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## Measurement by membrane feeding of reduction in *Plasmodium falciparum* transmission induced by endemic sera

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### Abstract

The standard laboratory test for reduction in malaria transmission is based on the measurement of oocyst numbers in mosquitoes fed on blood meals containing test and control sera. Interpretation of the results, however, is often hampered by the large variation in numbers of infected mosquitoes and oocysts. The objective of this study was to compare 3 measures for the assessment of transmission reduction (so-called R values) and to define the experimental criteria that allow interpretation of the results. To determine variability in R values of control sera, a replicate experiment was performed with 10 non-endemic sera of Dutch blood donors. Furthermore, 2 measures for calculation of transmission reduction were compared in a triplicate experiment using *Plasmodium falciparum*, *Anopheles gambiae* and malaria endemic sera. Calculations using the geometric mean of Williams are currently used to identify blocking and non-blocking sera. However, calculations using log-transformed data could distinguish more gradual levels of transmission reduction activity by endemic sera—i.e. blocking, reducing and non-blocking activity. Grading of transmission reduction activity is important for epidemiological studies on transmission immunity and for validation of future transmission-blocking vaccines.

**Keywords:** malaria, *Plasmodium falciparum*, *Anopheles gambiae*, transmission reduction by serum, measurement

### Introduction

Malaria transmission reduction assays are performed by feeding *Anopheles* mosquitoes on cultured fertile gametocytes of *Plasmodium falciparum*, using membrane feeders (PONNUDURAI *et al.*, 1987). Reproducible measurement of transmission reduction requires a reliable source of infectious gametocytes and mosquitoes of standard quality (PONNUDURAI *et al.*, 1989). In the assay, test sera are included in the infectious feed and transmission levels are expressed as oocyst numbers in the mosquito. A reduction in the oocyst number can be measured by a so-called R value, which is obtained by comparing the oocyst level resulting from use of a test serum (T) with that obtained with a control serum ( $T_c$ ):

$$R = \frac{T_c - T}{T_c}$$

For evaluation of transmission reduction, the R value then assumes a value between nought (no reduction,  $T=T_c$ ) and one (maximal reduction,  $T=0$ ).

There are a number of ways to assess the oocyst numbers as follows.

(i) The arithmetic mean:

$$\bar{x} = \sum_{i=1}^n x_i / n$$

(ii) The geometric mean of Williams:

$$\bar{x}_g = \sqrt[n]{\prod_{i=1}^n (x_i + 1)} - 1$$

(iii) The arithmetic mean on a logarithmic scale:

$$\bar{y} = \sum_{i=1}^n \frac{y_i}{n}$$

In all these equations,  $x_i$  is the number of oocysts in mosquito  $i$ ,  $n$  is the number of mosquitoes in the assay, and  $y = \log_n (x_i + 1)$ . Each method leads to a different R value for measuring transmission reduction.

Williams's geometric mean is generally used to accommodate the relatively great influence on the mean of individual high oocyst counts (ICHIMORI *et al.*, 1990). In this study we investigated which type of R value is most suitable for expressing transmission reduction.

### Materials and Methods

#### Study design

The study was based on the data from a replicate experiment using 10 non-endemic sera obtained from Dutch blood donors without malaria experience as test sera. For the determination of the R values of each serum, a pool of the 10 sera was used as the control. The transmission capacity of each serum was measured twice in the bioassay. R values were calculated using, respectively, the arithmetic mean, the geometric mean, and the arithmetic mean on a  $\log_n$  scale.

#### Cultures

Gametocytes (*P. falciparum*, strain NF54) were routinely produced in the semi-automated 'tipper' system (PONNUDURAI *et al.*, 1982). Gametocytes were harvested for the transmission test after 14 d in culture.

