Comparative Analysis of Haematological Parameters in Hookworm and *Plasmodium falciparum* Co-Infected Individuals in Kintampo North Municipality, Ghana

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Background: Hookworm and *Plasmodium falciparum* are endemic in Ghana, especially in the north-east and middle belt areas. Haematological parameters have been shown to predict the presence of these infections in patients. This study investigated the effect of hookworm-*P. falciparum* co-infection on haematological parameters among the patients.
Methodology: Stool and blood samples from 984 participants in a cross-sectional study conducted

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in the Kintampo North Municipality of Ghana were examined for hookworm and *Plasmodium falciparum* parasites. Malaria parasitaemia was estimated by microscopy and *Plasmodium falciparum*-specific 18S rRNA gene by polymerase chain reaction. Hookworm eggs in faecal samples were analyzed using Kato-Katz and formol-ether concentration methods. Hookworm speciation was done by PCR. Estimation of haematological parameters was done by automated haematology analyzer. Tukey multiple comparisons test was used to compare continuous variables among the infected groups and Spearman's rank correlation test determined the relationships between variables.

Results: The overall mean (SD) age of the study participants was 22.8 (17.4). Mean lymphocytes and eosinophils counts were higher while mean neutrophil and monocytes counts were lower in the co-infected individuals compared to the single infections. The mean red blood cell (RBC) count and haemoglobin (Hb) levels were higher in the co-infected individuals than in those with malaria only infection. However, white blood cell count and platelet counts were not statistically significant among the groups. There was a significant negative correlation between malaria parasite density and Hb level ($r = -0.239$, $p = 0.001$)

Conclusion: Hookworm and *Plasmodium falciparum* co-infections showed an increased in lymphocyte, eosinophil count and Haemoglobin levels, but a decrease in neutrophil and monocyte levels compared to malaria only infection. The possible mechanisms accounting for the variations in haematological levels remain to be elucidated.

1. INTRODUCTION

Hookworm is estimated to affect about 740 million people in the world [1], including 156 million children and most of these individuals are found in tropical regions of the world where such infections are linked to poverty [2]. In sub-Saharan Africa, hookworm prevalence is approximately 30 % [3]. However, in the north-eastern Ghana and the middle belt of Ghana (Kintampo North Municipality), the prevalence has been reported to be as high as 50 % [4] and 45 % [5]. The resultant effects of hookworm infection include growth delay, malnutrition, poor appetite and anemia, which, in pregnancy may result to poor birth outcomes [6]. Hookworm infection may also cause retardation in both physical and cognitive development in young children [7,8].

Malaria is one of the leading causes of morbidity and mortality in the developing world, especially sub-Saharan Africa. In endemic areas, about 60–70 % of the cases are attributable to *Plasmodium falciparum* infection while 30–40 % are attributable to other malaria parasite infections [9,10]. *P. falciparum* is responsible for 13–28 % of deaths in children under 5 years of age [11]. The high prevalence of both malaria and hookworm infections among individuals living in Africa means that a co-infection will be common [12]. However, little is known about the interaction between these widely distributed parasites. Hookworms cause chronic intestinal blood loss while acute haemolysis and depletion of haemoglobin are associated with *Plasmodium* infections [13-14]. Therefore, there is the need to investigate the haematological profiling in hookworm and malaria co-infection. Unfortunately, not enough studies have been done to investigate the pathological effects of *N. americanus* and *P. falciparum* infections to determine how their co-occurrence, as well as individual occurrences, may affect an individual's general blood cell levels. Results from studies addressing this effect would help to possibly predict the type as well as the level of infection, with respect to hookworm and malaria, which would go a long way to have profound implications for both malaria and hookworm control programmes in Ghana.

2. METHODS

2.1 Study Site, Design and Recruitment of Participants

The study was approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (FWA#: 00001824). All study participants provided written informed consent prior to their recruitment. This study was conducted in Kintampo North Municipality located within the forest-savannah transitional ecological zone in the middle belt of Ghana. The ages of the study participants ranges from 4yrs to 80 yrs.

