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ORIGINAL ARTICLE

Detection of *Plasmodium vivax* using Automated Hematology Analyzer in the Korean Army

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SUMMARY

Background: **This study aimed to evaluate whether our equation model developed from the Sysmex hematology analyzer can discriminate patients with *Plasmodium vivax* (*P. vivax*) infection from those with acute febrile illness (AFI) and healthy controls. Besides, we compared our model with the previously studied models.**
Methods: **A total of 312 blood samples were collected from the *P. vivax*, AFI, and healthy control groups. All samples were tested for routine complete blood count conducted by using a Sysmex XE-2100 or XE-5000 analyzer. We compared the reportable and research parameters generated from the Sysmex analyzer among the three groups. The selected parameters that showed a significant difference between the *P. vivax* and the other group were included in the logistic regression analysis to develop our model (N-OI$_{pv}$ model). Moreover, we analyzed the CBC data according to the previous models, such as the presence of abnormal blue coded events in the WBC/BASO scattergram called the observer-interpretation (OI$_{pv}$) model, and the previous equation model (N-OD1$_{pv}$ model) developed by Campuzano-Zuluaga et al.**
Results: **The N-OI$_{pv}$ model, which consists of three parameters, such as mean cell volume, plateletcrit, and Lymph-X, showed the best performance for detection of malaria (97.4% accuracy). Also, this model can increase the sensitivity by about 11.9% to 18.1% compared with the OI$_{pv}$ and N-OD1$_{pv}$ models, respectively.**
Conclusions: **We concluded that the N-OI$_{pv}$ model using the Sysmex hematology analyzer is a useful diagnostic tool in the routine laboratory workup for malaria.**
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INTRODUCTION

The automatic hematology analyzer has been studied for malaria diagnosis. Although the results from the hematology analyzer are not complete in comparison to microscopic examination, the hematology analyzer shows promise of future application to the diagnosis of malaria [1]. The automatic hematology analyzer has an

advantage in the rapid detection of malaria. Automated hematology analyzers are used for performing complete blood count (CBC), which is a routine laboratory test ordered for patients with fever, and therefore, the automatic hematology analyzer will be an ideal screening method for malaria detection. Currently, the standard method for malaria diagnosis is the microscopic examination of a Giemsa-stained thick and thin blood smear [2]. However, microscopic examination for malaria is time-consuming, and its accuracy varies depending on the examiner [3]. Therefore, it is necessary to develop an automatic detection system using the hematology analyzer to improve the shortcomings of microscopic diagnosis.

For the past 20 years, various automatic hematology analyzers such as Cell-Dyn (Abbott Diagnostics, Santa Clara, CA, USA), Coulter GEN·S, LH 750 and DxH800 (Beckman Coulter Inc., Miami, FL, USA), and the Sysmex XE-2100 analyzers (Sysmex Corporation, Kobe, Kansai, Japan) have been studied for malaria diagnosis [1]. These analyzers can differentiate cell components of the blood by their size and internal granularity, and nuclear parts mainly based on the flow-cytometry technology [4]. Various parameters based on flow-cytometry detection may indicate the presence of malarial parasites or parasite debris. In the case of the Cell-Dyn analyzer, there are abnormal depolarization events during flow cytometry analysis because malaria-infected blood contains the hemozoin pigment in parasitized red blood cells and phagocytic cells [5,6]. Studies of detection of malaria by the Sysmex analyzer have shown abnormal patterns or numbers on flow cytometry analysis, such as unusual patterns in DIFF, WBC/BASO, and RET-EXT scatter-plots, and pseudo eosinophilia [7-11]. However, these parameters do not provide numerical data; namely, detection of malaria was manually performed by trained personnel [7,9,10,12]. To establish an automated model applied for a 'malaria alarm' in a routine laboratory setting, a recent study developed two non-observer dependent models (N-OD$_{PV}$) for *Plasmodium vivax* [8]. One model (N-OD1$_{PV}$) used three numerical variables, including Delta DIFF/WBC, LYM-PH-Y, and plateletcrit (PCT), and the other model (N-OD2$_{PV}$) consisted of PLT-O and counting the number of abnormal dots in the WBC/BASO scattergram defined by the authors. They found that the diagnostic accuracy of the N-OD1$_{PV}$ and N-OD2$_{PV}$ models was 94.7% and 96.8%, respectively. Between the models mentioned above, only the N-OD1$_{PV}$ can be processed by the laboratory information system (LIS) because it includes all factors that constitute the analyzer's numerical data. It was crucial in terms of screening where all clinical samples can provide a 'malaria alarm' by LIS without a request for malaria examination.

This study aimed to develop and evaluate the diagnostic performance of our equation model (N-OI$_{pv}$ model) for automated *P. vivax* detection. We also compared our model with the previous two models, N-OD1$_{PV}$ and abnormal dots in the WBC/BASO scattergram called observer-interpretation (OI$_{pv}$) model.

