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ACCURATE LIGHT MICROSCOPIC DIAGNOSIS OF SOUTHEAST ASIAN OVALOCYTOSIS

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ABSTRACT

Introduction: Southeast Asian ovalocytosis (SAO) is a common inherited red blood cell polymorphism in Southeast Asian and Melanesian populations, coinciding with areas of malaria endemicity. Validation of light microscopy as a diagnostic alternative to molecular genotyping may allow for its cost-effective use either prospectively or retrospectively by analysis of archived blood smears.

Methods: We assessed light microscopic diagnosis of SAO compared to standard PCR genotyping. Three trained microscopists, each assessed the same 971 Giemsa-stained thin blood films for which SAO genotypic confirmation was available by PCR. Generalized mixed modeling was used to estimate the sensitivity, specificity, positive predictive value, and negative predictive value of light microscopy versus "gold-standard" PCR.

Results: Among red cell morphologic parameters evaluated, knizocytes, rather than ovalocytic morphology, proved the strongest predictor of SAO status (odds ratio [OR] = 19.2; 95% confidence interval [95% CI] = 14.6-25.3; $P = <.0001$). The diagnostic performance of a knizocyte-centric microscopic approach was microscopist-dependent: two microscopists applied this approach with a sensitivity of 0.89 and a specificity of 0.93. Inter-rater reliability among the microscopists ($\kappa = .20$) as well as between gold standard and microscopist ($\kappa = .36$) underperformed due to misclassification of stomatocytes as knizocytes by one microscopist, but improved substantially when excluding the error-prone reader ($\kappa = .65$ and $= .74$, respectively).

Conclusion: Light microscopic diagnosis of SAO by knizocyte visual cue performed comparable to time-consuming and costlier molecular methods, but requires specific training that includes successful differentiation of knizocytes from stomatocytes.

Keywords: Southeast Asian ovalocytosis, knizocytes, light microscopy, inter-rater diagnostic performance, malaria

INTRODUCTION

Most of the common Mendelian diseases of humankind (e.g. sickle-cell disease, the thalassemias, and glucose-6-phosphate dehydrogenase deficiency [G6PD]) – are considered to offer a survival advantage against malaria, the strongest known selective pressure impacting the human genome.[1] The inherited red blood cell (RBC) polymorphism Southeast Asian ovalocytosis (SAO) arose in humans approximately 10,000 years ago likely as a consequence of malaria selection pressure.[2] Despite homozygous lethality *in utero*, the balanced polymorphism affords heterozygotes survival advantage against both falciparum[3,4] and vivax malarias,[5] and occurs on the Malay Peninsula, the Indonesian and Philippine archipelagos, and through much of Melanesia at prevalence rates as high as 30%.[6-9]

SAO is genotypically defined by a 27-base pair (bp) deletion in the erythrocyte band 3 gene (*SLC4A1*Δ*27*).[10] The locus encodes the band 3/anion exchanger AE1, an abundant transmembrane RBC glycoprotein mediating the electro-neutral exchange of chloride and bicarbonate ions.[11,12] Located at the boundary between the cytosolic domain and the first transmembrane segment of band 3,[10,13,14] the corresponding nine amino acid deletion severely abrogates anion transport.[13,15] Although the resultant RBC exhibits markedly reduced membrane mobility presumably due to increased band 3 binding to ankyrin,[16,17] clinical symptoms in children after three years of age[18] and adults is largely confined to sporadic associations with anemia.[8,19-20] The impact of this polymorphism on the clinical course of *P. falciparum* and *P. vivax* infection and transmission dynamics, including possible interaction with other co-inherited RBC variants (e.g. α-thalassemia,

G6PD, and Gerbich blood group) has been scarcely explored. A cost-effective, sensitive and specific tool to reliably diagnose SAO in retro- and prospective studies would expand practical pursuit of those explorations.

