Supraorbital Postmortem Brain Sampling for Definitive Quantitative Confirmation of Cerebral Sequestration of Plasmodium falciparum Parasites

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Background. The conventional clinical case definition of cerebral malaria (CM) is imprecise but specificity is improved by a definitive clinical feature such as retinopathy or confirming sequestration of parasites in a post-mortem examination of the brain. A full autopsy is often not possible, since it is costly and may encounter resistance of the deceased’s family.

Methods. We have assessed the use of a cytological smear of brain tissue, obtained post-mortem by supraorbital sampling, for the purpose of quantifying cerebral sequestration in children with fatal malaria in Blantyre, Malawi. We have compared this method to histological quantification of parasites at autopsy.

Results. The number of parasites present on cytological smears correlated with the proportion of vessels parasitized as assessed by histology of fixed and stained brain tissue. Use of cytological results in addition to the standard clinical case definition increases the specificity of the clinical case definition alone from 48.3% to 100% with a minimal change in sensitivity.

Conclusions. Post-mortem supraorbital sampling of brain tissue improves the specificity of the diagnosis of fatal cerebral malaria and provides accurate quantitative estimates of cerebral sequestration. This tool can be of great value in clinical, pathogenetic, and epidemiological research studies on cerebral malaria.

Malaria infection estimates per year have dropped from 500 million to 225 million in the last decade; similarly during the same time period, the number of malaria deaths per year has plummeted from 3 million to 791000 [1, 2]. Despite this progress, 90% of malaria deaths in occur in sub-Saharan Africa, with deaths in children aged <5 accounting for 85% of all deaths [1, 2]. The clinical case definition of cerebral malaria (CM)—a common cause of fatal malaria—includes the presence of parasites in peripheral blood, coma, and no other discernible cause for coma [2–4]. Among 31 children who fulfilled the clinical case definition and came to autopsy in a previous study, 7 (23%) were found to have died of causes other than malaria and to have little or no sequestration of parasites in the brain [5]. The use of ophthalmoscopy to detect malaria retinopathy can increase the specificity of this diagnosis to 98% [6–11]. Ophthalmoscopy can contribute to more accurate diagnosis of CM during life. For patients who succumb to disease, the procedure for most accurate diagnosis is autopsy examination of the brain with histological examination. The presence of large numbers of sequestered parasites in cerebral vessels and the absence of other significant pathology strengthens the likelihood that the patient’s disease was caused by malaria and was not merely accompanied by parasitemia. An alternative and simpler method of examining brain vasculature after death.
involves passing a needle through the supraorbital plate to obtain a small sample of brain tissue, which can be smeared between 2 glass slides, stained, and immediately examined for parasites sequestered in microvessels [12]. This method can be performed by nonpathologists and uses the same staining techniques as peripheral blood smear samples. It is therefore a method of postmortem confirmation of CM that is rapid, less expensive, and more universally available than formal postmortem histopathology.

We have demonstrated elsewhere that in a clinicopathological study of patients dying from malaria and other causes, postmortem supraorbital brain sampling assessed qualitatively provides an accurate prediction of cerebral sequestration [13]. To further validate this method for use as a definitive tool when no autopsy is possible, we have quantitatively evaluated the histological and cytological samples from a larger set of cases.

MATERIALS AND METHODS

Patients
From 1996 to 2010, children with severe Plasmodium falciparum infections were admitted to the Paediatric Research Ward in the Queen Elizabeth Central Hospital, Blantyre, Malawi, in a study of the clinicopathological correlates of severe malaria. Clinical management, laboratory investigations, and treatment protocols have been described elsewhere [5]. In the event of death, a Malawian clinician or nurse met with key family members to seek consent for an autopsy. Clinical diagnoses were determined before each autopsy. Patients’ diagnoses were categorized as either clinical CM (ie, fulfilling the clinical case definition of CM including Blantyre coma score ≤2, parasitemia, no other cause of coma), or nonmalarial coma (ie, another cause of death evident clinically, with or without peripheral parasitemia) [5]. If permission was granted, a postmortem examination was performed with minimal delay. The research ethics committees at Michigan State University, the University of Liverpool, the University of Malawi College of Medicine, and the Brigham & Women’s Hospital approved all or appropriate portions of this study.