MATERIALS AND METHODS

Subjects

A total of 312 blood samples, consisting of 94 samples from primary *P. vivax* infection cases and 5 samples from *P. vivax* relapse cases, 107 samples from acute febrile illness (AFI) cases, and 106 samples from healthy controls, were included between April 2015 and September 2015. *P. vivax* and AFI cases among soldier patients deployed to malaria-endemic areas were recruited at Armed Forces Yangju Hospital, Armed Forces IL-dong Hospital, and Armed Forces Goyang Hospital. Two experienced laboratory physicians diagnosed *P. vivax* cases by reviewing Giemsa-stained blood smears. The examiner observed two hundred high power fields and the following formula calculated parasitemia:

$$\text{Parasitemia (number/}\mu\text{L)} = \frac{\text{Number of parasites oberved} \times \text{total WBC from the Sysmex analyzer}}{200}$$

Although all malaria-positive patients were treated with the WHO standard regimen, including oral administration of hydroxychloroquine (600 mg base at 0 hour and 300 mg base at 6, 24, and 48 hours) followed by primaquine (15 mg/day, for 2 weeks), five cases of *P. vivax* relapse between 24 and 44 days after treatment were also included in the *P. vivax* group. The AFI cases that had fever were requested to undergo malaria workup through a clinician and were diagnosed with malaria negative. In the case of healthy controls, we randomly selected the patients who visited the Korea University Anam hospital for a medical check-up, lived in malaria nonendemic areas, and retrospectively collected their CBC data. Subjects with *P. vivax* infection and AFI were asked for their informed consent under the protocol approved by the Ethics Committee of the Armed Forces Medical Command (AFMC-15003-IRB-15-003).

Automated hematology analyzer

All samples were collected into K_2EDTA tubes and tested for CBC with Sysmex XE-2100 or XE-5000 analyzers within 1 hour after blood draw. The device performs blood cell analyses according to the radiofrequency (RF)/direct current (DC) detection method, sheath flow DC detection, flow cytometry method, and SLS-hemoglobin method. Briefly, hemoglobin (HGB) is measured after lysis of red blood cells (RBCs) and then it is converted into SLS-hemoglobin and the number of RBCs and platelets (PLT) is determined using sheath flow DC detection. The PLT count is also determined using sheath flow DC detection or fluorescence technology, allowing for an optical PLT count (PLT-O).

The white blood cell (WBC) count is performed in two different channels (WBC/BASO and WBC/DIFF) with a flow cytometry method using a semiconductor laser, and both results are compared in each case. A WBC 4-part differential (DIFF scattergram) is obtained (lymphocytes, monocytes, eosinophils, and neutrophils + basophils) by flow cytometry using their light scattering and fluorescence characteristics. When an abnormal signal appears in several scattergrams, including the WBC/BASO and DIFF scattergram, suspect flags (manufacturer defined) are generated, related to qualitative changes. In the DIFF scattergram, the central part of the neutrophils and the lymphocyte areas are determined according to the x-axis and y-axis (NEUT-X, NEUT-Y, LYMPH-X, and LYMPH-Y) and reported as research parameters [11].

Malaria detection model using Sysmex XE-2100 analyzers

The equation type model, called the N-OI$_{pv}$ model, was designed to compute the predicted probability (PP) of malaria from LnR by following the methods transformed by a previous study [8]. Briefly, the numerical data from the Sysmex analyzer, including 24 clinical variables and 14 technical variables, were used to compare between the *P. vivax* group vs. the AFI group and the *P. vivax* group vs. the healthy control group using one-way ANOVA with posthoc Tukey HSD test. The variables selected through ANOVA analysis according to $p < 0.05$ of inclusion criteria were included in the Logistic regression analysis (LR) to predict whether a patient has a *P. vivax* infection. The PP was calculated using a stepwise analysis with the forward-conditional method, which was executed by a p-value of 0.1 as the criterion for exclusion and a p-value of 0.05 as the criterion for entry of variables. Moreover, we evaluated the diagnostic performance of the two previously reported models [8]. First, the OI model, referred to as the visually determined parameter, was the presence of prominent blue coded events in the WBC/BASO scattergram, located in low FSC and medium SSC areas (area II and area III) (Figure 1). It was applied to the *P. vivax* and AFI groups to assess their diagnostic ability. Second, we adopted the N-OD1$_{PV}$ model to evaluate the diagnostic performance. The N-OD1$_{PV}$ model also provides the PP according to the following formula:

$$\mathrm{PP} = \frac{1}{1+e^{-\left(-74.13-(20.5\times \mathrm{PCT})+(46\times \mathrm{Delta-WBC})+(0.45\times \mathrm{LYMPH-Y})\right)}}$$

Validation of N-OI$_{pv}$ model

The new N-OI*pv* model was validated in 5,493 hospitalized patients with no malaria infection in non-endemic areas. For model validation samples, microscopic exam was performed using the remaining EDTA samples after CBC test, and we confirmed that there was no malaria infection.