The presence of ovalocytes (RBCs with a length:width ratio >1:1 and <2:1), stomatocytes (RBCs with a single, slit-like central region of pallor) and knizocytes (triconcave RBCs with two or less frequently three pale regions separated by a narrow, well-hemoglobinized ridge)[8,21] are classic red cell morphologic alterations described in blood films of individuals with SAO. Morphologic diagnosis of SAO in epidemiologic studies typically relies upon a single parameter, ie. the degree of ovalocytosis, as the sole SAO-defining criteria. Wide variation in diagnostic criteria (cut-off values ranging from 25-50%),[22-27] along with inter- and intra-observer variability, were cited as some of the reasons that molecular probes,[28] employed in epidemiologic studies since the elucidation of the molecular defect underlying SAO in the early 1990s,[10] were deemed superior to peripheral smears. Few published studies since then have analyzed RBC morphologic parameters with genotypic diagnosis in hand. In the current retrospective study of 971 peripheral smears, each represented by PCR confirmation of SAO mutant or wildtype genotype, we set out to validate the most sensitive and specific methodology for the light microscopic diagnosis of SAO.

SUBJECTS, MATERIALS AND METHODS

Ethics Statement. This study is a secondary analysis of data and blood samples that were collected under a separate approved protocol. Both were approved by the Eijkman Institute Research Ethics Commission, and conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all subjects. Parents or guardians signed the informed consent for minors.

Study Design and Study Site. Study samples (blood smears and venous blood samples from 2,056 females) collected in hamlets surrounding two health centers in Southwest Sumba (Kodi Balaghar and Kodi Bangedo sub-districts), East Nusa Tenggara Province, Indonesia, were screened for malaria and G6PD deficiency in the field. Later at our laboratory in Jakarta, an inter-rater diagnostic performance design with cross-validation was used, whereby slides were held constant across microscopist, to assess both the diagnostic performance of light microscopic detection of SAO and the variability of this diagnostic performance among microscopist.

SAO Genotyping. The *SLC4A1* gene region (17q21.31) was amplified from genomic DNA isolated from EDTA anticoagulated blood using a modified 'salting out' method adapted from Lahiri and Nurnberger.[29] PCR amplification was as follows using the band 3-specific primer pairs originally described by *Jarolim et al*.[10] that span the deletion; 5'-GGGCCCAGATGACCCTCTGC-3' (bases 1098-1117); and 5'-GCCCGAAGGTGATGGCGGGTG-3 (bases 1271-1253). Briefly, 150ng DNA was

amplified in a 25µL PCR reaction containing GC buffer from KAPA PCR kit (Kapa Biosystems, Wilmington, MA, USA), 2.5mM of each deoxyribonucleotide phosphate, 0.25µM of each primer, and 0.125 units of Taq DNA polymerase (Kapa Biosystem). Initial denaturation was performed for 3 minutes at 94°C, followed by 30 cycles of the following; 30 seconds at 95°C for denaturation, 1 minute at 54°C for annealing, and 30 seconds at 72°C for extension. PCR products were separated on 2% agarose gels and visualized with UV light. DNA from normal individuals yielded the expected 175 bp band 3 product, whereas ovalocytic individuals had a 148 bp band indicating the deletion derived from the abnormal chromosome and the 175 bp band derived from the wild-type allele.

Thin Film Preparation and Slide Inclusion Criteria. Thin blood films were prepared immediately from venous blood without anticoagulant, air dried, methanol fixed, and stained with Giemsa. Low power (10x eye piece; 40x objective) light microscopy criteria used to evaluate the adequacy of thin films for RBC morphology includes; a) the presence of a feathered edge with a single cell layer suspension, b) appropriate Giemsa staining defined by the ability to discriminate distinct morphologic cytoplasmic and/or nuclear features of leukocytes, and c) freedom from considerable water/drying/staining artifact that could interfere with RBC morphology assessment. Of the 2,056 blood smears and venous samples collected in the field, 1,033 were randomly chosen for PCR confirmation (Figure 1). After excluding 29 samples due to non-diagnostic SAO PCRs, and excluding another 33 slides due to poor slide quality, 971 high quality malaria blood smears with SAO genotypic diagnosis remained.