#### Feeding procedure

All serum samples were initially inactivated by heating at 56°C for 30 min. In order to restore complement activity, 40  $\mu$ L of complement-rich serum were added to 80  $\mu$ L of each serum. The culture material from one 'tipper' vessel, containing about 0.3 mL packed cells with a gametocytaemia of 0.5–1%, was centrifuged at 500g for 2 min. The supernatant was removed and the pellet, containing parasitized red blood cells (RBC), was carefully mixed with 3.75 mL freshly washed and prewarmed group O RBC. Meanwhile, 120  $\mu$ L of test or control serum were warmed to 37°C in Eppendorf tubes and 150  $\mu$ L of the parasitized RBC suspension were quickly added to each serum sample. The mixture (270  $\mu$ L) was placed in a prewarmed (37°C) 'minifeeder' (PONNUDURAI *et al.*, 1989), on which 50 female *A. gambiae* (3–5 d old) were allowed to feed for 15 min. All unfed and partially fed mosquitoes were removed, and the fully fed ones kept at 26°C and fed on 5% glucose. Twenty mosquitoes per feeder were examined for oocysts after 6 d.

### Results

For standardization purposes we studied the inter- and intra-assay variations using sera from individuals with no previous malaria experience.

The arithmetic means and standard deviations (SDs of oocyst distributions in the duplicate experiment are shown in Fig. 1A. The SDs were all greater than half of the mean, strongly suggesting that the distributions are skewed. Oocyst numbers did not differ significantly between the 10 sera in either measurement (Kruskal–Wallis test:  $P=0.11$  and  $P=0.46$ ). Comparison of oocyst dis-