Statistical analysis of the results

Data entry was performed using Excel 2007 (Microsoft Corporation, Redmond, WA, USA). Thirty-eight numerical variables generated from the Sysmex analyzer among the three groups were subjected to a one-way ANOVA analysis with the posthoc Tukey HSD test to determine the factors for entry into the LR analysis. One-way ANOVA and LR were analyzed using SPSS v12 (SPSS Inc., Chicago, IL, USA). The optimal cutoff values of the PP in the N-OI$_{pv}$ model to differentiate the *P. vivax* group from the malaria-negative groups, including the AFI and healthy control groups, were determined by the receiver-operating characteristic (ROC) curve analysis. A comparison of ROC curve analysis between the N-OI$_{pv}$ and N-OD1$_{PV}$ models was performed with MedCalc version 9.5.2.0 (MedCalc Software, Mariakerke, Belgium). The sensitivity and specificity of malaria detection models were evaluated based on the microscopic examination as a reference method. We used the chi-squared test for the comparison of proportions between the *P. vivax* and AFI groups.

RESULTS

A total of 312 samples were included in this study. All participants ware men. The demographics of the subjects covered in each group are presented in Table 1. In the *P. vivax* infected samples, parasitemia ranged from 39/µL to 19,564/µL (mean 2,435/µL). Malarial parasites were not found in any of the AFI cases and healthy controls. Among the AFI cases, 62.3% of the patients were diagnosed with lower respiratory tract infection, 22.6% of the patients were diagnosed with nonspecific febrile syndrome, 6.6% of the patients were diagnosed with upper respiratory tract infections, 4.7% of the patients were diagnosed with gastroenteritis, 1.9% of the patients were diagnosed with respiratory tuberculosis infection, and 1.9% of the patients were diagnosed with urinary tract infections.

The CBC parameters in the *P. vivax* group were compared with those in the malaria-negative groups using a one-way ANOVA test. Among them, statistically significant variables are presented in Table 2. Briefly, there was a substantial decrease in the WBC, PLT, and lymphocyte counts, and an increase in eosinophil counts in the *P. vivax* group compared with the malaria-negative group. The PDW, MPV, and P-LCR were significantly higher in the *P. vivax* group. In contrast, mean corpuscular volume (MCV) and PCT in the *P. vivax* group were substantially lower than those in the malaria-negative groups [13]. The mean of the MCHC in the *P. vivax* group was located between those in the AFI and healthy control groups. There were significant differences in the X and Y axes values (side scatter of light; LYMPH-X and side fluorescence of light; LYMPH-Y) in the WBC-diff scattergram for lymphocytes in the *P. vivax* group compared with the malaria-negative group. The ratio between the WBC count via the DIFF-channel

Table 1. Demographics of the subjects in the *P. vivax*, acute febrile illness, and healthy control groups.

Feature	*P. vivax*	Acute febrile illness	Healthy controls
No. of subjects	99	107	106
Age			
Mean ± SD	21.7 ± 2.19	20.6 ± 2.47	28.4 ± 4.38
Range	19 - 33	18 - 37	19 - 35

Table 2. Variables included in the logistic regression analysis and comparison of CBC variables among the three groups.

Variables	*P. vivax* (n = 99)		*P. vivax* vs. AFI (p-value)	AFI (n = 107)		*P. vivax* vs. Healthy controls (p-value)	Healthy controls (n = 106)		Overall ANOVA
	Mean	S.D.		Mean	S.D.		Mean	S.D.	
White blood cells (× 10^3/μL)	4.67	1.83	< 0.001	7.79	4.10	0.002	5.99	1.49	< 0.001
MCV (fL)	84.68	2.75	< 0.001	87.46	2.96	< 0.001	88.42	3.63	< 0.001
MCHC (g/dL)	35.15	0.84	< 0.001	33.99	0.85	< 0.001	34.44	0.77	< 0.001
PLT (× 10^3/μL)	70.84	36.20	< 0.001	197.54	67.91	< 0.001	250.61	50.72	< 0.001
PDW (fL) *	13.28*	2.90	< 0.001	11.69	2.17	< 0.001	11.85	1.42	< 0.001
MPV (fL) *	10.70*	1.04	< 0.001	10.02	0.93	0.002	10.25	0.78	< 0.001
P-LCR (%)*	30.98*	8.57	< 0.001	24.98	7.63	< 0.001	26.55	6.48	< 0.001
PCT (%)*	0.08*	0.03	< 0.001	0.19	0.06	< 0.001	0.26	0.05	< 0.001
Lymphocyte (× 10^3/μL)	0.83	0.53	< 0.001	1.30	0.58	< 0.001	2.14	0.59	< 0.001
Eosinophil (× 10^3/μL)	0.33	0.53	< 0.001	0.06	0.15	0.047	0.22	0.15	< 0.001
LYMPH-X (arbitrary units)	87.05	3.87	< 0.001	82.42	1.41	< 0.001	84.86	1.29	< 0.001
LYMPH-Y (arbitrary units)	72.14	7.61	< 0.001	67.86	2.72	< 0.001	68.87	2.64	< 0.001
Delta-WBC (DIFF/WBC) ratio	1.13	0.24	< 0.001	0.99	0.03	< 0.001	1.01	0.03	< 0.001

* Ninety-four samples were included because 5 samples with very low platelet counts could not produce the data for these variables by the Sysmex hematology analyzer.