RBC Morphologic Evaluation. Three suitable slide collections for which genomic DNA was available (primary proficiency test, n = 21; secondary proficiency test, n = 50; main study set, n = 971) were selected. Three expert microscopists systematically evaluated each blinded slide according to a standard operating procedure (SOP) that was refined at each of the three phases. RBC morphology was primarily assessed using the 40x objective, employing the 100x objective under oil immersion only to confirm borderline cases. Cell counting was performed with a graticule/cell counter and reported per 1000 RBCs as the denominator. The RBC morphology of the following variants was evaluated and recorded; ovalocytes, non-ovalocytic stomatocytes, and knizocytes, which are defined as follows; a) ovalocytes; RBCs with a length:width ratio >1:1 and <2:1; b) non-ovalocytic stomatocytes: RBCs with multiple linear and/or non-linear ('S', 'C', Greek letter [θ], or bizarre-shaped) pale regions; and c) knizocytes: triconcave RBCs with two or less frequently three pale regions separated by a narrow, well-hemoglobinized ridge.[8,21] The number of ovalocytic red cells was reported as either present (Yes) or absent (No) and further stratified as none (< 1%), Low (1-25%), or High (≥ 25%). Knizocytes and non-ovalocytic stomatocytes were recorded as either present (Yes) or absent (No). In the first proficiency test only, the exact percentage of ovalocytes and non-ovalocytic stomatocytes was recorded in lieu of a binary Yes/No response.

SAO Diagnostic Criteria & Reporting of Results. To render a diagnosis of SAO, the following diagnostic criteria were applied in the first proficiency test; a) ≥1% ovalocytosis and ≥25% non-ovalocytic stomatocytosis, or b) ≥1% ovalocytosis plus the

presence of knizocytes. In the second proficiency test, a diagnosis of SAO was rendered if all three morphologic features recorded (ovalocytes/stomatocytes/knizocytes) were present. In the main study set, a diagnosis of SAO was made if one or more knizocytes were detected in at least eight out of ten (80%) high powered fields screened at 100x. The presence of non-ovalocytic stomatocytosis although recorded, was excluded as part of the final diagnostic criteria. Equivocal results for a diagnosis of SAO were not allowed. All slides were independently read by all three microscopists, who entered their reports by hand into case forms.

Statistical Analysis. Analyses were conducted using SAS Software 9.4 (SAS Inc., Cary, NC). A sample of 971 slides (true positive [TP]: 167, true negative [TN]: 804) were read by three separate microscopists, totaling an overall sample of 2,913 observations. Positive and negative cases were randomly split into a training set (1,455) and a validation set (1,458), using the holdout method for cross-validation. Because slides were held constant across microscopist, generalized mixed modeling with sandwich estimation assuming a binary distribution was used to estimate sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), using PROC GLIMMIX. Other diagnostic performance markers calculated include positive and negative likelihood ratio (PLR and NLR), Youden's J, and ROC area under the curve (c-statistic). Agreement between microscopist and gold standard was estimated using Cohen's Kappa with PROC FREQ, because "gold standard" contains some error. Agreement among microscopists was estimated using Weighted Kappa with the SAS MACRO MAGREE. The relationship between independent categorical and dichotomous outcomes in bivariate analysis was

assessed with χ^2 and Fisher's exact (2-tail) tests. For continuous covariates, we derived Pearson or Spearman correlations for normally and non-normally distributed variables, respectively. Either *t*-tests or Wilcoxon rank sum test were used in bivariate analyses addressing between group differences for continuous normal and non-normal data, respectively. Significance was established *a priori* at the 0.05 level and all interval estimates were calculated for the 95% confidence.