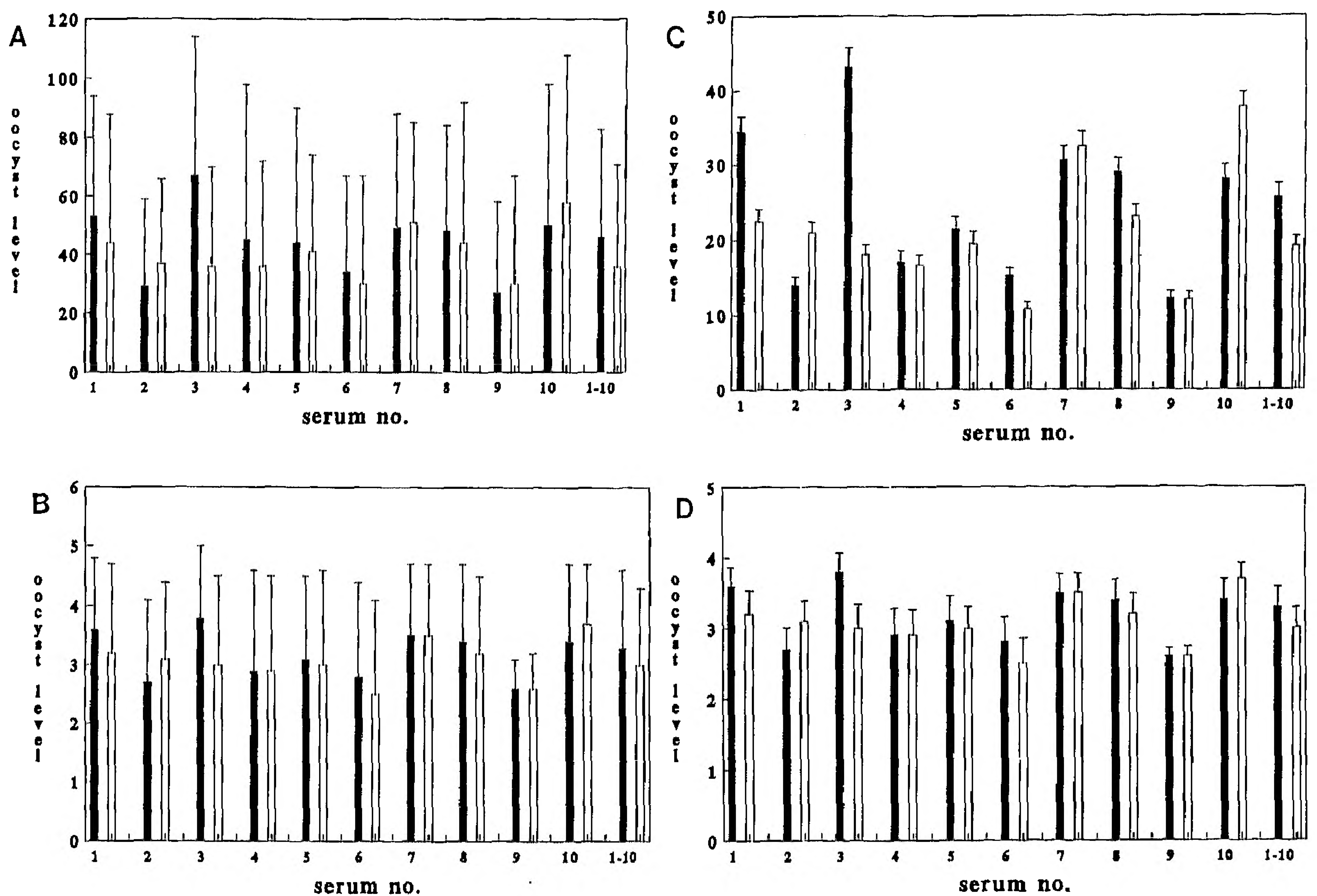


Fig. 1. Numbers of oocysts of *Plasmodium falciparum* developing in *Anopheles gambiae* when the infecting blood meal was suspended in one of 10 non-endemic sera and a pool of these sera (1-10) to serve as a control. The bars represent means of duplicate experiments plus one standard deviation (A, B) or one standard error (C, D). A, arithmetic mean; B, arithmetic mean on a logarithmic scale; C, Williams's geometric mean; D, arithmetic mean on a logarithmic scale. Solid bars indicate the first determination, open bars the second.