Cytology
After death and before the autopsy, a large-bore needle with trochar was used to obtain a sample of the frontal lobe via the supraorbital plate, as described elsewhere [13]. From the core of frontal lobe brain tissue, smear samples were obtained and stained with either reverse Field’s or Giemsa stain. For each smear sample, the slide was assessed to be adequate (≥3 vessel segments present on the slide) or inadequate (<3 segments). For adequate smear samples, the quantity of parasites within the capillary was assessed by orienting a length of vessel across the diameter of an oil immersion field (×1000) so that the length of vessel in which parasites were counted was equal to 1 field diameter. All parasites in the high-powered field within the vessel were counted and staged. This was repeated 9 times for a total of 10 vessel segments of a length equal to the diameter of a high-powered field. The stage of parasites present in the vessel segments of all cases was recorded (unpigmented rings, trophozoites, and/or schizonts). Free pigment globules (hemazoin after schizogony) and pigment within macrophages were not counted on cytological smear samples because these elements (unlike sequestered parasites) may be disrupted by the smearing process. All vessel segments counted were digitally photographed and rechecked by a second observer. In prior work, 10 vessel segments, each with ≥5 parasites per segment, were used as a qualitative determinant of true cerebral sequestration; therefore, we estimated that a cutoff of 50 parasites per 10 vessel segments would delineate CM from non-CM on a continuous scale [13].

Autopsy Procedures
Gross examination, documentation, and histological assessment were performed as described elsewhere [5]. For each autopsy case, a final anatomic diagnosis was determined, and the patient was classified as CM1 (ie, clinical CM with sequestration only), CM2 (ie, clinical CM with sequestration and extravascular pathology), CM3 (ie, clinical CM with no evidence of sequestration and the finding of another anatomical cause of death), or non-CM (ie, not clinical CM with another anatomical cause of death) [5].

Histology
Using routine hematoxylin–eosin–stained histological sections, the degree of malaria parasite sequestration in the brain, including the percentage of vessels parasitized, was quantified by counting all parasite elements, as described elsewhere [5]. As part of this method, parasite elements that were quantified included unpigmented parasites, pigmented parasites, pigment globules, and pigment within macrophages. In a previous study, a threshold of ≥23.1% of vessels parasitized with any of these elements was calculated (using Classification and regression tree [CART] analysis) for confirming the clinical diagnosis of CM (n = 50) [5]. A repeated analysis of this histologically determined threshold using the total data set (N = 103) showed a similar cutoff (Milner et al). For this study, we used the published 23.1% threshold for all calculations.

Statistics
The raw cytological counts (range, 0–1186 parasites in 10 vessel segments in smeared brain tissue) and the normalized percentage of vessels parasitized (range, 0%–100% of vessels parasitized in histological samples) were both log transformed and plotted. Linear regression was used to assess the association and the correlation between these 2 tests. If one test is a good
surrogate of another, the 2 tests are expected to be highly associated with most observations falling on the regression line (large $R^2$). Using histological examination as the reference standard, sensitivity, specificity, and area under the receiver operating characteristic curve (AUC-ROC) were determined for the cytological examination, for the clinical case definition alone, and for the clinical case definition in combination with the cytological results. Confidence intervals were estimated based on the logit-transformation of the proportions and bootstrap (for the AUC-ROC). All statistics were performed using S-plus 8.0 software (Insightful).

RESULTS

Adequacy of Smear Samples
During the period of the study, 103 autopsies were performed (among 2147 admitted patients, of whom 327 died), and tissue obtained by postmortem supraorbital collection before the autopsy was adequate for assessment by the working definitions in 71 cases [5, 13]. The 71 cases included 56 patients meeting the clinical case definition of CM (including 40 with and 16 without histological criteria for CM), 2 meeting the clinical case definition of severe malarial anemia (in coma), and 13 who had coma of other causes (with or without parasitemia). Cytology was positive for parasites in 38 of these 71 patients (53.5%). Parasitological staging demonstrated predominantly mid- to late-stage trophozoites in 33 of 38 patients (86.8%). A mixture of schizonts and trophozoites were seen in 5 (13.5%), with variable proportions (1.9%-10.8%) of schizonts (Figure 1). Sequestration of ring-stage parasites was not observed.

