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Contraindications of Batch Staining in Malaria Diagnosis and its implications on Drug and Vaccine Protective Efficacy

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ABSTRACT

The primary objective was to evaluate if batch staining of malaria blood films results in false positive smears. False positive smears (>1%) may cause a serious underestimate of a drug's or vaccine's protective efficacy, as well as affect evaluation of diagnostics, estimates of malaria prevalence, and clinical management. Thick blood films may float from a glass slide during staining and adhere to other films if batch staining is used resulting in false positive readings. Venous blood in EDTA anticoagulant from malaria positive samples of ≥ 20 parasites per high power field and a true negative sample was utilized to make thick and thin smears. Two true negative smears were stained with Giemsa stain with eight positive smears in batch in Coplin jars for 10 minutes or overnight. Two control negatives were stained alone with the same batch of stain. Blinded microscopists read these slides using a rereading paradigm. Thick film loss was graded by gross appearance ranging from 0 (none) to 4+ (> ¾ loss). A total of 602 slides were evaluated in this study, of which 392 were true positives (65%) and 210 (35%) were true negatives. Of the true negatives, 110 were batch stained with true positives, and 100 were true negative controls stained alone. Of the initial readings, 11-20% were reported as falsely positive. "Fishing" or cross-contamination was infrequently noted by one of the microscopists, but was uniformly present in these smears on reexamination. Of the true positive smears (high density), 1-3% were read falsely negative. On reexamination of these slides, the cause was found to be reporting of results from very poor quality smears. Thick film loss was clearly more severe for the positive slides with 10 minute versus overnight drying (means score 0.97 vs 1.97, p <0.001). This experiment confirmed that false positive smears result from cross-contamination during batch staining using methods employed today. Since low frequencies of false positive smears can adversely impact research and product development results, single slide staining should become the norm in this setting. Reporting of false negative results occurred in malaria smears with high densities of parasites. Microscopists should be trained not to report results when smear quality is not adequate.

Introduction

Background

Malaria microscopy remains the reference or "gold" standard for malaria diagnosis in clinical trials (drug and vaccine), new diagnostic evaluation, and as well as in clinical care for much of the world today. It is known that microscopy is an imperfect gold standard [1,2]. A 1% false positive rate with serially collected smears can underestimate protective efficacy by ≥10% in malaria prevention studies [3]. As long as microscopy is used to determine the primary endpoint in malaria prevention and treatment trials, great lengths must be taken to ensure that good products are not inadvertently discarded.

Cross-contamination resulting in false positive malaria films has been described many decades ago. James in 1911 [4] first report that malaria parasites are occasionally found deposited on other smears, indicating that there is some parasite loss from thick film during staining. In 1924, Barber and Komp [5] conducted and experimented with true negative blood collected from pigeons and true positive smears collected from malaria-infected humans. They showed that 47% of negative smears became positive, and that the use of a surface active agent in the stain reduced the rate to 6%. In 1948, Brooke and Donaldson [6] reported that when thick blood films are stained together by mass procedures, blood elements may transfer from one film to another. In an attempt to prevent or at least to reduce material transfer of blood elements during mass staining, various modifications of the staining procedures were assessed by these authors. Most of these modifications did not eliminate the transfer. In 1960, Grant and colleagues [7] also described cross-contamination when multiple thick smears were made on the same slide. They concluded this was likely due to movement from one thick film to another while standing upright on a drying rack. In 1963, Hoo et al [8,9] identified that cross-contamination could be reduced when the scum on the surface of the stain was carefully removed. In 1966, Dowling and Shute [10] compared parasite counts between thick and thin films. They found parasite counts in thin films were greater than in

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thick smears made with same blood, and therefore deduced that 60% of the parasites were being lost from the thick films. In the last 40 years, only one article has addressed the issue of cross-contamination with malaria microscopy. In 2004, Aubouy and Carme [11] describes DNA contamination across malaria slides by the oil from the oil-immersion lens.

Despite these findings, most laboratories in the developing world do not employ procedures to prevent cross-contamination. In the clinical setting, many malaria smears are often stained repeatedly using the same batch of Fields stain. In clinical trials, epidemiology studies and other research studies, large numbers malaria blood films are often collected in EDTA anticoagulant and stained in batches. A visiting expert microscopist recommended a change from batch staining to single slide staining to prevent cross-contamination. To confirm if this was needed, this experiment was conducted to determine if cross-contamination is a problem using standard methods commonly employed today. This is one of several experiments the Malaria Diagnostics Center of Excellence has conducted in order to improve the quality of malaria microscopy.

Methods

Slide collection and preparation

Slides were prepared from EDTA-preserved venous blood (purple top tubes). True negative blood was collected from a newly arriving visitor to Western Kenya who was not ill and who had never had malaria. With a micropipetter, two microlitres of blood were smeared to produce a thin film, while 12 µl blood was spread in a circle with 15mm diameter using a slide template. Afterwards the thin film was fixed with absolute methanol, and allowed to air dry. They were stored overnight and were used within 5 days.