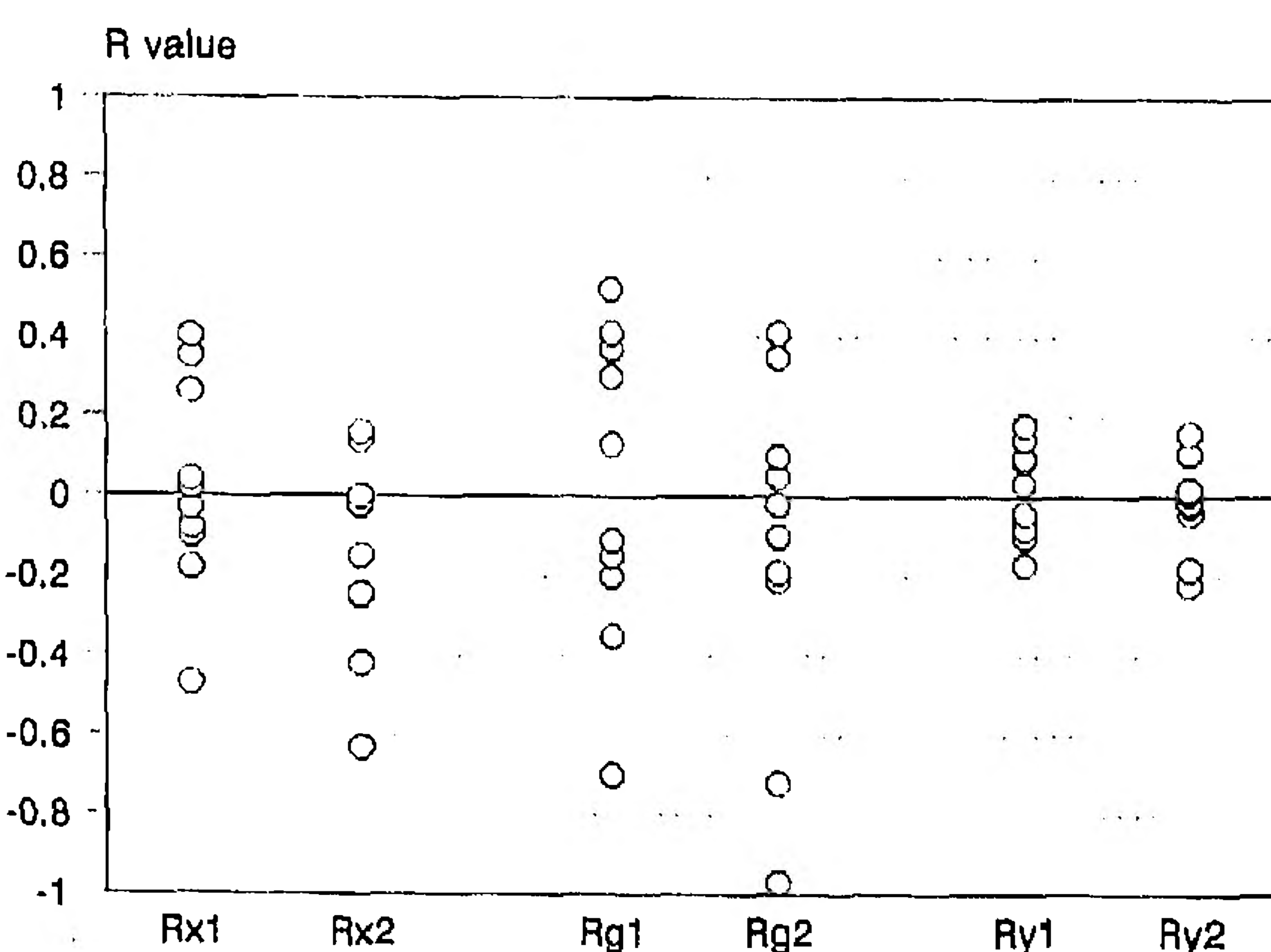


Fig. 2. Distribution of R values (ability to reduce transmission of malaria) of 10 non-endemic sera in a duplicate experiment:  $R_x$  is based on the arithmetic mean  $\bar{x}$ ,  $R_g$  is based on the geometric mean  $\bar{x}_g$ , and  $R_y$  is based on the arithmetic mean ( $y$ ) on a logarithmic scale.  $R_{x1}$ ,  $R_{g1}$  and  $R_{y1}$  refer to the first measurement,  $R_{x2}$ ,  $R_{g2}$  and  $R_{y2}$  to the second measurement.

tributions between measurements 1 and 2 for each serum showed that they differed only for serum 3 (Wilcoxon test:  $P=0.03$ ). Logarithmic transformation is a well accepted method to reduce skewness and reduce variances. The means and SDs of the transformed data  $y_i = \log_n(x_i + 1)$ , where  $x_i$  is the number of oocysts found in mosquito  $i$  ( $i=1, \dots, 20$ ), are shown in Fig. 1B. Using the log scale, the arithmetic means of the distributions associated with the pooled sera in experiments 1 and 2 were respectively 3.3 and 3.0 and the SDs were both 1.3. These SDs are less than half the associated means, indicating that the logarithmic transformation has substantially improved the skewness of the oocyst distributions.

The geometric (Williams's) mean and the arithmetic mean on a log scale of the oocyst numbers and their standard errors are shown in Fig. 1C and D. The geometric mean accommodates skewed distributions and has been recommended for the calculation of transmission blocking capacity (PONNUDURAI *et al.*, 1989; ICHIMORI *et al.*, 1990). However, it did not reduce the variation in oocyst numbers (Fig. 1C). The variation was smallest with the arithmetic mean on a log scale (Fig. 1D). The R values for each of the 3 transmission reduction measures are compared in Fig. 2, which clearly shows the relative small distribution of R values obtained when using the log scale. The geometric mean showed the largest variation.

The standard deviations of the R values for non-endemic control sera measured on the log scale were stable ( $SD=0.12$  in both measurements). Therefore we may construct a reference interval for this type of R value. The critical one-sided R values associated with a 0.99 reference interval (normal approximation on a log scale) are respectively 0.32 and 0.27. This means that  $R_y$  values of test sera above 0.30 indicate a transmission capacity lower than that of non-endemic sera; this contrasts with the R values based on the geometric mean, values of which up to 0.90 may be obtained with the non-endemic sera.

R values based on the geometric means and the means on a log scale were compared in triplicate experiments using endemic sera (Table 1). In the 3 experiments, different batches of mosquitoes and parasites were used. The blocking sera (A, B and C) were clearly identified in each experiment using either calculation method. Using the geometric mean all sera, apart from A, B and C, would be regarded as 'non-blockers'. However, since the log scale suggested a cut-off value for transmission reduction of 0.30, sera D and E should be classified as 'reducers' and sera F and G as 'non-blockers'.