RESULTS

Proficiency Testing. In the first proficiency test, microscopists evaluated the slides, 57% of which were SAO positive by PCR (12/21); with a sensitivity =1.0 and specificity =0.29. As the exact percentage of ovalocytosis and non-ovalocytic stomatocytosis was recorded in lieu of a binary Yes/No response in the first proficiency test, we began by comparing the mean percentages of each morphologic feature in SAO PCR positive versus negative cases. The mean percentage of ovalocytosis in this slide set in PCR positive and negative cases was 3.8% (95% confidence interval [95% CI]: 2.59-5.07) and 1.5% (95% CI: 0.07-2.93) respectively, and this difference was significant ($P = 0.012$). The mean levels of non-ovalocytic stomatocytosis, although not signficantly different ($P = 0.24$) between PCR positive (40.0%; 95% CI: 36.73-43.32) and negative cases (37.0%; 95% CI: 33.21-40.82), was much higher than the level of ovalocytosis. All slides met the criteria of either None (≤ 1%) or Low (1-25%) degree of ovalocytosis, as no slides with a high degree of ovalocytosis (≥25%) were recorded.

In the second proficiency test, diagnostic criteria were expanded to require knizocytes (due to the low specificity achieved in the first round). Microscopic evaluation of the slides (50% SAO positive by PCR [25/50]); yielded a sensitivity =0.71 and specificity =0.89. The two approaches employed in the first and second proficiency tests resulted in an unacceptable level of misclassification of SAO primarily due to the stomatocyte clause. Therefore, a knizocyte-dependent approach was chosen over the previous RBC morphology-centric approaches for the final analysis. For this approach, the presence or absence of knizocytes was the defining moprhologic criteria.

SAO Diagnostic Criteria Applied to Main Study Set.

Diagnostic performance. The genotypically confirmed prevalence of SAO in this study set (n = 971) was 17.2% (167/971). Table 1 lists RBC morphology and recorded SAO diagnosis of the genotypically defined thin smears. Using a knizocyte-centric diagnostic criteria, SAO was diagnosed if one or more knizocytes were identified in 100x fields in a minimum of 80% of 10 scanned fields. If stomatocytes or at least a low degree of ovalocytes were detected in the background and knizocytes were not detected in the requisite 80% of fields then an additional 10 fields were scanned at 100x. The detection of knizocytes in the majority of fields screened (>50%) in a background of stomatocytosis of ovalocytosis again prompted a diagnosis of SAO. Using this approach, the three microscopists evaluated the slides with a sensitivity of 0.92, a specificity of 0.74, a PPV of 0.28, and a NPV of 0.98 (Table 2). These results were confirmed with the validation set (also in Table 2). As a high rate of non-ovalocytic stomatocyte misclassfication as knizocytes was recorded by microscopist 1 compared to microscopists 2 and 3 (knizocytes were detected in 62.5% of non-SAO slides, compared to 7.2% and 5.8% detection in non-SAO slides by microscopists 2 and 3 [Table 1]), removal of microscopist 1 from the data set slightly decreased the sensitivity to 0.89, though NPV did not change, while dramatically increasing the specificity to 0.93 and the PPV to 0.69. As indicated in Table 2, PLR, NLR, and Youden's J all increased greatly after removal of the reports of microscopist 1.

Inter-rater reliability. Agreement among the three microscopists was slight to fair (κ = .20-.23) but became substantial (κ = .64-.66) when the findings of microscopist 1 were excluded. Because PCR or the "gold standard" has some error, agreement was also calculated between microscopists and PCR. The overall Kappa was 0.36 (Figure 2), which is substantially lower than the Kappa values achieved by microscopists 2 and 3 due to the relatively inaccurate findings of microscopist 1, without which the overall Kappa almost doubled to 0.65 (Table 2).