Table 3. Comparison of hematological findings among the *P. vivax*, AFI and healthy control groups.

Hematological finding	No. of cases (%)		
	P. vivax	AFI *	Healthy controls
Anemia (Hb < 12 g/dL), exclusively	1 (1.0)	3 (2.8)	1 (0.9)
Leukopenia (WBC < 4 × 10^3/μL), exclusively	0 (0.0)	9 (8.4)	2 (1.9
Thrombocytopenia (150 × 10^3/μL), exclusively	52 (52.5)	18 (16.8)	1 (0.9)
Bicytopenia	44 (44.4)	6 (5.6)	0 (0.0)
Pancytopenia	1 (1.0)	0 (0.0)	0 (0.0)
No specific finding	1 (1.0)	71 (66.4)	102 (96.2)
Total	99 (100)	107 (100)	106 (100)

*AFI: Acute febrile illness.

Table 4. Variables used in the malaria diagnostic model for *P. vivax* positive patients and regression equations of the previously established models and the newly developed model.

Variables entered	Diagnostic model	
	Previous N-OD1$_{pv}$ model mean ± standard error	New N-OI$_{pv}$ model mean ± standard error
MCV		84.68 ± 0.276
PCT (%)	0.077 ± 0.003	0.077 ± 0.003
LYMPH-X (arbitrary units)		87.05 ± 0.389
LYMPH-Y (arbitrary units)	72.14 ± 0.76	
Delta-WBC (DIFF/WBC) ratio	1.13 ± 0.025	

$$\text{Previous N} - \text{OD1}pv : \frac{1}{1 + e^{-(-74.13-(20.5\times\text{PCT})+(46\times\text{Delta}-\text{WBC})+(0.45\times\text{LYMPH}-\text{Y}))}}$$

$$\text{New N} - \text{OI}pv : \frac{1}{1 + e^{-(-17.209-(75.528\times\text{PCT})-(0.548\times\text{MCV})+(0.876\times\text{LYMPH}-\text{X}))}}$$

Table 5. Performance results of the diagnostic models generated for identifying *P. vivax* and distinguishing it from acute febrile illness.

Model	Cutoff	Area under the curve (95% CI)	TP[a]	TN[b]	Sensitivity (95% CI)	Specificity (95% CI)
New N-OI$_{pv}$ (n = 307) [c]	> 0.3441	0.992 (0.975 - 0.999)	89/94	210/213	94.7% (88.0 - 98.3)	98.6% (95.9 - 99.7)
Previous N-OD1$_{pv}$ (n = 307) [c]	> 0.8367	0.882 (0.840 – 0.916)	72/94	206/213	76.6% (66.7 – 84.7)	96.7% (93.3 – 98.7)
OI $_{pv}$ (n = 206) [d]	-	-	82/99	105/107	82.8% (73.9 - 89.7)	98.1% (93.4 - 99.8)

[a] True positive, [b] True negative, [c] After excluding the five samples with low platelet counts because no PCT was presented, [d] Without including healthy controls because we unfortunately did not collect the data.

Table 6. Sensitivity of new models (New N-OI$_{pv}$) according to parasitemia.

Parasitemia (/µL)	Cases	Sensitivity	95% Confidence Interval
<1,000	46/49	93.88 %	83.13% to 98.72%
>1,000	46/48	95.83%	85.75% to 99.49%

and the WBC count via the WBC/BASO channel, called Delta WBC (DIFF/WBC), was significantly higher in the *P. vivax* group than in the malaria-negative groups. Hematological findings of the *P. vivax* group and the malaria-negative groups are shown in Table 3. Isolated thrombocytopenia was observed in 52.5% of the cases followed by bicytopenia (44.4%), pancytopenia (1.0%), and isolated anemia (1.0%) in the *P. vivax* group. On the other hand, no specific finding was observed in 66.4% of the cases, followed by isolated thrombocytopenia (16.8%), isolated leukopenia (8.4%), bicytopenia (5.6%), and isolated anemia (2.8%) in the AFI group. The healthy control group rarely showed any abnormal hematological findings. The results for comparison of the proportion difference in thrombocytopenia (PLT < 150×10^3/µL) between the *P. vivax* and AFI groups was 75.6% (95% CI = 65.1% - 83.3%) and this difference was statistically significant (p-value < 0.0001). The proportion difference in leukopenia (WBC < $4{,}000 \times 10^3$/µL) between the *P. vivax* and AFI groups was 24.4% (95% CI = 11.8 - 36.3%), and this difference was also statistically significant (p-value = 0.0001). The pro-