2.2 Sample Collection and Processing

Trained field workers administered demographic and health questionnaires, and distributed labeled stool-collection containers to the participants. Stool samples were collected the following day and finger pricks were made to test for malaria using Rapid Diagnostic Test (RDT) kits (CareStart™ Malaria *Pf*HRP2/pLDH Ag RDT, Access Bio, Inc, USA) and to prepare thin and thick blood film on the same slide. About 5 mL of blood was drawn at the same time into EDTA vacutainers tubes for haematological analysis. Separate samples of blood were spotted on Whatman FTA Blood Stain Cards for storage until use in species identification using PCR. Prepared slides were stained with Giemsa and examined under the light microscope. Malaria parasitaemia was estimated by microscopy according to WHO protocols [15] and *P. falciparum*-specific 18S rRNA gene was detected in blood by PCR. Faecal samples were analyzed for the presence of helminth eggs on the day of collection using the Kato-Katz and formol-ether concentration methods. Hookworm speciation was carried out for hookworm positive cases by PCR using specific primers.

2.3 Hookworm Speciation by PCR

Hookworm species identification was determined using genomic DNA extracted from purified hookworm eggs samples of infected individuals using QIAamp DNA stool kit (QIAGEN, Hilden, Germany). Five microliters of purified gDNA (20-40 ng) was amplified in 1.25 mM each of deoxynucleotide triphosphate (dNTP), 1U of the *Taq* DNA polymerase enzyme (Sigma, Cat. #. D1806-250UN) and 0.3μL of each primer. The primers used were forward primer (NC2; 5'-TTA GTT TCT TTT CCT CCG CT-3'), with species specific reverse primers for *A. duodenale* (jmAD; 5'-TGC GAA GTT CGC GTT CGC TGA GC-3') or *N. americanus* (jmNA; 5'-CGT TAA CAT TGT ATA CCT GTA CAT AC-3') in separate reactions as described elsewhere [16]. The amplification conditions were initial heating at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute, with a final elongation step at 72°C for 5 minutes.

2.4 PCR Identification of *P. falciparum*

Total DNA was extracted from FTA cards using the Chelex method [17]. A 276 bp fragment of *P. falciparum* 18S rRNA gene sequence was amplified using the specific forward 5'-AAC AGA CGG GTA GTC ATG ATT GAG-3' and reverse 5'-GTA TCT GAT CGT CTT CAC TCCC-3' primers as used elsewhere [18]. The 20 μl reaction contained 20 – 40 ng total DNA, 0.25 mM of each primer, 1.25 mM of each dNTP, 1U of HotStar *Taq*® DNA polymerase (Biomol GmbH, Hamburg, Germany) and 1X reaction buffer. The PCR conditions were 34 cycles of denaturation at 94 °C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72 °C for 1 minute with a final elongation step at 72 °C for 5 minutes. DNA of the NF54 strain of *P. falciparum* extracted from culture was included on each PCR plate as positive control.

2.5 Visualization of Amplicons

The amplified products were visualized and the sizes determined by UV visualization after electrophoresis in a 2 % ethidium bromide stained-agarose gel. Products of the appropriate size (690 bp for *A. duodenale*, 870 bp for *N. americanus* and 276 bp for *P. falciparum*) were considered positive compared to standard controls.

2.6 Haematological Profiling

The haematological levels were determined using haematology analyser (ABX Pentra 60C+, HORIBA Medical, Rue du Caduce', France) by following the manufacturer's instructions.

2.7 Statistical Analysis

Statistical analysis was done by SPSS Version 24 (Chicago, IL, USA). Proportions such as prevalence were compared between groups (Pearson χ^2 test). Normalized variables were compared between groups using either the Welch two sample t test or One-Way analysis of variance (ANOVA) where appropriate. Pairwise differences between groups were compared using Post Hoc Test (Turkey's HSD). Spearman's rank correlation test determined the relationships between variables. P-value of <0.05 was considered statistically significant.