Correlation of Knizocytes with SAO status. Knizocytes alone are a strong predictor of SAO status irrespective of ovalocytic level or any other additional morphologic features (odds ratio [OR] = 19.2; 95% CI =14.6-25.3; *P* = <.0001). The high rate of misclassification of stomatocytes as knizocytes by microscopist 1, disqualified those reports from the analyses described here. Individual odds ratio assessment by microscopists 2 and 3 for the detection of SAO by knizocyte alone was 113.5 (95%CI: 64.3 – 200.4; P<0.0001) and 47.9 (95%CI: 30.3-75.7; P< 0.0001), respectively. The absence of knizocytes as the sole diagnostic criteria correlated with a NPV of 0.98 and 0.95, respectively for microscopists 2 and 3. Conversely, when knizocytes are present, the PPV for these microscopists was 0.72 and 0.73. When additional morphologic details such as level of ovalocytosis (irrespective of knizocytes) were added to derive a diagnosis of SAO, the NPV value either remained unchanged or increased slightly (0.98 and 0.96, respectively). The PPV in turn either remained unchanged 0.71 (microscopist 2) or declined to 0.62 (microscopist 3). Knizocytes are also strongly correlated with a high

level of ovalocytosis (Phi categorical correlation =0.85); these results were confirmed in the validation set.

Impact of Patent Parasitemia on the Prevalence of SAO and Knizocytes. The prevalence of patent *P. falciparum* parasitemia in this study set was 5.8% (56/971). Excluding rare *P. vivax, P. malariae,* and mixed *P. falciparum* and *P. vivax* cases, the prevalence of SAO or knizocytes in slides with and without patent *P. falcipaum* parasitemia was not significantly different (P = 0.11 and 0.49, respectively).

DISCUSSION

The RBCs of SAO are often described as being stomatocytic elliptocytes with a slit-like area of central pallor or resembling the Greek letter theta (θ). In a small proportion of these stomatocytes, two or less frequently three pale regions separated by a well hemoglobinized ridge are apparent, giving the appearance of double stomatocytes,[21] ie. knizocytes (Figure 3). Although, not pathognomonic for SAO, this study highlights the fact that knizocytes (and their correct classification), and not ovalocytic morphology are key to the accurate diagnosis of SAO by a stained peripheral blood smear. O'Donnell *et al*[30] in Papua New Guinea found red cells with two or more linear or irregularly-shaped pale regions (ie. knizocytes) also were the most consistent feature of SAO in microscopy. These predicted SAO with high sensitivity (93.8%) and specificity (99.1%). Our findings

corroborate those and both studies demonstrate the good diagnostic performance achieved with a specific focus on knizocytes.

Knizocytes are very rarely encountered in the blood of healthy adults in routine practice (even though their percentage was once estimated at 0.6% ± 0.5 in healthy controls [min-max values: 0-2.5%]).[31] These RBC morphologic variants however are frequently detected in the peripheral smears of newborns,[32] in the context of acute and chronic liver dieseas secondary to alcohol and viral hepatitis where they can account for up to 15% of red cells,[33-35] and in patients with familial lechitin/cholesterol acyltransferase deficiency.[36] Membrane deformability appears to be a common denominator in all of these conditions/disorders and therefore an important determinant of the knizocyte morphology. SAO is associated with decreased lateral and rotational mobility of the membrane due to increased band 3 binding to ankyrin,[16,17] neonatal RBCs exhibit impaired membrane deformability, liver disease can influence RBC morphology by alterating membrane lipid content,[35,37] and decreased membrane deformability is associated with familial lechitin/cholestrol acyltransferase deficiency.[36]

The PPV associated with the knizocyte-centric diagnostic approach, never exceeded 0.75. This could in part be explained by the high prevalence of α-thalassemia in Southeast Asia.[38,39] Knizocytes have been described in association with this disorder of hemoglobin synthesis, although this microcytic hypochromic anemia is usually accompanied on the peripheral smear by target cells (codocytes), schistocytes, and in some cases heinz bodies.[40] This study did not capture additional red cell morphologic variants such as target cells, schistocytes, and macro-ovalocytes which if incorporated into the diagnostic criteria, may have greatly reduced the number of false positives.