True positive blood was selected from EDTA containing whole blood that was to be discarded that was reported to have >20 parasites/high power field (n = 5). No identifiers were retained. Positive slides were prepared as above, except they were either dried for 10 minutes or overnight before staining. All slides were placed in 10-slot Coplin jars, stained using 10% Giemsa for 10 minutes, and rinsed carefully. Slides were then stood upright to dry in a drying rack. A total of 61 batches of smears were stained. Each batch contained 10 slides (8 true positive, 2 true negative). With each batch, two true negatives were stained alone in a separate Coplin jar as controls.

Evaluation of thick film loss

Thick film loss (flaking) was graded by gross appearance ranging from 0 (none) to 4+ (> ¾ loss). Grading was as follows by the amount of thick film loss: none = 0; 0-1/4 = 1+, 1/4-1/2 = 2+, 1/2-3/4 = 3+, > 3/4 = 4+.

Reading of malaria blood films

The distribution of malaria blood films to the readers was conducted by a trained and experienced slide coordinator. Trained microscopists reading the smears were unaware of the study being conducted and completely blinded to the study code, as well as each others results. All slides were read using a rereading paradigm.

All slides were first read twice by one of four qualified R1/R2 readers. Those that were discrepant in terms of positivity, species, or density were reread by one of two expert (R3) readers. The R3 also confirmed a sample of concordant readings. All false positive and false negative smears were later reassessed unblinded as well. Those results are reported separately.

If malaria was not identified, slides were reported negative after counting 200 high power fields. If malaria was identified, parasites and WBCs were counted simultaneously on a thick film using tally counter and 100X oil-immersion objective unless > 20 parasites per high power field were present on the thick film. In this case, microscopists turned to the thin blood film and counted parasites per 2000 red blood cells (RBCs). Parasites per microliter were calculated as parasites/200WBC x 8000 or parasites/2000RBC x 4,000,000.

Data analysis

Data were entered into, verified in, and calculations performed with Microsoft Office Excel 2003. Statistical analyses were performed and tables generated with SPSS 15.0 for Windows.

Means with confidence intervals were determined using the explore function with confidence interval for mean set at 95%. Means with *p* values for continuous variables were determined with the means function and Anova table selected. Means with p values for nominal variables were determined with the crosstabs function and Chi-Square and Fisher's Exact test selected.

Results

In total, 602 slides were stained and read by the R1 and R2 readers. Of these, 392 were true positives, 110 were true negatives stained with true positives, and 100 were true negative controls. 240 positives were from slides dried 10 minutes, while 152 were from slides dried overnight (Table). The R3 readers read 282 slides, including all discordant readings and a sample of concordant readings.

Four-five percent of the R1 and R2 reading and 3% of R3 readings had no results reported because of unacceptable smear quality. Of the 381 true positive smears in which counts were reported, the mean parasite densities using the rereading paradigm with R3 reader final was 181868 parasites/µl (median 126000, 95% CI 1627621 -199913). Of the true negatives reported as false positive, the mean parasite count was 3926 parasites/µl (95% CI 1949 - 5902, range 600-17680).

Of the true negatives smears that were batch stained with positive smears, 11-33% were read falsely positive while none of the singly stained negative smears were ($p < 0.001$, Table). On re-review of these slides, "fishing" or cross-contamination was clearly present on all of them, although it was only uncommonly noted in the blinded reading. The appearance was patches of many parasites in an otherwise thick film background without parasites. Thin films also had patches of parasites not clearly in red blood cells.

Of the true positive smears, 1-3% was read falsely negative (Table 1). On re-review of these slides, the cause was found to be reporting results on very poor quality smears and possibly cross contamination from negative smears.

All smears were assessed for the degree of thick smear loss (flaking) as reported in ((figure 1). As expected, thick film loss was clearly more severe for the slides with 10 minute versus overnight drying (means score 0.97 vs 1.97, $p < 0.001$). However, the problem was not completely resolved with overnight drying, as 14% still had 3 or 4+ thick film loss.

The table details the results of the reading paradigms for the different types of smears. The paradigm used affects the final sensitivity and specificity reported.

