Submicroscopic Infection in Plasmodium falciparum—Endemic Populations: A Systematic Review and Meta-Analysis

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Introduction. Light microscopy examination of blood slides is the main method of detecting malaria infection; however, it has limited sensitivity. Low-density infections are most likely to be missed, but they contribute to the infectious reservoir. Quantifying these submicroscopic infections is therefore key to understanding transmission dynamics and successfully reducing parasite transmission.

Methods. We conducted a systematic review of endemic population surveys in which P. falciparum prevalence had been measured by both microscopy and a more-sensitive polymerase chain reaction (PCR)–based technique. The combined microscopy:PCR prevalence ratio was estimated by random-effects meta-analysis, and the effect of covariates was determined by meta-regression.

Results. Seventy-two pairs of prevalence measurements were included in the study. The prevalence of infection measured by microscopy was, on average, 50.8% (95% confidence interval [CI], 45.2%–57.1%) of that measured by PCR. For gametocyte-specific detection, the microscopy prevalence was, on average, 8.7% (95% CI, 2.8%–26.6%) of the prevalence measured by PCR. A significantly higher percentage of total infections was detected by microscopy in areas of high, compared with low, transmission (74.5% when the prevalence determined by PCR was >75% versus 12.0% when the prevalence determined by PCR was <10%).

Discussion. Microscopy can miss a substantial proportion of P. falciparum infections in surveys of endemic populations, especially in areas with low transmission of infection. The extent of the submicroscopic reservoir needs to be taken into account for effective surveillance and control.

Light microscopy examination of blood slides is the mainstay for detection of malaria, both in clinical diagnosis and in epidemiological surveys. However, it has long been established that a proportion of individuals with malaria have low-density infections that are unlikely to be detected by conventional microscopy [1]. Such submicroscopic infections only occasionally cause acute disease [2], but they are capable of infecting mosquitoes and contributing to transmission [3–7]. Quantifying the extent of submicroscopic infections and the underlying true prevalence of parasites in different settings is therefore central to understanding the dynamics of malaria transmission and the stability of parasite populations.

Polymerase chain reaction (PCR) and other molecular techniques have enabled measurement of infections of substantially lower density than those measured by microscopy [8]. The sensitivity of a malaria test—that is, the probability that at least 1 parasitized red blood cell is detected—is directly related to both the volume of blood that the test can screen and the density of parasites in the blood sample [9]. When standard microscopy techniques [10, 11] are used, the volume of blood examined is ~0.06–0.2 µL, but when a PCR assay is used, the volume examined can be several microliters, giving theoretical limits of detection of ~5–16 parasites/µL versus 0.02–1 parasites/µL for each technique, respectively [12]. In practice, the detection limit of routine microscopy has been estimated to
be only $\sim 100$ parasites/µL [13], which may be the result of operational constraints or technical factors, such as loss of parasites during the staining procedure [10]. Therefore, microscopy is likely to miss lower-density infections during screening of endemic populations; however, the proportion of infections in this range has not been well characterized.

Experiments measuring human-to-mosquito transmission have shown that submicroscopically infected individuals can significantly contribute to the total human infectious reservoir—that is, the parasite population within humans that can be transmitted onward to mosquitoes [5, 12]. Two studies testing the infectivity of randomly selected community samples found that individuals who were negative for gametocytes by blood slide examination were equally likely to transmit infection to mosquitoes as slide-positive individuals [6, 7]. The infectivity of individuals who were slide negative for all parasite stages was investigated in one of these studies, which was performed in a setting where transmission of infection was low; these individuals infected $\sim$10-fold fewer mosquitoes than did individuals who had positive results of blood slide examination, but they represented $\geq 95\%$ of the population and therefore constituted the greater part of the infectious reservoir [7]. Submicroscopic infections have been implicated in seedling transmission in areas of strongly seasonal transmission at the start of the rains, when prevalence, as determined by microscopy, may be zero [14, 15]. Malaria parasite densities are known to vary according to several factors that are likely to be correlated with transmission intensity: immunity [16], rates of reinfection [17], and multiplicity of clonal subtypes [18]. Assessment of whether the sensitivity of microscopy therefore also varies across settings is a key question to understanding the importance of the submicroscopic reservoir in areas of different endemicities and informing malaria control programs accordingly [19].