The markers of diagnostic performance and concordance in this study draw one clear conclusion: accuracy hinges on the training and judgement of the microscopist, which will inherently vary among individuals. Nonetheless, provided appropriate and relatively simple visual training and practice, the light microscopic diagnostic criteria described herein proved powerful predictors of SAO status, comparable to far more expensive and laborious molecular diagnostics. These data in aggregate suggest that recognition of knizocytes is key to the diagnosis of SAO, and that the inclusion of additional morphologic criteria such as percent non-ovalocytic stomatocytosis or degree of ovalocytosis provides only slight incremental improvement to the NPV, with either no improvement or a declination in the PPV. The capture of additional morphologic parameters (e.g. target cells and schistocytes) would likely boost the specificity of this approach, which is ultimately limited by the variable morphologic expression of SAO genotype and the not insignificant number of morphologically normal, SAO positive slides. Furthermore, additional RBC morphologic variants that may be associated with SAO such as macro-ovalocytes, macro-ovalostomatocytes and/or macro-ovaloknizocytes (personal communication) require further investigation as they were not studied in depth in our analysis.

In summary, a systematic analysis of microscopic diagnostic approaches to SAO-known specimens led us to focus on the knizocyte phenotype of this inherited blood disorder. Doing so allowed trained microscopists to provide a diagnosis of SAO with high sensitivity and specificity, albeit imperfectly with 1 of 3 microscopists not realizing accurate recognition of knizocytes. The microscopic diagnosis of SAO may allow

relatively simple and inexpensive surveys of this disorder whenever mass blood surveys are undertaken.

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CONFLICT OF INTEREST

None of the authors have any potential conflicts of interest to report.

AUTHOR CONTRIBUTION

AWS, AH, LVP, LLE, and IS performed the research; CPN, AWS, GLB, and JKB designed the research study; AWS, AH, DS, and JKB contributed the malaria blood smears and SAO genotypic data; CPN, GLB, and JKB analyzed the data; CPN, AWS, GLB, and JKB wrote the paper.

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TABLES

Table 1: Red blood cell morphologic parameters assessed by 3 microscopists in n = 971 blinded slides genotypically screened for SAO.

	Microscopist #1		**Microscopist #2**		**Microscopist #3**	
	Non-SAO	SAO	Non-SAO	SAO	Non-SAO	SAO
N	804	167	804	167	804	167
Ovalocyte	584 (72.6) *	163 (97.6)	794 (98.7)	167 (100)	122 (15.1)	142 (85.0)
Non-ovalocytic stomatocyte	804 (100)	167 (100)	770 (95.7)	167 (100)	803 (99.8)	167 (100)
Knizocyte	503 (62.5)	159 (95.2)	58 (7.2)	150 (89.8)	47 (5.8)	125 (74.8)
Ovalocytic Level None (<1%) Low (1-25%) High (>25%)	 318 (39.5) 481 (59.8) 5 (0.6)	 9 (5.4) 136 (81.4) 22 (13.2)	 4 (0.5) 743 (92.4) 57 (7.1)	 0 19 (11.4) 148 (88.6)	 0 781 (97.1) 23 (2.8)	 0 83 (49.7) 84 (50.3)
Diagnosis SAO Negative Positive	 302 (37.5) TN† 502 (62.4) FP‡	 8 (4.8) FN§ 159 (95.2) TPψ	 742 (92.3) TN 62 (7.7) FP	 14 (8.4) FN 153 (91.6) TP	 718 (89.3) TN 86 (10.7) FP	 29 (17.4) FN 138 (82.6) TP

* Numbers in parentheses represent percentage of total (N) for each column.
† TN = true negative
‡ FP = false positive
§ FN = false negative
ψ TP = true positive

Table 2. Training and validation diagnostics, for all and selected microscopists.