Table 1. Effect of batch staining and drying time on percentage of smears read positive

	Time True Positive Smears Dried	n	Reader 1	Reader 2	Reader 3*	Rereading Paradigm**
True Negatives Batch Stained	*10 minutes*	72	20%	20%	59%	25%
	Overnight	38	11%	14%	71%	16%
True Negatives Single Stained		100	0%	0%	0%	0%
True Positives		240	98%	99%	99%	100%

* Read discordant and a sample of concordant slides from reader one and reader 2

** Concordant readings from reader 1 and 2 or reader three result final

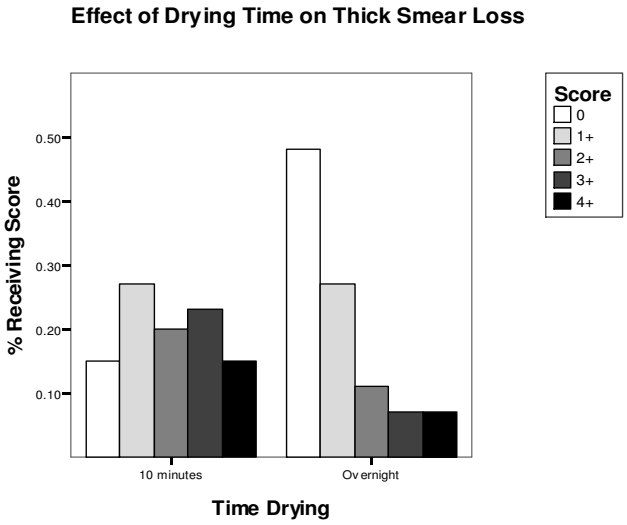

Figure 1. Thick film loss was graded by gross appearance ranging from 0 (none) to 4+ (> ¾ loss).

Discussion

An objective for the Malaria Diagnostics Center of Excellence in Kisumu, Kenya is to assess factors that will improve malaria diagnosis. This is one of a series of such experiments to improve malaria microscopy and to study alternative methods of malaria diagnosis. The findings in this experiment confirm the work of several investigators conducted over 40 years ago. Despite it being known that cross-contamination causes false positive smears, the practice of batch staining continues, even in the research setting. We hope our findings change practice in the research setting so that all slides are singly stained when false positive smears could impact interpretation of study results. Alternatively, if single staining of slides in not possible in some settings, microscopists' work forms should include a space to enter if cross-contamination or "fishing" is present.

With 8 high density positive smears stained with 2 true negative smears, our results likely overestimate the rate that parasite transfer occurs in the normal setting. However, since a 1% false positive rate with serially collected smears can underestimate protective efficacy by $\geq$10% [3] in malaria prevention trials, single-slide staining must be done in this setting. It should also be done in all research settings when practical. In the clinical setting, malaria smears are often repeatedly dipped into Field's stain. The effect of cross-contamination in this setting should be studied, and the practicality and cost of single slide staining explored.

Clusters of parasites are commonly noted in our experience. However, we have not until recently realized that this may represent cross-contamination from other smears. We suspect this issue may contribute to the lower than expected sensitivity in reported results from new diagnostic assessments where microscopy is used as the gold standard. Batch staining is commonly used in this setting.

EDTA-anticoagulated blood is believed to worsen the ability of thick smears to adhere to glass slides. Heparin-anticoagulated blood is believed to adversely alter parasite staining and therefore is not routinely used. The best anticoagulant to use for making malaria smears should be systematically studied. Unanticoagulated blood adheres better to slides, but is often not used for practical reasons. The effects of Unanticoagulated versus anticoagulated blood on smear quality and reported results should also be assessed.

Thin smears, while the standard of care for malaria diagnosis in the Western world, are infrequently examined in the research setting, and often not even prepared in the clinical setting [12]. Thin smears should always be examined and results reported in most research settings. Thin smears will help confirm a positive smear is positive if batch staining is used, as parasites will not be intracellular. Microscopists will need to be specifically trained to identify this problem, based on the low reporting of this finding in this exercise. Thin smear reading will always help microscopists separate artifact from parasites, and confirm species when densities are adequate. Thin smears are usually not used in the developing world because of claim of poor sensitivity and time required to read them. We have conducted a pilot experiment to better define the sensitivity of the thin smear by parasite density, as well as time to identify parasites to better define how to interpret results [12]. The usefulness of the thin smear should continue to be better defined.

Ten minutes of drying is routinely used when results are rapidly needed for patient management. Overnight drying is routinely used to improve adherence of films to the slides when rapid turn around is not required. We confirmed that it clearly improved adherence of thick films. However, the effect on false positive rates was not as large as expected, and thick film loss continued to occur (table).

List of abbreviations

EDTA: Ethylene Diamine Tetra-Acetic Acid (purple top tube)
R1, R2, R3, Reader 1-3 (see methods)
WBCs: White Blood Cells
RBCs: Red Blood Cells

Authors' contributions

JO conceived of the design and conducted the experiments, analyzed and interpreted the data, and assisted with manuscript preparation. PO is lead microscopist/ phlebotomist for Malaria Diagnostics Center of Excellence who assisted data collection, interpretation of results, and editing of the manuscript. JSO is the expert microscopist who served as a R3 (reference reader)

and assisted with data interpretation. BO is Director of the Malaria Diagnostics Center of Excellence. He assisted with interpretation of data and manuscript preparation. AN was the lead visiting expert microscopist, who pointed out the batch staining methods being used were potentially problematic. He assisted data interpretation. CO served as mentor for experiment design, data collection, analysis, and interpretation of data. He analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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