3. RESULTS

3.1 Demographic and Parasitological Characteristics of the Study Population

Table 1 shows the demographic characteristics of the study population. The study population consisted of 48.9 % males and 51.1 % females. The overall mean (SD) age of the study participants was 22.8 (17.4). There was no

significant difference in the geometric mean of egg per gram (EPG) of stool for individuals who were infected with only hookworm and those with *P.falciparum* and hookworm co-infection, though the co-infected group showed a marginal increased in intensity of hookworm infection. The mean *P. falciparum* density of individuals with malaria infection mono-infection was also significantly higher than those individuals co-infected with hookworm (p=0.001). All the PCR speciation for hookworm showed positive for *Necator americanus.*

3.2 Relationship between Haematological Parameters and Infection Status

Significant changes were found in the haematological parameters of the control, healthy subjects and the hookworm and malaria infected individuals. Mean white blood cell (WBC) counts did not generally vary significantly among the various infection statuses (p = 0.056) even though, within the various infected groups, the mean WBC level was significantly higher in the malaria-mono infected group than in the hookworm-mono infected group (p = 0.0348) which had the least mean WBC count (Table 2). Mean neutrophil, monocyte, eosinophil, lymphocyte, red blood cell (RBC) and haemoglobin levels all exhibited a significant variations among the various groups generally. It was also observed that co-infection caused an increase in lymphocyte, eosinophil, red blood cell, and haemoglobin levels compared to the malaria only. However, the co-infection led to significant reduction in neutrophil and monocyte level compared to malaria-mono infected group. Basophil levels were found to be increased in infected individuals with the highest level found to be associated with the hookworm-only infected individuals. Platelet counts, showed no significant variations among the groups.

3.3 Association of Intensity of Infection with Haematological Parameter

Table 3 shows the relationship between laboratory parameters and intensity of infection in individuals with hookworm and malaria infections. Lymphocytes, neutrophil and basophil levels correlated negatively, and weak (r = -0.020; r = -0.103; r = -0.017) with *P. falciparum* intensity and statistically not significant. But, correlation between *P. falciparum* intensity and haemoglobin (Hb) levels was negative, medium and statistically significant (r = -0.237, p=0.001). The relationship of *P. falciparum* intensity with relative eosinophil count and monocytes showed medium, positive (r = 0.281, p=0.036; r = 0.154, p<0.001) and a statistically significant correlation. The relationship of *N. americanus* intensity with WBC, neutrophils and monocytes showed a medium and strong, negative respectively (r = -0.235; r = -0.437; -0.562) and a statistically highly significant correlation. Though there was also a negative correlation between *N. americanus* intensity and Hb, it was not significant.

4. DISCUSSION

Complete blood count is a routine haematological test frequently used to help diagnose a number of diseases, such as anaemia, various acute infections, immune disorders, cancers and in health screening [19]. Hookworm infection and malaria are both known to cause anemia [20, 21]. Both infections will characteristically induce immune responses in the body like all other infections. However, due to differences in the anatomical position of the parasites and the mechanism of feeding or infecting of RBCs within the host. Hookworm harbours in the small intestine to obtain its food, thus lives outside the body cells, whilst *P. falciparum* infects the hepatic and the RBCs, thus lives within the cells. Therefore, different specific immune responses mediated by specific immune cells are expected to be elicited towards these infections.