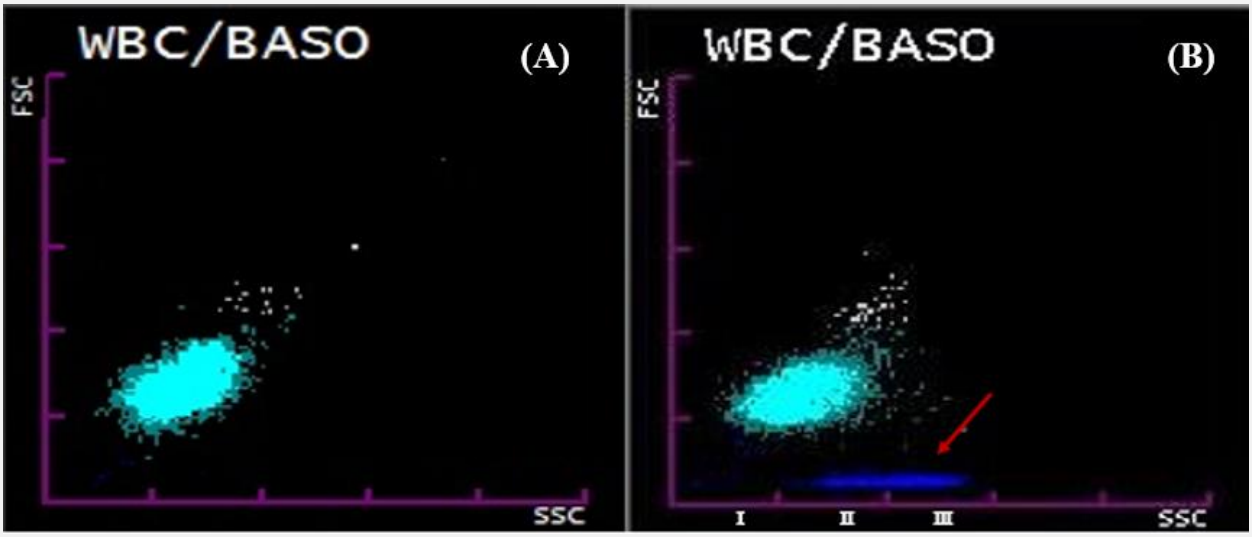

Figure 1. Representative WBC/BASO scattergram images in the AFI (A) and *P. vivax* (B) groups. Horizontal axis represents the side-scattered light and vertical axis represents the forward-scattered light. The low to medium SSC areas are divided arbitrarily into three parts, called area I, II, and III. The red arrow indicates the presence of the prominent blue coded events that are part of the OI_{pv} model.

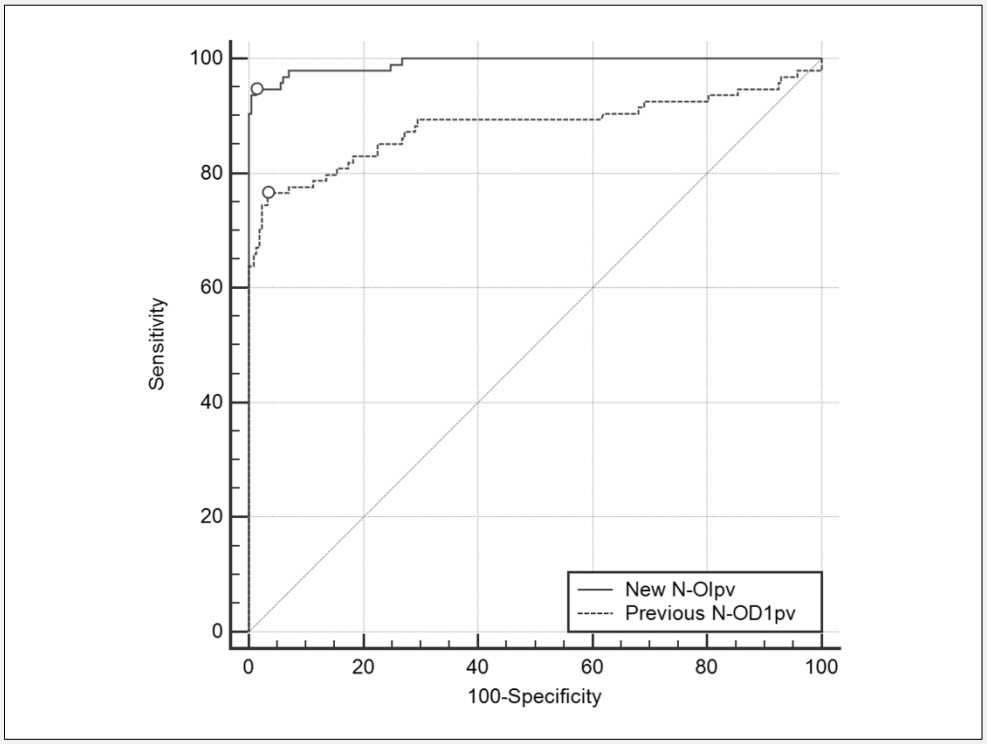

Figure 2. Diagnostic performance of the N-OI_{pv} model and the N-$OD1_{PV}$ model by comparison of the receiver-operating characteristic (ROC) curve analysis.