	All*				Removed†			
Training Set	**Estimate**	**SE‡**	**95% CI§**		**Estimate**	**SE**	**95% CI**	
Sensitivity	0.92	0.02	0.88	0.95	0.89	0.03	0.82	0.93
Specificity	0.74	0.01	0.72	0.76	0.93	0.01	0.9	0.94
PPV	0.28	0.03	0.22	0.34	0.69	0.04	0.59	0.76
NPV	0.98	0.005	0.97	0.99	0.98	0.01	0.96	0.99
Kappa (Test by Truth)	0.36	0.02	0.33	0.39	0.74	0.03	0.68	0.79
Kappa (Microscopist)	0.2	0.03			0.65	0.05		
Positive LR	3.52				11.8			
Negative LR	0.1				0.12			
ROC-AUC	0.70	0.01	0.68	0.72	0.84	0.02	0.80	0.87
Youden's J	0.66				0.81			
Validation Set	**Estimate**	**SE**	**95% CI**		**Estimate**	**SE**	**95% CI**	
Sensitivity	0.90	0.02	0.85	0.94	0.87	0.03	0.81	0.92
Specificity	0.72	0.01	0.7	0.75	0.90	0.01	0.88	0.92
PPV	0.27	0.03	0.21	0.33	0.61	0.05	0.52	0.7
NPV	0.98	0.01	0.96	0.99	0.97	0.01	0.96	0.98
Kappa (Test by Truth)	0.34	0.02	0.31	0.38	0.67	0.03	0.61	0.73
Kappa (Microscopist)	0.23	0.03			0.65	0.05		
Positive LR	3.27				9.16			
Negative LR	0.13				0.14			
ROC-AUC	0.68	0.01	0.66	0.71	0.80	0.02	0.77	0.83
Youden's J	0.62				0.77			

* All three microscopists.
† Analysis restricted to microscopists 2 & 3 only.
‡ SE = standard error
§ 95% CI = 95% confidence interval

FIGURE CAPTIONS

Figure 1. Flow chart of samples used in our inter-rater diagnostic performance study.

Figure 2. Kappa coefficients of diagnostic performance by PCR with 95% confidence limits for each microscopist.

Figure 3. Morphologic consequence of the variable expression of SAO as observed on Giemsa stained peripheral smears with examples of true positives (A & B), a false negative (C) and a false positive (D). A) High degree of ovalocytosis along with red cells with multiple pale regions separated by a well-hemoglobinized ridge, e.g. knizocytes. B) Low degree of ovalocytosis along with the presence of a knizocyte. C) Genotypically positive for SAO, but a high proportion of normocytes without evidence of ovalocytosis or knizocytes. D) Genotypically negative for SAO, but positive for a knizocyte. Knizocytes are highlighted in panels A, B, and D with blue arrows.

FIGURES

Figure 1.