We present a systematic review of surveys of endemic populations in which P. falciparum prevalence is measured by both microscopy and a more sensitive PCR-based technique. The proportion of submicroscopic infections is estimated, and its association with transmission intensity is investigated.

METHODS

Literature review and data extraction. Relevant studies were identified in PubMed and Medline, by use of the search terms “malaria” and “PCR,” and by searching the bibliographies of included studies for additional references. Searches were performed up to 13 January 2009. Studies were eligible for inclusion if they reported or allowed calculation of the prevalence of P. falciparum by both standard microscopy and PCR in the same sample of individuals. Inclusion criteria were as follows: (1) the study participants consisted of a population sample of individuals in an endemic area who were not chosen on the basis of malaria symptoms or test results, (2) the study population came from a defined geographical area, and (3) there were no large-scale interventions for malaria (for example, treatment or use of bed nets) as part of the study before measurement. If only a subsample of those tested by microscopy were also tested by PCR, the study was included as long as the subsample was randomly selected. Studies that excluded symptomatic malaria cases detected in the survey were retained in the analysis because the prevalence of symptomatic cases at any one time is very small [20].

From each study, the following information was extracted by 2 independent researchers and double entered into records for analysis: location, citation details, study sample, laboratory methods (PCR gene target and method, reported use of negative controls, and criteria for declaring a negative result for a microscope slide), and outcomes (prevalence of P. falciparum, as determined by microscopy and PCR; discordant results of microscopy and PCR measurement; and parasite density in the sample for which results of microscopy were positive). Information on season (ie, rainy versus dry season) was also collected if >2 measurements were made at different times in the same population and area.

Statistical analysis. The prevalence of infection detected by microscopy was compared with the prevalence of infection determined by PCR, by use of 2 different methods. The first method analyzed the ratio of the infection prevalence measured by microscopy to that measured by PCR (ie, the “prevalence ratio”). The second method additionally used information on discordant results (ie, the number of false-positive and negative results obtained by microscopy, compared with PCR “reference standard” results). Discordant results were published only in a subset of studies, which is why the second method was not used alone. The aim of this second analysis was to provide combined estimates of the sensitivity and specificity of microscopy relative to PCR and to compare the sensitivity estimate with the result obtained by the first analysis (ie, the “microscopy:PCR prevalence ratio”).

Using the first method, the log microscopy:PCR prevalence ratios and the sample sizes in each study were analyzed using random-effects meta-analysis, to obtain a combined estimate [21]. Before log transformation, any zero values for prevalence of infection determined by microscopy or PCR were set to equal one-half of the detection threshold of the sample (0.5 individuals with a positive result divided by the total number of individuals assessed). Meta-regression was used to test the association between the microscopy:PCR prevalence ratios and the PCR prevalence of infection, age of the study sample, season of transmission, PCR method used, detection of the microscopy protocol, and parasite density, allowing for random study effects. Each study population was given an age category as follows: “children” if the study included only individuals <15
years of age, “adults” if only individuals $\geq 15$ years of age were included, or “all ages” if individuals $<15$ years and those $\geq 15$ years of age were included. The detection limit of microscopy was calculated as 1 parasite/volume of blood examined, under the assumption that there were 8000 leukocytes/μL of blood and 0.2 μL of blood per 100 fields of a thick smear [10, 11].

The PCR prevalence of infection was used as a marker of transmission intensity and was explored both as a linear variable and according to 5 prevalence categories (<10%, 10%–24%, 25%–50%, 50%–74%, and $\geq 75\%$) that included an approximately equal number of studies in each group. PCR prevalence, rather than microscopy prevalence, was used as a marker of transmission intensity in this analysis, because the sensitivity of any imperfect screening test would be expected to be correlated with the prevalence values that it measures, due to random variability in test sensitivity (for example, because of variable reagent quality in any setting). For example, if multiple microscopy measurements were made for samples with the same PCR prevalence, those which, by chance, had higher sensitivity would also record a higher microscopy prevalence, creating a correlation even though the underlying PCR prevalence was the same. Such factors would not create a correlation between microscopy sensitivity and PCR prevalence.