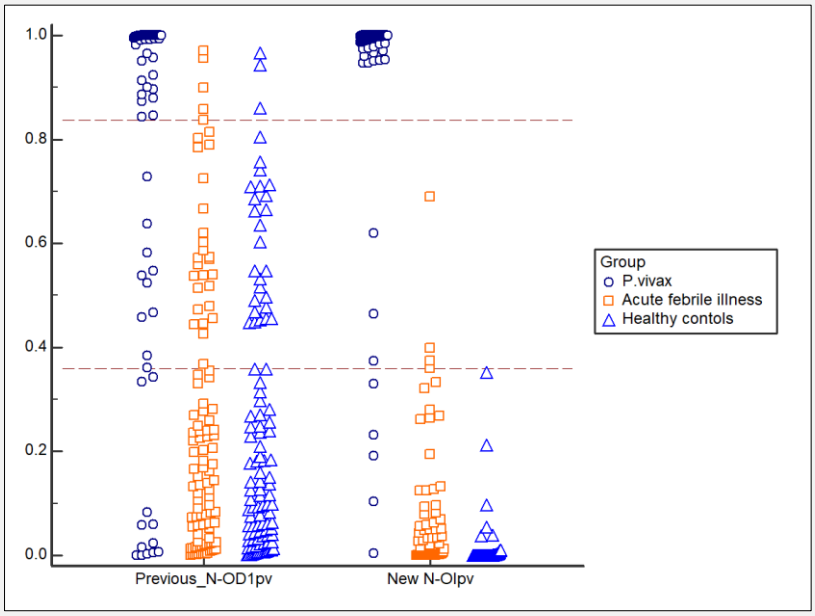

Figure 3. Distribution of the predicted probability from the N-OI$_{pv}$ and N-OD1$_{pv}$ models among the *P. vivax*, AFI, and healthy control groups. The upper transverse dotted line represents the cutoff value (> 0.8367) of the N-OD1$_{pv}$ model, and the lower transverse dotted line represents the cutoff value (> 0.3441) of the N-OI$_{pv}$ model.

portion of leukocytosis (WBC > 10,000 × 10^3/μL) was higher in the AFI group than in the *P. vivax* group (23.4% vs 2.0%, p-value < 0.0001).

According to the inclusion criteria (p < 0.01), the CBC parameters that were significantly discriminatory between the *P. vivax* and malaria-negative groups were selected, and then the equation model was generated by LR using SPSS software (Table 4). The new N-OI$_{pv}$ model consisted of three variables, MCV, PCT, and LYMPH-X. The previous N-OD1$_{pv}$ model also included three variables, the PCT, the Delta-WBC (DIFF/WBC) ratio, and the LYMPH-Y. Inevitably, five samples in *P. vivax* group were excluded from the equation model evaluation because the PCT value was missing due to low platelet count.

Model characteristics with the optimal cutoff value, the area under the curve, sensitivity, and specificity are shown in Table 5 and are described below. Briefly, the N-OI$_{pv}$ model showed superior performance in distinguishing the *P. vivax* group from the malaria-negative groups. It was also shown to demonstrate a significant difference in the area under the curve (AUC) in comparison to the previous N-OD1$_{pv}$ model (p < 0.001, Figure 2). Also, the N-OI$_{pv}$ model had fewer false positive and false negative results for ascertaining the distribution of the PP value dividing the optimal cutoff line (Figure 3). When the developed N-OI$_{pv}$ was applied to the validation samples (n = 5,493), specificity was 92.4% (95% CI = 91.7% to 93.1%). Considering the low-incidence rate in Korea, the malaria prevalence of 1% was assumed, the negative predictive value of N-OI$_{pv}$ was 99.94% (95% CI = 99.83% to 99.99%). In addition, the sensitivity of the N-OD1$_{pv}$ model according to parasitemia is presented in Table 6. Contrary to expectations, there was no significant difference in sensitivity according to concentration.

The OI$_{pv}$ model showed positive signs in 82 out of the 99 samples in the *P. vivax* group, but it showed positive signs in only 2 out of the 107 samples in the AFI group. Because the OI$_{pv}$ model had an equivalent specificity to the N-OI$_{pv}$ model, it suggests the possibility of its use as a complement if the N-OI$_{pv}$ model produces false positive. Indeed, in our sample, the OI$_{pv}$ model could correctly reclassify the four false-positive samples misdiagnosed by the N-OI$_{pv}$ model.