In this study, significant changes in RBC counts and haemoglobin levels were found with malaria-mono infected subjects having the least RBC and haemoglobin levels compared to the co-infected group. We observed that increased in parasite density led to a significant reduction in Hb level. The destruction of RBCs by malaria parasite rapid proliferation and clearance of malaria-infected RBCs by the immune system are contributory factors to the severity of malarial anaemia [22,23]. Hookworm infection on the other hand, results in only intestinal blood loss, where the parasite resides and feeds on blood, as the cause of anemia [24. Therefore, we expected co-infections to experience reduced Hb and RBCs level [25]. However, our result showed that the levels of RBCs and Hb were higher in the co-infected individual than the malaria-mono infected individuals, thus, suggesting some protective effect in the presence of co-infection [4,26,27]. The mechanism by which hookworm apparently protect against a decrease in haemoglobin in *P. falciparum* malaria is unknown. The high levels of the Th2 cytokines (IL-10) produce during helminth infection may

counteract the Th1 cytokines (TNF-alpha) induced by malaria to prevent the development of severe anemia [28]. The overall Th2/Th1 balance, the homeostatic role of interleukin 10 and TGF-β as modulators of the immune response [29], and the role of the CD23/NO pathway in reducing sequestration [30] are additional possible mechanisms of protection against severe malaria [27].

It was also found that lymphocyte numbers were significantly increased in the co-infected individuals compared to malaria only infected individual, however, similar to those with hookworm mono-infection. The reason for this may be due to the complexity of the hookworm life-cycle which offers numerous opportunities for parasite-host interaction at the molecular level. Additionally, natural attrition of larvae at critical barriers, such as during skin invasion, and transit through lung tissues, as well as arrival in the gut and penetration of its mucosa, presents the host with an extensive diversity of antigenic challenge, immune stimulation and modulation [21]. This is opposed to the relatively simple life-cycle of the malaria parasites which only involves the hepatic and erythrocytic stages in the host.

Neutropenia was observed in individuals with co-infection infection compared to malaria only infected individuals. This possibly may be due to a decrease in production, increased destruction or an accelerated usage of neutrophils which usually occurs during concurrent infections. Secreted proteins by the infective larvae of *N. americanus* (*Na*-ASP-2) has been found to induce significant leukocyte (mostly comprised of 60% neutrophils and 30% monocytes) influx to the skin [31]. Furthermore, Neutrophil Inhibitory Factor (NIF), a glycoprotein secreted by the adult *N. americanus* may also be a possible contributor to the low neutrophil numbers due to its ability to potently inhibit CD11b/CD18-dependent neutrophil function and recruiting at worm attachment sites [32, 33]. Also, other factors such as vitamin B12 deficiency or unmeasured infections may have accounted for

this low neutrophil count as these factors are known to negatively impact neutrophil levels [34]. Monocyte counts was significantly found to be higher in malaria only infected individuals compared to those with co-infection or single infected with hookworm, which corroborate with other studies [35,36]. Mononuclear cells, which are activated by *Plasmodium* during malarial infection, produce inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) which stimulate the hepatic synthesis of acute phase inflammatory proteins, including CRP, which increase during malarial infection [37]. Eosinophil count was found to be elevated in both the co-infected and single infected (Na or Pf) cases compared to the negative control. Eosinophils are known to feature prominently in the leukocytic response to larval and adult stages of hookworm which is reflected by peripheral eosinophilia [38]. In general, nematode infections drive a strong Th2 response, promoting IgE synthesis [39]. Mast cell degranulation in response to IgE-allergen interaction plays a critical role in the activation and the local mobilization of eosinophils [40]. A cohort study by Kurtzhals and other [41] among children in Ghana found out that seven out of nine children with asymptomatic *P. falciparum* infection showed eosinophilia. Eosinophils have also been suggested to play a role in protection against malaria by induction of parasite killing [42]. These may account for the rise in eosinophil levels among the malaria infected subjects.

We found basophil levels to be higher in co-infected and hookworm only infected individuals compared to the malaria only or negative control. However, basophil level was higher in malaria only infected individuals than the negative control. Basophils have been poorly studied in the context of malaria with our study finding an increase in basophil count in malaria cases. It will be of paramount interest to investigate the possible mechanism accounting and the role play by basophil in malaria infection among Ghanaians.

