Original Research Article

Comparative analysis of haematological parameters in hookworm and *Plasmodium falciparum* co-infected individuals in Kintampo North Municipality, Ghana

Abstract

Background: Hookworm and malaria 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. The main aim of the study was to compare the haematological parameters of these infections in a co-infection state with haematological parameters of these same infections occurring as single infections.

Methodology: Human subjects from Kintampo North municipality, Ghana were enrolled in this study. Malaria parasitaemia was estimated by microscopy and *Plasmodium falciparum*-specific 18S rRNA gene by polymerase chain reaction. Helminth 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 (ABX Pentra 60C+, Horiba Medical, Rue du Caduce, France). Tukey multiple comparisons test was used to compare continuous variables among the infected groups. Spearman's rank correlation test determined the relationships between variables. P-value <0.05 was considered statistically significant.

Results: Mean lymphocytes and mean eosinophils counts were higher while mean neutrophil counts were lower in the co-infected individuals than those with single infections. We also found that red blood cell count and haemoglobin levels were higher in the co-infected individuals than in those with malaria infection only. Generally, white blood cell count and platelet counts were deranged as well, even though the differences were not statistically significant.

Conclusions: Hookworm and malaria mono-infections and co-infections presented different haematological profiling. Haematological levels in the co-infections are fairly different and not merely the sum of levels associated with individual hookworm and malaria infections.

The possible mechanisms remain to be elucidated and could potentially have implications on control strategies in areas where both infections are endemic.

Keywords: *Necator americanus, Plasmodium falciparum,* Parasitaemia**,** infection intensity, Kato-Katz

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 (*Ancylostoma duodenale* and *Necator americanus*) 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]. Hook worm 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 state. Unfortunately, not enough studies have been done in Ghana 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.

Methods

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 80yrs.

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.

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.

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.

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.

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.

Statistical analysis

Statistical analysis was done by SPSS Version 24 (Chicago, IL, USA). Tukey multiple comparisons test was used to compare continuous variables among the infected groups. Spearman's rank correlation test determined the relationships between variables. P-value of <0.05 was considered statistically significant.

Results

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 mean eggs per gram (epg) of stool of

individuals with mono- hookworm infection was insignificantly higher than those co-infected with malaria (p = 0.8240**)**. The mean *P. falciparum* density of individuals with malaria infection mono-infection was also significantly higher than those individuals co-infected with hookworm (p<0.05). All the PCR speciation for hookworm showed positive for *Necator americanus*.

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

Characteristic	**Control**	***Na***	***Pf***	**Na+Pf**	**P-Value**
Number	27	39	53	63	
Sex Male	11	16	19	43	
Female	16	23	34	20	
Mean epg (range)	0	3235(144, 23328)	0	2626(144, 29952)	0.8240
Mean PD (range)	0	0	1632(16, 22000)	794(16, 12720)	<0.05

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

Relationship between haematological parameters and infection status

Significant changes were found in the haematological parameters of the control, healthy subjects and the 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. Changes in the mean platelet levels were however, not statistically significant.

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

Variables	Control	*Na*	*Na+Pf*	*Pf*	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[a]	11.76±2.5[b]	0.003
Platelet	220.9±92.2	195.3±83.9	184.8±65.8	203.9±89.7	0.251

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

149 It was also observed that co-infection caused the extreme changes in lymphocyte, neutrophil,
150 eosinophil, red blood cell as well as platelet levels where the maximum levels where found in
151 terms of lymphocytes and eosinophils and the minimum levels were found in terms of red
152 blood cells, neutrophils and platelets. Malaria-mono infected groups recorded the highest
153 levels of general WBC and monocyte counts among the various groups. Conversely, the least
154 count of RBCs and haemoglobin levels were observed in the malaria-mono infected group.
155 Basophil levels were found to be increased in infected individuals with the highest level
156 found associated with the hookworm-only infected individuals. Platelet counts, as opposed to
157 basophil counts, were found to be diminished in infected subjects with the least recorded
158 among the co-infected individuals.

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.