In the second method of analysis, sensitivity and specificity values for each study were calculated as the percentage of PCR-positive and -negative individuals, respectively, who were correctly identified as such by microscopy. Combined estimates of sensitivity and specificity were obtained using the meta-analysis method recommended by Hamza et al [22]. In brief, pairs of sensitivity and specificity values from each study were analyzed jointly within a bivariate random effects model, which assumes a binomial distribution of the within-study distribution of sensitivity and specificity and which explicitly models the correlation between these 2 measurements. For studies in which any of the outcome categories (true positive, false positive, true negative, or false negative) included 0 individuals, this number was modified to 0.5.

All analyses were performed using stata software (version 10.0; stataCorp) or SAS software (version 9.1; SAS Institute).

RESULTS

Literature search. The literature search generated 2216 results in PubMed and 1211 in Medline. After duplicate results were discarded, the total number of articles considered was 2441. Following screening of titles and abstracts, 100 articles were retained for more detailed evaluation. The most common reasons for excluding the other studies were that they (1) did not perform PCR on malaria parasites; (2) included only clinical samples or parasite-positive samples; (3) were not done in humans; (4) concerned a Plasmodium species other than P. falciparum, or (5) were not performed in P. falciparum–endemic populations. Of the 100 articles that underwent further evaluation, 42 were included in the study; their main characteristics are shown in Table S1 in the Appendix, which appears only in the electronic version of the Journal. Searches of the bibliographies of the included studies identified 12 additional articles for detailed evaluation, although none of these additional articles were included after full-text examination, resulting in a total of 70 excluded studies. These studies are presented in Table S2 in the Appendix (which appears only in the electronic version of the Journal) according to the main reason for their exclusion.

The 42 articles included in the evaluation yielded 70 pairs of microscopy and PCR prevalence estimates: 50 from Africa, 8 from Asia, 7 from the Americas, and 5 from other areas. They covered 45 different locations in 23 countries. The most referenced PCR method was that of Snounou et al [8]. The large majority of microscopy protocols used thick smears ($n = 57$); 2 estimates report using thin smears only, and 11 did not report the type of blood smear used. Because the geometric mean was the most commonly reported summary measurement of parasite density ($n = 14$), it was used for analysis.

Comparison of microscopy and PCR prevalences. The prevalence of P. falciparum infection measured by PCR was consistently higher than that determined by microscopy in all but 3 estimates [23–25] (Figure 1). The combined estimate of the P. falciparum microscopy:PCR prevalence ratio, excluding gametocyte-specific estimates ($n = 65$), was 0.508 (95% confidence interval [CI], 0.452–0.571) (Figure 2), indicating that the prevalence of infection detected by microscopy was, on average, 49.2% lower than that detected by PCR. The prevalence ratio values were highly heterogeneous between studies ($P<.001$) and ranged from 0.000 to 1.230 (Figure 1). The combined estimate of the ratio of the microscopy:PCR prevalence of gametocytes was 0.087 (95% CI, 0.028–0.266) (Figure 3), which was significantly lower than the combined estimate for studies detecting nonspecific parasite stages ($P = .002$); again, the heterogeneity between estimates was significant ($P<.001$). This prevalence ratio indicates that 91.3% of gametocyte carriers were missed by microscopy. These gametocyte-specific data were not analyzed to determine the effect of covariates, because there were only 5 estimates.

Thirty-three study samples also allowed estimation of the sensitivity and specificity of microscopy relative to PCR (ie, they reported which results were discordant between the 2 techniques). None of these estimates were gametocyte specific. The combined estimate of the sensitivity of microscopy was 53.1% (95% CI, 40.2%–65.6%), and the specificity was 98.4% (95% CI, 96.7%–99.3%). The combined sensitivity estimate closely corresponded to the combined estimate of the microscopy:PCR prevalence ratio, mainly because of the very high specificity of microscopy (ie, low numbers of false-positive results). Therefore,
the subsequent analyses presented use the prevalence ratio as an outcome, because this enables inclusion of a greater number of studies (65 studies instead of 33 studies). The specificity estimate should be interpreted considering that PCR is also imperfect, and it may be as likely that PCR would sometimes fail to detect an infection as that a slide result would be recorded as positive without parasites present.