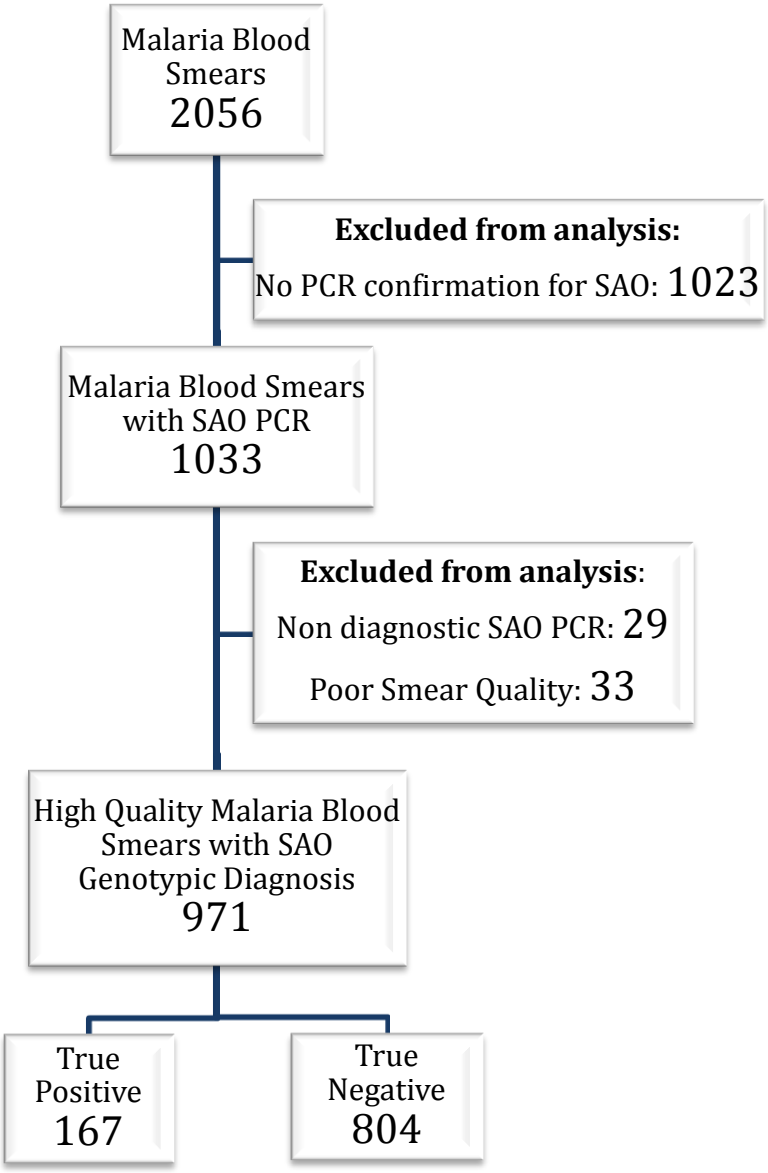

Figure 2.

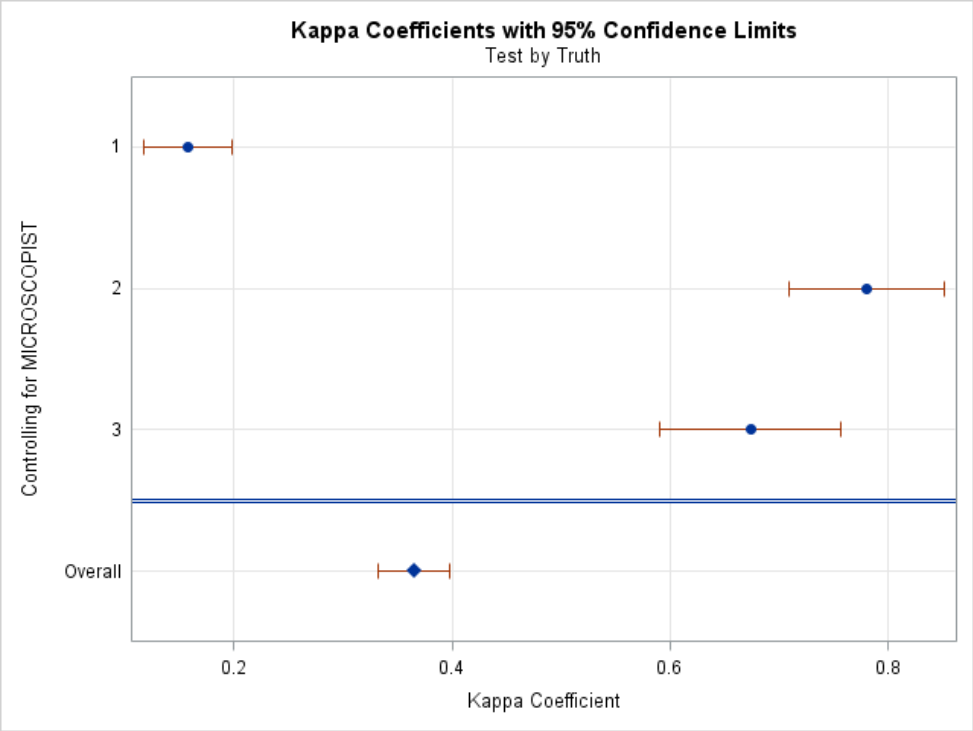
Kappa Coefficients with 95% Confidence Limits
Test by Truth
Controlling for MICROSCOPIST
1
2
3
Overall
0.2
0.4
0.6
0.8
Kappa Coefficient

Figure 3.