale for examining fixed and stained liver slices for the presence of EEF schizonts following experimental infection. This approach is more laborious since a better estimation of the number of EEF schizonts involves counting more slices. Preparing a whole rodent liver would lead to examining a surface area that is impractical (7,500 cm^2 for *Grammomys* and 2,500 cm^2 for C57BL6 mouse), so that usually less than 100 cm^2 is screened. An approximate value used in the calculation should be as close as possible to the real value, since it is multiplied manyfold. The screening of 100 cm^2 of liver sections is already a tremendous and time-consuming job.

Our improved method for the quantification of *P. berghei* liver stages may reflect more closely the absolute numbers of EEFs per infected liver. Our method may be criticised as not being suitable in sporozoite challenge experiments, due to its tediousness and time-consuming nature, as well as the requirement for well-trained experts to carry it out. In this context, the efficiency (in terms of time control) of the RT-PCR method needs to be highlighted. Many consider real-time RT-PCR of liver-stage specific transcripts to be the gold standard for *Plasmodium* sporozoite challenge and cell biological experiments (Bruna-Romero et al. 2001). We believe that, although modern molecular biological tools can be used to reach a more precise estimate of the total number of dividing parasites in the whole liver, thereby giving a more precise assessment, this has yet to be carefully evaluated in comparison with microscopic methods. In our opinion microscopy gives an actual visualisation of the parasite and, moreover, it can distinguish mature parasites from others that are blocked in their division. This latter point could lead to bias while using RT-PCR.

In this context we favor the use of our technique. It is completely based on microscopy as are the other previously used methods. Although we agree that only a few researchers may actually follow our new method, we think it suitable for documenting EEF number. Although our method is time consuming, we believe that it is more efficient than the methods used by Scheller et al. and Garnham and Bray.

In conclusion, given the inherent variability of biological systems and the crudeness of existing methods (only 1/250th-1/500th of the liver screened), our results suggest that the methods employed up to now have substantial deficits and show that improved ones can be and need to be designed and evaluated.

Table 4 An overview of the methods used to extrapolate EEF counted in slices to the totel number of EEF in the whole liver

Methods	EEF slices to EEF	Problems	Conclusions
Garnham and Bray	EEF slices/mean EEF diameter (ø)	The measurements done on EEF slices under the microscope are different from the EEF slices thickness EEF is not a spherical structure	Since EEF ø is usually less than the EEF thickness, this method lead to an underestimation of the number of EEF
Scheller et al. 1994	Examine and count EEF slices in every 10th section. The EEF slices counted are then likened to whole EEF	By counting EEF slices in every 10th section and liken these slices to whole EEF assumes that EEF appears in ten consecutive sections	Knowing that EEF appears in less than ten consecutive sections, this method leads to an underestimation of EEF.
This paper	EEF slices/mean number of sections in which a schizont appears.		

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