Association between transmission intensity and submicroscopic parasitemia. The association between transmission intensity and the proportion of infections that are submicroscopic was examined using PCR prevalence as a measurement of transmission intensity. In univariate analysis, as the underlying PCR prevalence increased, the microscopy:PCR prevalence ratio also increased (Figure 4 and Table 1) (the prevalence ratio was 1.135 times higher per 10% increase in PCR prevalence [95% CI, 1.051–1.226]; P = .002). This finding suggests that a higher proportion of infections are undetected by microscopy in areas with low levels of transmission. Thus, in studies where the PCR prevalence in the study population was <10%, microscopy detected only 12.0% of the infections that were identified by PCR (95% CI, 4.8%–29.9%), whereas in studies in which the PCR prevalence was ≥75%, microscopy detected 74.5% of infections (95% CI, 67.1%–82.8%).

PCR contamination was explored as a potential confounding factor in the association between transmission intensity and apparent microscopy sensitivity. Because PCR is a highly sensitive amplification-based procedure, it is prone to false-positive results [26]. If a constant proportion of samples assessed by PCR were contaminated in all settings, microscopy sensitivity would appear to increase in association with observed PCR prevalence, because in the low-transmission settings, the probability of contaminated samples being true-negative results would be higher. We could not directly estimate contamination levels, but we categorized studies according to whether they reported the use of negative controls as a potential indicator for contamination rates. Excluding gametocyte-specific estimates, use of a negative control during PCR was reported for 32 of 70 estimates. Reporting the use of a negative control was not associated with any significant difference in the overall combined microscopy:PCR prevalence ratio (P = .732) (Table 1). However, when areas with different transmission levels were examined separately, in low-transmission areas recording a PCR prevalence of <10%, microscopy appeared to detect a significantly higher proportion of infections if a negative control was reported in the study (P = .005). The pooled microscopy:PCR prevalence ratio in these areas was 0.009 (95% CI, 0.000–0.193) in studies that did not report use of a negative control (n = 4) and 0.311 (95% CI, 0.137–0.702) in studies that did report use of a negative control (n = 7). No effect of negative controls was seen in studies with a higher PCR prevalence (P > .25, for all the following prevalence bands: 10%–24%, 25%–49%, 50%–74%, and ≥75%). This finding suggests a possible effect of contamination in low-transmission settings, although our criteria for identifying the use of negative controls are imprecise, and although there are small numbers of studies in the subgroups. Excluding estimates for which the PCR prevalence is <10%, the microscopy:PCR prevalence ratio still increased significantly with PCR prevalence (the prevalence ratio was 1.112 times higher per 10% increase in PCR prevalence [95% CI, 1.038–1.191]; P = .003).

Effect of study sample and laboratory methods. Table 1 shows associations between study characteristics and the microscopy:PCR prevalence estimated by meta-regression analysis. The microscopy:PCR prevalence ratio was, to some extent, lower among studies that included only adults (individuals ≥15 years of age) than among studies that included only children (individuals <15 years of age), although this as-
Figure 2. Forest plot showing ratios of the prevalence of *Plasmodium falciparum* determined by microscopy to that determined by polymerase chain reaction (PCR) (excluding gametocyte-specific estimates) separately by study. The combined estimate from the random-effects meta-analysis is labeled “Overall” (studies are labeled with the study identification [ID] number [see Table S1 in the Appendix, which appears only in the electronic version of the Journal]).

Association was nonsignificant. This finding would be consistent with parasite densities decreasing with age and immunity. Neither the PCR method nor the detection limit of the microscopy method, nor the season in which the survey was performed (rainy versus dry season), nor parasite density was associated with the microscopy:PCR prevalence ratio.