DISCUSSION

Many malaria studies using various automated hematology analyzers, such as Cell-Dyn, Coulter GEN·S, LH 750, DxH800, and the Sysmex XE-2100 analyzers, have shown the substantial diagnostic performance of the models in their way [1,5,6,9,13-15]. For Cell-Dyn analyzers, detection of abnormal depolarization events using the side-scatter and the depolarized side scatter

plot have shown overall sensitivities and specificities of 48.6% to 100% and 25.3% to 100%, respectively [1,5, 6]. For the Coulter analyzers, Briggs et al. and Fourcade et al. have proposed the 'malarial factor' equation model that consists of a combination of the lymphocyte volume standard deviation (SD) and the monocyte volume SD, and it had a sensitivity and specificity of 96.9% to 98% and 82.5% to 94%, respectively [14,15]. In 2014, P. Sharma et al. reported an 'improved malaria factor' equation model, which was composed of platelet, lymphocyte volume SD, and lymphocyte conductivity SD, and that model showed better performance than the previous factor model [13]. Lee et al. have reported distinctive malaria signals on the nucleated red blood cell plots from a newly developed DxH800 analyzer with a sensitivity and specificity of 100% [16].

Previous reports of malaria detection with the Sysmex hematology analyzer have applied various parameters [1]. Both visual models and equation models have been reported. Abnormal patterns in DIFF, WBC/BASO, and RET-EXT scattergrams in *P. vivax* infected patients can be visually observed. Among these scattergrams, the abnormal WBC/BASO scattergram that we named OI_{pv} was excellent because it showed a simple unusual pattern which nonprofessionals could easily detect and it had the highest performance value in some studies [8, 17]. It had sensitivities ranging from 96.8% to 100% and specificities ranging from 93.9% to 99.9%.

There was only one study assessing the equation models to diagnose *P. vivax* using a Sysmex analyzer. The first developed equation models by Campuzano et al. had a higher diagnostic accuracy for detecting *P. vivax* in comparison with the visual model [8]. An equation model also has the advantage of enabling automation processes to connect with the LIS or the hospital information system. Besides, since hematological changes in malaria depend on the population characteristics, new models need to be developed instead of applying the previous model [18,19]. Thus, we developed a new equation model ($N\text{-}OI_{pv}$) for *P. vivax* and compared it with the previous models. In addition, the OI_{pv} model, which has the characteristic of quickly recognizing positive malaria signs through visual interpretation without requiring extensive experience or knowledge, was selected by reviewing the previous studies and its diagnostic performance was evaluated [1, 7, 9, 10].

In this study, the $N\text{-}OI_{pv}$ model showed improved diagnostic performance as compared with the previous $N\text{-}OD1_{pv}$ model in Korea (Figure 2). In the study by Campuzano et al., the accuracy of the $N\text{-}OD1_{pv}$ model was 94.7%, whereas its accuracy in this study was 86.6% [8]. In contrast, the $N\text{-}OI_{pv}$ model had an accuracy of 97.5% and it showed a statistically significant difference compared to the $OD1_{pv}$ model ($p < 0.0001$). $N\text{-}OD1_{pv}$ and this $N\text{-}OI_{pv}$ model do not directly detect malaria, but rather basically reflect changes in immune cells and blood components of infected individuals through statistical methods. Therefore, the difference in performance as described above occurred due to the fundamental characteristics of the model being dependent on the immune response to malaria of the infected person. Each individual's immune system is very specific, but the response to the malaria varies from person to person depending on several factors. The most influencing factors are age, time, sex, hereditary, non-hereditary, non-pathogenic, pathogenic microorganisms, genetic makeup, and environment. Many studies have suggested that they could be due to the difference in factors for the study subjects, such as the level of immune status according to malaria endemism, the prevalence of hereditary hemoglobinopathy and nutritional status, or in the parasite strain related to the geographical location [18-22].

In the $N\text{-}OI_{pv}$ model, the PCT reflecting the decrease in platelet count caused by *P. vivax* infection can be a significant factor. Thrombocytopenia is a significantly more common complication in the malaria group compared to the non-infected 'control' population [23,24]. The exact mechanism of thrombocytopenia in malaria is not known, but several theories have been suggested [23]. Both immunological and non-immunological destruction of platelets has been reported. The immunological approach involves specific platelet associated IgG antibodies that bind directly to malarial antigens in the platelets, and it can explain antibody-mediated thrombocytopenia in malaria [25-27]. According to the non-immunological theory, oxidative stress damage of platelets in malaria is mediated by low levels of superoxide-dismutase and glutathione peroxidase activity, and it can lead to the destruction of platelets [28,29]. Thrombocytopenia is known to have a high frequency in *P. vivax* infected patients [23]. It was observed in 97 (98.0%) of the 99 patients with malaria in this study. A similar result was obtained by Lee et al. in 2008, which showed that thrombocytopenia was observed in 95.4% of patients with malaria from the same areas evaluated in the current study [24]. Even though the presence of thrombocytopenia was more common in the *P. vivax* group than in the AFI group, it has a limited role as the only parameter for the diagnosis of malaria due to its low specificity.