**Table.** Comparison of two measures of *Plasmodium falciparum* transmission reduction, based on Williams's geometric mean and the arithmetic mean measured on a logarithmic scale, in a triplicate experiment using 7 endemic sera

Serum	Experiment 1 <sup>a</sup>			Experiment 2 <sup>a</sup>			Experiment 3 <sup>a</sup>		
	Inf%	R <sub>g</sub>	R <sub>y</sub>	Inf%	R <sub>g</sub>	R <sub>y</sub>	Inf%	R <sub>g</sub>	R <sub>y</sub>
A	0	1.0	1.0	0	1.0	1.0	0	1.0	1.0
B	0	1.0	1.0	0	1.0	1.0	0	1.0	1.0
C	5	0.99	0.97	0	1.0	1.0	0	1.0	1.0
D	26	0.92	0.84	95	0.85	0.45	90	0.83	0.46
E	53	0.74	0.57	95	0.61	0.33	85	0.88	0.54
F	70	0.49	0.30	95	0.63	0.24	100	-0.21	-0.01
G	80	0.29	0.15	95	0.59	0.21	100	0.13	0.03

<sup>a</sup>R<sub>g</sub>=R value based on the geometric (Williams's) mean; R<sub>y</sub>=R value based on the arithmetic mean on a logarithmic scale; Inf%=percentage of *A. gambiae* infected with *P. falciparum*.

## Discussion

There are 2 ways to assess the transmission blocking capacity of sera from the field by membrane feeding. One possibility is to perform the bioassay as described, using cultured gametocytes and a standardized mosquito colony. The other possibility is to feed whole blood from gametocyte carriers by membrane feeding but with serum replacement as described by MULDER *et al.* (1994). The advantage of the latter method is that transmission reduction is measured under more natural conditions. The complication, however, is that, due to the relatively low oocyst levels and therefore low mosquito infections, one has to use sample sizes of at least 50 to 100 mosquitoes (MEDLEY *et al.*, 1993). This makes testing of sera in epidemiological studies extremely difficult. Moreover, the intrinsic capacity of gametocytes to infect mosquitoes also influences infectivity and may depend on factors such as numbers, maturity, age, viability, the use of drugs, or even the genetic background of the parasite strain. Finally the choice of control serum used as replacement, as shown in our work, influences the final outcome of the test. Therefore, in order to obtain a better standardized control serum, 10 sera were pooled. From this pool of non-endemic sera we may expect to obtain an oocyst level which is in the centre of the range of values obtained with the individual non-endemic sera. The results of the present study were in agreement with this expectation. We consider the bioassay using standardized cultured gametocytes to be preferable. The advantages of the laboratory assay are the relatively high oocyst numbers in infected mosquitoes and the possibility of repeating the experiments. The assay is considered successful if at least 90% of the control mosquitoes are infected. Because of this high infection rate, genuine transmission blocking sera (sera A, B, C) can be detected using only 20 mosquitoes. We have always aimed at the highest oocyst level to ensure obtaining reduction over as great a range as possible. In theory this gives the possibility of discriminating between field sera and creating different categories of transmission reduction capacity. However, due to large variations, only R values (based on the Williams geometric mean) of more than 85% gave reliable results—i.e. could be confirmed in repeated experiments. This study showed that this high cut-off level was due to wide distribution of R values associated with non-endemic control sera. The range of R values obtained with non-endemic sera provides a basis for classifying the test results of other sera. The R values of control sera should be close to zero: only those values based on a logarithmic scale met this criterion (Fig. 2). In fact, R values based on the geometric mean were the least satisfactory for the measurement of transmission reduction.

The practical aspects of these results are demonstrated in the Table. It has been suggested that only the percentage of infected mosquitoes should be used for the determination of transmission reduction. However in experiments with high oocyst levels it is possible to have a

high mosquito infection rate combined with a high R value, as shown in experiments 2 and 3. Since it is still difficult to predict the transmission level in the feeder assay in advance, we have to aim at the highest oocyst levels for practical reasons.

In conclusion, we would like to suggest the use of the log scale in determining R values of endemic sera using feeder assays with high oocyst levels. This enables one to define 3 categories of sera: blocking ( $R > 0.9$ ), reducing ( $0.9 < R < 0.3$ ), and non-blocking ( $R < 0.3$ ).

We have compared 3 commonly used measures of transmission reduction. Apart from these measures, others might be constructed based on other transformations, e.g. the square root transformation, or one might use a parametric approach, e.g. fitting the negative binomial distribution to oocyst counts (MEDLEY *et al.*, 1993). These approaches would lead to other measures of transmission reduction. Further research should be directed towards these and perhaps other possibilities for constructing R values.

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