**DISCUSSION**

This meta-analysis finds a limited sensitivity of microscopy for the detection of *P. falciparum* in surveys of endemic populations, with the prevalence of infection detected by microscopy being, on average, 50.8% of the prevalence detected by PCR.
We provided a breakdown of PCR prevalence values at different levels of microscopy prevalence, to indicate the likely extent of submicroscopic infection in areas for which only microscopy data are available (Figure 1). As noted during the first cross-sectional surveys to use molecular techniques, the higher prevalence detected by PCR indicates a reservoir of infection that is larger and more stable than suggested by the microscopy prevalence [12, 27]. Microscopy appears to miss a higher proportion of infections in low-transmission settings, which is of particular relevance to elimination programs. Submicroscopic infections can persist for a number of months without any symptoms that would prompt treatment seeking [14] and therefore would need to be detected and targeted. We restricted this review to samples from *P. falciparum*-endemic populations; however, similar studies of other groups, such as symptomatic cases and pregnant women, also show a significant submicroscopically infected proportion [28, 29].

Rapid diagnostic tests, based on the detection of antibodies to parasites, are becoming more widely used in the field and have a sensitivity similar to or lower than that of microscopy [30]; therefore, we would expect to obtain similar results if these tests were compared with PCR. The low sensitivity of microscopy versus PCR has been attributed to a number of factors, both biological and experimental. Substantial heterogeneity between results was unexplained by the differences between studies that we examined in this analysis. The much lower apparent sensitivity of microscopy to detect gametocytes is likely to be a result of their well-documented lower density, compared with asexual parasite stages, although there were too few data on gametocyte density to test this in our analysis [31]. Variability in the results between microscopy readers and in microscopy protocol, as well as a loss of parasites during staining, have been well documented in other studies [10, 32]. These factors may override the influence of the variables on the microscopy:PCR prevalence association that we could investigate in this analysis.

In our analysis, we found that a higher proportion of infections are detected by microscopy as transmission intensity increases. A number of epidemiological factors could create this association. Some studies have shown that mean parasite densities among infected individuals increase with transmission intensity, with this association being strongest in younger individuals [16, 33]. More frequent reinfection, higher multiplicity of infection, and lower rates of symptoms and treatment per infection in areas where transmission is higher might all be expected to result in a higher mean population parasite density. In the present review, the mean parasite density in the study populations was associated with only a small, nonsignificant increase in the microscopy:PCR prevalence ratio. However, parasite density was found to be strongly associated with microscopy sensitivity in previous studies [34]; this analysis probably misses the importance of this factor, because of using mean, rather than individual, densities and because of the small number of studies (n = 14) reporting comparable density outcomes.

Submicroscopic infections are of particular relevance in low-transmission areas aiming for elimination, where they are like-
Table 1. Meta-Regression Results Showing Univariate Association of the Microscopy:Polymerase Chain Reaction (PCR) Prevalence Ratio with Study Setting, Methods, and Sample