Cytological Correlation With Histological Counts
The total number of parasites counted (TPC) within 10 vessel segments from the frontal lobe of the cerebral cortex for a single slide by cytology was compared with the proportion of vessels parasitized at histology (VPH) after log transformation of both variables (Figure 2). For all levels, histology and cytology counts were strongly associated; changes of 1 unit in histology counts were associated with a change of 0.58 units in cytology counts ($P < .0001$), and the 2 tests were strongly correlated ($R^2 = 0.84$).

All but 2 patients with CM1 or CM2 had both $\geq 23.1\%$ VPH and $\geq 50$ TPC. The 2 discordant cases (Figure 2, open circles) classified as CM at histology (based on $\geq 23.1\%$ VPH cutoff) and missed at cytology (based on $>50$ TPC cutoff) had 4.9 and 5.7 total parasites per 100 capillaries. All cases of CM3 or non-CM had both $<23.1\%$ VPH and $<50$ TPC. Two additional patients (Figure 2, open squares) who met the clinical case definition of CM were classified as negative based on the 23.1% VPH (histological) count threshold and the 50 TPC (cytological) count threshold despite having the classic gross appearance of ring hemorrhages and a discolored brain. The VPH for these 2 patients was very low (6.3% and 1.9%).

Validity of Cytology to Detect True CM
Against the histological reference standard, cytology counts based on the cutoff value of $\geq 50$ TPC had specificity twice as high as the application of the case definition alone to diagnose true CM (Table 1). The accuracy of a criterion combining the cytology results and the clinical case definition was much better, with an AUC-ROC of 0.98, specificity of 100%, and sensitivity of 95.0%.

Figure 1. Representative paired histological ($A$, $C$, $E$, $G$) and cytological ($B$, $D$, $F$, $H$) images, demonstrating 2 of the 5 cases ($A/B$ and $C/D$) in which a mixture of trophozoites and schizonts were seen by histology and cytology; $E/F$ and $G/H$ demonstrate the more common finding of a pure mid- to late-stage trophozoites population (all images, hematoxylin-eosin [$A$, $C$, $E$, $G$] or Giemsa [$B$, $D$, $F$, $H$] stain; ×1000 oil immersion, digitally magnified to ×5555).
DISCUSSION

Measuring the impact of interventions against malaria can be improved by more accurate ascertainment of malaria-associated mortality. Cerebral malaria is often misdiagnosed, because its clinical features are nonspecific and common to many other diseases [5]. Advances in the clinical diagnosis of CM, such as ocular funduscopic examination, may play an important role in stratifying patients for intervention studies [8, 9]. Determining the actual cause of death in a patient with clinically defined CM can be made more accurate by some form of postmortem brain examination.

The use of cytology to identify parasites in the brain was first described in 1922 and has since played a valuable role in several adult studies in which full autopsies were not available [12, 14–19]. We have demonstrated elsewhere that this method is an accurate way of assessing cerebral sequestration qualitatively in our pediatric autopsy series [5, 13]. Parallel studies have also shown that cerebral sequestration is similar between different parts of the brain, so that sampling from a frontal lobe provides tissue that is representative of the brain as a whole (Milner et al, in process).

The present quantitative analysis, carried out in more patients than our qualitative analysis, supports the qualitative findings overall. There were 4 misclassification in a total of 71 cases. Two were in patients who satisfied the clinical case definition of CM and had histologically confirmed CM (≥23.1% VPH) but in whom no parasites were seen at cytology. For all patients meeting the clinical case definition of CM that went to autopsy and were found to have ≥23.1% VPH, the mean total parasite count per 100 capillaries was 246.4 ± 170.3, with these 2 cases representing the 2 lowest total parasite counts per 100 capillaries in the data set. These 2 patients had the lowest number of total parasites per 100 vessels identified histologically in the CM group. Because parasite elements such as free pigment and pigment within macrophages contribute to the VPH, when the actual burden of intact parasites is low, cytology becomes less sensitive because the probability of a positive smear sample decreases.