Table 1. Characteristics of the study population recruited for the study

Characteristic	Control (n=36)	*Na* (n=40)	*Pf* (n=59)	*Na+Pf* (n=63)	P-Value
Sex Male	16 (44.4)	17 (42.5)	20 (33.9)	43 (68.3)	
Female	20 (55.6)	23 (57.5)	39 (66.1)	20 (31.7)	
Mean epg (range)	0	3235(144, 23328)	0	2626(144, 29952)	0.824
Mean PD (range)	0	0	1632(16, 22000)	794(16, 12720)	0.001

N. americanus (Na) cases, P. falciparum (Pf), N. americanus - P. falciparum co-infection (Na+Pf), N. americanus egg per gram (epg), parasite density (PD)

Table 2. Haematological parameters (mean ± SD) among study population

Variables	Control (n=36)	*Na* (n=40)	*Na+Pf* (n=63)	*Pf* (n=59)	P –Value
White blood cell	5.40±1.3	4.85±1.7[a]	5.31±2.4	5.93± 1.5[a]	0.056
Lymphocyte	51.06±8.1[bd]	64.69±9.3[cd]	66.07±8.1[ab]	53.19±10.4[ac]	<0.001
Neutrophil	34.29±10.1[bde]	21.48±8.9[cd]	17.50±9.8[ab]	27.09±11.3[ace]	<0.001
Monocyte	9.23±2.4[bd]	4.17±2.7[cd]	5.25±3.9[ab]	10.27±3.3[ac]	<0.001
Eosinophil	4.50±4.2[a]	6.57±6.3	8.22±5.8[a]	7.91±6.1	0.031
Basophil	0.93±0.3[bd]	3.09±1.8[cd]	2.96±1.6[ab]	1.55±1.9[ac]	0.01
Red blood cell	4.82±0.8[ab]	4.36±0.7	4.36±0.6[a]	4.31±0.9[b]	0.022
Haemoglobin	13.61±2.4[ab]	12.67±2.3	12.13±1.6[ab]	11.76±2.[5ab]	0.003
Platelet	220.9±92.2	195.3±83.9	184.8±65.8	203.9±89.7	0.251

P-values were calculated using ANOVA. Means that share a common letter are significantly different (Using the Tukey multiple comparisons test); N. americanus (Na); P. falciparum (Pf); N. americanus-P.falciparum co-infection (Na+Pf); Negative endemic control (control)

Table 3. Spearman's rank correlation coefficients for laboratory parameters and intensity of infection

Variables	*Na* intensity/epg		*Pf* intensity/Density	
	r	*P*	*r*	*P*
White blood cells	-0.235	**0.001**	0.080	0.277
Lymphocytes	0.534	**<0.001**	-0.020	0.785
Neutrophil	-0.437	**<0.001**	-0.103	0.163
Monocytes	-0.562	**<0.001**	0.281	**<0.001**
Eosinophils	0.103	0.161	0.154	**0.036**
Basophils	0.614	**<0.001**	-0.017	0.815
Haemoglobin	-0.052	0.481	-0.239	**0.001**

r, Spearmans rank coefficient; Na, N. americanus; Pf, P. falciparum

The function of eosinophilia against *N. americanus* infections in the present study population remains unclear as no significant reduction in intensity of hookworm infection was observed with an increased in eosinophil count. This is surprising, since, eosinophilia play a vital role in keeping the intensities of STH infections low by killing incoming larval stages [29, 43]. Our study finding strongly indicated that *P. falciparum* infections induce eosinophilia among Ghanaians and confirms previous study [44]. Hence, further investigation is needed to elucidate the possible protective or pathological role of eosinophil in malaria among Ghanaians.

5. CONCLUSION

The study shows that hookworm-malaria co-infection is associated with decrease in neutrophil and monocyte level but an increased in haemoglobin, eosinophil count and lymphocyte levels compared to malaria only infection. The possible mechanisms accounting for the variations in the haematological levels remain to be elucidated and could potentially have implications on control strategies in areas where both infections are endemic.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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