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*

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 classification of their causative organisms; thus, *N. americanus* which is a helminth and *P. falciparum*, a protozoan; 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. 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]. There is, therefore, a clear suggestion that pathological effects exhibited by malaria parasites on RBCs of the host outweigh that of hookworm. Co-infections are suspected to enhance severity of anaemia as have been earlier proposed [25]. However, this study found that the levels of RBCs and haemoglobin were no worse than the levels observed in malaria-mono infected individuals but rather similar with individuals infected with only hookworm and thus, suggesting some protective effect in the presence of co-infection. There have been reports of a larger negative effect on blood hemoglobin and RBC levels, especially in children, with malaria infection alone than in those who were co-infected with helminths [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 infected groups with a notable rise in hookworm-infected cases more than in the malaria-infected cases. 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 also observed in the infected groups and this variation was statistically significant. Reduced neutrophil numbers can be due to a decrease in production, increased destruction or an accelerated usage of neutrophils which usually occurs during most infections. It was observed that neutropenia was greater in hookworm-related cases than in malaria-related cases. Ancylostoma Secreted Protein-2 secreted 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 *Ancylostoma caninum* and *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]. There is little knowledge about the interaction between *Plasmodium sp.* and neutrophils with previous studies suggesting an increased in neutrophil count in malaria which was in contrast with this study [34]. However, it should also be considered that 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 [35].Monocyte counts differed with increased monocyte numbers, above the negative endemic control, found in malaria-only infected subjects and reduced numbers in hookworm-only infected subjects. Previous studies suggested increase in monocytes numbers in malaria cases which is congruent with our study [34, 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]. Previous studies suggested increased in monocyte levels in hookworm only infections which contrast with our findings [3]. Possibly, sequestration of monocytes (together with neutrophils) into the skin by *Na*-ASP-2, as stated earlier, produced by the infective larval stages of *N. americanus* may play a role in the lowered monocyte counts observed in our study[39]. There was a variation in eosinophil counts with, eosinophilia observed in the infected individuals, and this variation was generally significant. Eosinophils are known to feature prominently in the leukocytic response to larval and adult stages of hookworm which is reflected by peripheral eosinophilia [40]. In general, nematode infections drive a strong Th2 response, promoting IgE synthesis [41]. Mast cell degranulation in response to IgE-allergen interaction plays a critical role in the activation and the local mobilization of eosinophils [42]. The individuals co-infected with hookworm and

malaria had the greatest mean eosinophil count, surprisingly, the mean eosinophil count in the hookworm-mono infected subjects trailed the mean eosinophil count in the malaria-only infected individuals even though there was no significant difference between these means. Acute malaria in adults and children from endemic areas of Africa has been known to usually be associated with low eosinophil count although at the same time the bone marrow is rich in eosinophil precursors [36, 43]. However, a cohort study by Kurtzhals and other [44] among children in Ghana found out that seven out of nine children with asymptomatic *P. falciparum* infection showed eosinophilia while a decrease in eosinophil count was observed in the same proportion of children with symptomatic *P. falciparum* infection. They also argued that it appears the low eosinophil count, commonly associated with malaria, may be due to tissue sequestration and destruction of eosinophils and not necessarily a decrease in production. Eosinophils have also been suggested to play a role in protection against malaria by induction of parasite killing [45]. These reasons may account for the rise in eosinophil levels among the malaria-only infected subjects observed, however, all the malaria cases were asymptomatic. It appears the greater parasitic burden in co-infected state causes the rapid and greater infiltration of eosinophils into tissues as well as their destruction which outruns the supply of eosinophils by the bone marrow resulting in low eosinophil counts

In general, helminths have been shown to induce a strong type 2 immune response characterized by increased numbers of Th2 cells, mast cells, eosinophils and basophils owing to the recorded increase in basophil numbers associated with hookworm both as a single infection and as a co-infection with malaria observed in our study [46]. Basophils have been poorly studied in the context of malaria with our study finding an increase in basophil count in malaria cases. Pelleau and others [47] reported that reduced basophil numbers in malaria cases was due to the

recruitment and accumulation of these basophils in tissues especially during the complications of malaria.

The study observed variations in the blood cell levels among the various infection status and the possible explanation could be due to the 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 it's food, thus lives outside the body cells, whilst *P. falciparum* infects the hepatic and the RBCs, thus lives within the cells. 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, 48]. Our study finding strongly indicated that *P. falciparum* infections induce eosinophilia among Ghanaians and confirms previous study [49]. Hence, further investigation is needed to elucidate the possible protective or pathological role of eosinophil in malaria among Ghanaians.

Conclusion

The study shows different haematological profile in individuals co-infected with hookworm and malaria parasite and individuals infected with single parasite. The possible mechanisms remain to be elucidated and could potentially have implications on control strategies in areas where both infections are endemic.

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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.

Competing interests

Authors have declared that no competing interests exist.

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