The MCV was included in the $N\text{-}OI_{pv}$ model. Decreased MCV was observed in the *P. vivax* group in comparison to the malaria-negative groups. This finding was in contrast with the previous studies [1,13,19]. We do not know the exact reason why this difference occurred. However, we assume that it is due to the effect of the hemoglobin level between our study and other studies. *P. vivax* infected persons tend to have a lower hemoglobin level; however, we found that the mean hemoglobin level (mean ± SD: 14.34 ± 1.76 g/dL) was within the normal range in the *P. vivax* group [30]. The results seem to have partly contributed to the fact that all study subjects were male. Anemia induced by *P. vivax* infection is not only due to hemolysis of both infected RBCs and non-infected RBCs but also due to decreased production of erythrocytes in the bone marrow [30,31]. High hemoglobin concentration seen in patients in this

study may be attributed to the reduced activity of cytokines that induce erythropoiesis in the bone marrow compared to patients with low hemoglobin concentrations. This suggests that the production of reticulocytes and their circulation in the peripheral blood are reduced, leading to a relative decrease in the mean red blood cell volume in the *P. vivax* group. However, we have mentioned our hypothesis above, and we think that further research is needed.

The LYMPH-X value was a good predictor variable in the N-OI_{pv} model. Increased LYMPH-X value in the *P. vivax* group may be associated with hemozoin-laden macrophages [8]. Hemozoin-laden macrophages could cause a high side-scatter signal to be erroneously detected as the X-axis in the DIFF scattergram and then possibly producing a high value of LYMPH-X.

Several authors have reported studies about the diagnosis of *P. vivax* using Sysmex XE-2100 in endemic regions, and they showed that the DIFF scattergrams demonstrated abnormal areas for mature neutrophils and/or for eosinophils, which were enough to suggest the diagnosis of malaria in *P. vivax*-infected patients [7-12]. Huh, et al. and You et al. have reported distinctive malaria signals on the DIFF scattergram using Sysmex analyzers with a sensitivity ranging from 46.2% to 69.4% and a specificity ranging from 99.7% to 100.0% [7,9]. In recent studies, the sensitivity ranged from 80% to 87.5% and the specificity ranged from 92.9% to 99.9% [8,10]. As reported in several studies, the *P. vivax* infected patients who did not show any DIFF scattergram abnormality tend to have low parasitemia. Thus, we did not investigate it due to the difficulty in recognizing malaria patterns and the difference in sensitivity depending on the researchers. Detecting an abnormal pattern in the DIFF scattergram is more complicated than identifying it as an abnormal pattern in the WBC/BASO scattergram because it shows various patterns related to *P. vivax* infection. However, the OI_{pv} model based on abnormal signals in the WBC/BASO scattergram that are easy to interpret demonstrates considerable diagnostic performance [8,17]. Abnormal signals on the WBC/BASO scattergram are known to be due to the hemozoin-containing RBCs and reticulocytes, and it has been reported to have a significant correlation with the concentration of some blood stages of *P. vivax*, such as mature trophozoites, schizonts, and gametocytes [8]. The accuracy of the OI_{pv} model was 90.8% in this study, which was intermediate between that of the N-OI_{pv} model and the N-$OD1_{pv}$ model.

The limitations of this study are the small size of the participating group and an uneven distribution of age and gender. Second, the N-OI_{pv} model showed higher accuracy than the other model, but it could not adjust for the low platelet counts (below 35,000/μL) in patients infected with *P. vivax* because the Sysmex analyzer cannot generate the PCT for those samples. Third, since N-OI_{pv} reflects the immunological characteristics of malaria infection, its application may be limited in groups of different sexes or ages. As previously seen, the performance of the N-$OD1_{pv}$ model developed in a similar manner can be seen to be inferior in this study. Despite such shortcomings, the new model demonstrates excellent negative predictive value, so it is considered to be practically useful in regions with low malaria prevalence such as Korea.

CBC tests by automated hematology analyzers are necessary laboratory tests in patients with fever. Laboratory technicians and hematopathologists should pay careful attention to analyzing the data from CBC tests for interpretation and flagging. Concerning clinical laboratory services, Sysmex hematology analyzers can provide convenient parameters to detect malaria. The analyzer can detect blood cells that have ingested malarial pigment and hematological changes caused by malarial infection via the developed models that we have studied. We suggest that the N-OI_{pv} model provides an opportunity to detect unsuspected cases of malaria during routine tests without additional costs or reagents. In conclusion, we recommend that the equation model developed with the use of the Sysmex hematology analyzer is indicated as a rapid test in the laboratory workup for malaria.

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Declaration of Interest:

The authors have declared that they have no competing interests.

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