Two additional patients had grossly discolored brains and obvious ring hemorrhages, typical of CM2, but the numbers of parasites and pigment globules were very low. One had evidence of early formation of Dürck granulomas. When the clinical data, including entire length of reported illness and

Figure 2. Graph of the total number of parasites counted at cytology versus the percentage of vessels found to be parasitized at histology (filled circles). Two patients were misclassified by both cytology and histology (open squares). The 2 discordant cases (open circles), in which results were positive at histology but negative at cytology, lay close to the predetermined cutoff for CM. The 23.1% cutoff (gray horizontal line) for parasitized vessels was previously estimated by Taylor et al [5], and the 50-parasite cutoff (gray vertical line) was previously estimated by Milner et al [13].
Table 1. Accuracy of Cytological and Clinical Criteria in Detecting True Cerebral Malaria (CM), as Defined Based on Histological Examination of the Brain.

<table>
<thead>
<tr>
<th>Means of CM Diagnosis</th>
<th>Sensitivity (95% CI), %</th>
<th>Specificity (95% CI), %</th>
<th>AUC-ROC (95% CI)</th>
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<tbody>
<tr>
<td>Clinical criteria</td>
<td></td>
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<tr>
<td>(BCS ≤ 2, parasitemia, no other cause of coma identified)</td>
<td>100 (...)</td>
<td>48 (31–65)</td>
<td>0.74 (.65–.83)</td>
</tr>
<tr>
<td>Cytological and clinical criteria</td>
<td>95 (82–99)</td>
<td>100 (...)</td>
<td>0.98 (.94–1.00)</td>
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The reference standard for a diagnosis of CM was ≥23.1% of vessels parasitized at histological examination. Data are shown for 71 patients, including 56 who met the clinical case definition of CM. Of these, 40 showed cerebral sequestration above the cutoff; 16, below the cutoff. Fifteen patients did not meet the clinical case definition.

Abbreviations: AUC-ROC, area under the receiver operating characteristic curve; BCS, Blantyre coma score; CI, confidence interval.

* Positive for CM according to both cytological and clinical criteria. The cytological criterion for CM was a total parasite count of ≥50 in 10 vessel segments, with the length of each segment equal to the diameter of a ×1000 oil immersion field.

coma were reviewed, these 2 patients had the longest and second longest coma times of all patients with confirmed CM deaths, suggesting that they were at the very last stages of CM, after macrophages had phagocytized sequestered parasites and debris, leaving only healing ring hemorrhages. The finding of only 6.3% and 1.9% of vessels with any parasite elements is consistent with this hypothesis. The exact event(s) leading to death in the CM group is not yet clearly defined.

We have shown that supraorbital sampling can be used to identify intracerebral sequestration of P. falciparum with a degree of accuracy very close to that of routine histology. The benefits of cytological preparations compared with complete autopsies include the speed of obtaining and preparing tissue, simplicity of training clinicians and staff, and potentially greater cultural acceptance than full autopsy. The primary limitation of this tool is the inability to determine a nonneurological cause of death in patients without CM. If, however, a field site employing this tool determines that a large number of deaths clinically attributed to malaria are not in fact due to malaria, further investigations (such as an autopsy study) may be warranted. In this era of declining malaria incidence, this becomes an extremely important concept. In instances of fatal cerebral processes (ie, meningitis, hemorrhage, encephalitis), cytological sampling may be of value. For evaluating patients with coinfection (eg, Plasmodium vivax with P. falciparum), the tool may prove very useful in sorting out the source of neurological symptoms, but these studies have not yet been performed.

Notes

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D. A. M. designed the study, collected and analyzed the data, and wrote the manuscript. C. V. analyzed the data and wrote the manuscript. R. L., K. B. P., and S. K. collected and analyzed the data. M. E. M., K. B. S., and T. E. T. designed the study, collected the data, and edited the manuscript.

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References