<table>
<thead>
<tr>
<th>Variable</th>
<th>Studies, no.</th>
<th>Relative prevalence ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR prevalence of infection per 10% increase*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All studies</td>
<td>65</td>
<td>1.112 (1.038–1.191)</td>
<td>.003</td>
</tr>
<tr>
<td>Studies in which PCR prevalence was &gt;10%</td>
<td>54</td>
<td>1.135 (1.051–1.226)</td>
<td>.002</td>
</tr>
<tr>
<td>PCR method*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snounou (1993) [8]</td>
<td>27</td>
<td>1</td>
<td>.411</td>
</tr>
<tr>
<td>Other</td>
<td>38</td>
<td>1.235 (0.742–2.054)</td>
<td></td>
</tr>
<tr>
<td>Report using PCR-negative control*</td>
<td></td>
<td>.732</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>31</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>0.919 (0.564–1.499)</td>
<td></td>
</tr>
<tr>
<td>Detection limit of microscopy, no. of parasites/μL*</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;5</td>
<td>19</td>
<td>1</td>
<td>.786</td>
</tr>
<tr>
<td>5–40</td>
<td>33</td>
<td>1.072 (0.643–1.788)</td>
<td></td>
</tr>
<tr>
<td>Parasite density per 1-unit increase in log geometric mean*</td>
<td>13</td>
<td>1.072 (0.642–1.788)</td>
<td>.786</td>
</tr>
<tr>
<td>Parasite stage detected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonspecific</td>
<td>65</td>
<td>1</td>
<td>.002</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>5</td>
<td>0.206 (0.077–0.550)</td>
<td></td>
</tr>
<tr>
<td>Season*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>12</td>
<td>1</td>
<td>.626</td>
</tr>
<tr>
<td>Rainy</td>
<td>10</td>
<td>1.279 (0.453–3.606)</td>
<td></td>
</tr>
<tr>
<td>Age group of study sample*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>40</td>
<td>1</td>
<td>.879</td>
</tr>
<tr>
<td>&lt;15 years (children)</td>
<td>17</td>
<td>1.507 (0.862–2.634)</td>
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</tr>
<tr>
<td>≥15 years (adults)</td>
<td>4</td>
<td>0.729 (0.269–1.973)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. CI, confidence interval.
* Gametocyte-specific measurements excluded.
** Estimated as 1 parasite per volume of blood examined.

ly to sustain transmission if not detected [7]. We estimated that an average of 88% of infections are not detected by microscopy in areas with a PCR prevalence of <10%. This finding has considerable implications for control agencies that are considering mass screening and treatment programs in low-transmission settings [35]. However, it is in these low-transmission areas that the estimation of microscopy sensitivity is also most problematic. First, estimates have the largest variability, because the numbers of PCR-positive results and microscopy-positive results used in calculating sensitivity are the smallest. Second, we identified a potential influence of contamination in these low-transmission areas. PCR contamination is a common problem, with rates of 0.7%–10% reported by laboratories [26], and it would be expected to have the biggest effect in low-transmission settings, where a higher proportion of samples have true-negative results. This is consistent with our finding that the reported use of negative controls had a significant effect on estimates in areas where the PCR prevalence is <10%, with the caveat that many authors would consider negative controls to be routine and would not report them. This result emphasizes the need for development of a standardized PCR protocol for parasite detection. Another experimental explanation for the observed association could be that microscopists are less experienced in identifying malaria in settings where transmission is lower. Our finding should be confirmed using other validated measures of transmission, such as the entomological inoculation rate. This would also allow a useful recalibration of malaria endemicity according to PCR prevalence. However, at present, there are few paired PCR and entomological inoculation rate measurements available.

The extent of low-density parasite carriage has wide-reaching implications in areas of malaria transmission dynamics and immunity. Mathematical models frequently predict higher prevalences than are detected by slide examination if long durations of infection, which include submicroscopic periods, are taken into account [36, 37]. The larger estimates of the reservoir of infection imply a more stable parasite population, making reduction of transmission more difficult. However, it will be important to gain an accurate estimate of the infectiousness of the submicroscopic reservoir, which currently relies on a small number of studies [5–7]. Low-density infections have not been found to be significantly infectious in all studies—for example, in gametocyte slide–negative individuals in the Gambia [38] and in gametocyte slide–negative individuals in Cameroon who were...
slide negative for all parasite stages [39]. In contrast, other studies have shown that even PCR-negative individuals can be infectious [4]. Our analysis also demonstrates that submicroscopic parasitemia is common in settings where transmission is low, indicating that those with little previous exposure are able to control parasite densities (also seen in experimental infections of malaria-naive individuals [17, 40]). This may be the result of clone-specific immunity [41] or partially successful treatment [42] and could be important for maintaining immune responses [29, 33, 43]. It is not yet feasible to use PCR routinely, because of the resources required [44]; however, rapid, simplified PCR methods are likely to become widely available in the near future [45] and should be considered in studies where the true reservoir of infection needs to be estimated accurately. As control programs scale up in the coming years, the extent of submicroscopic infection must be taken into account to ensure accurate estimation of the chances of successful malaria elimination.

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