Real-time PCR Demonstrates *Ancylostoma duodenale* Is a Key Factor in the Etiology of Severe Anemia and Iron Deficiency in Malawian Pre-school Children

Femkje A. M. Jonker1*, Job C. J. Calis1, Kamija Phiri2, Eric A. T. Brienen3, Harriet Khoffi2, Bernard J. Brabin1, Jaco J. Verweij3, Michael Boele van Hensbroek1, Lisette van Lieshout3

1 Global Child Health Group, Emma Children’s Hospital, Academic Medical Centre, Amsterdam, The Netherlands, 2 Community Health Department, College of Medicine, Blantyre, Malawi, 3 Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands

**Abstract**

**Background:** Hookworm infections are an important cause of (severe) anemia and iron deficiency in children in the tropics. Type of hookworm species (*Ancylostoma duodenale* or *Necator americanus*) and infection load are considered associated with disease burden, although these parameters are rarely assessed due to limitations of currently used diagnostic methods. Using multiplex real-time PCR, we evaluated hookworm species-specific prevalence, infection load and their contribution towards severe anemia and iron deficiency in pre-school children in Malawi.

**Methodology and Findings:** *A. duodenale* and *N. americanus* DNA loads were determined in 830 fecal samples of pre-school children participating in a case control study investigating severe anemia. Using multiplex real-time PCR, hookworm infections were found in 34.1% of the severely anemic cases and in 27.0% of the non-severely anemic controls (p<0.05) whereas a 5.6% hookworm prevalence was detected by microscopy. Prevalence of *A. duodenale* and *N. americanus* was 26.1% and 4.9% respectively. Moderate and high load *A. duodenale* infections were positively associated with severe anemia (adjusted odds ratio: 2.49 (95%CI 1.16–5.33) and 9.04 (95%CI 2.52–32.47) respectively). Iron deficiency (assessed through bone marrow examination) was positively associated with intensity of *A. duodenale* infection (adjusted odds ratio: 3.63 (95%CI 1.18–11.20); 16.98 (95%CI 3.88–74.35) and 44.91 (95%CI 5.23–385.77) for low, moderate and high load respectively).

**Conclusions/Significance:** This is the first report assessing the association of hookworm load and species differentiation with severe anemia and bone marrow iron deficiency. By revealing a much higher than expected prevalence of *A. duodenale* and its significant and load-dependent association with severe anemia and iron deficiency in pre-school children in Malawi, we demonstrated the need for quantitative and species-specific screening of hookworm infections. Multiplex real-time PCR is a powerful diagnostic tool for public health research to combat (severe) anemia and iron deficiency in children living in resource poor settings.

---

**Introduction**

*Ancylostoma duodenale* and *Necator americanus* are soil transmitted nematodes responsible for an estimated 576–740 million human hookworm infections worldwide [1–3]. Hookworm infection often leads to anemia and iron deficiency, major causes of sickness and delayed cognitive development, especially in pre-school children [4]. Hookworm, therefore, is one of the most important infections in terms of disease burden in developing countries [5–7] with a major impact on public health [8–11].

The intensity of the hookworm infection is related to the severity of disease [5,10]. As adult hookworms attach to and feed from the bowel mucosa of the infected host, they are the direct cause of intestinal blood loss, which often gives rise to iron deficiency and anemia. Crompton and Whitehead proposed a model describing the relationship between the actual number of adult worms present in the intestines, and the hosts iron status; increased infection load was associated with lower iron levels [12,13]. The conventional method for determination of infection load is done with a proxy marker, number of eggs, using Kato-Katz microscopy slides with a fixed amount of feces [14]. However, although useful for estimating prevalence in highly endemic areas, the use of Kato-Katz microscopy for estimating intensity is laborious and frequently omitted.

*N. americanus* and *A. duodenale* are considered to have distinct geographical distribution; *N. americanus* is predominant in tropical environments, whereas *A. duodenale* is adapted to colder and drier circumstances [15,16]. Based on studies of experimental human hookworm infections using labeled erythrocytes, daily blood loss is estimated to range from 0.03 to 0.30 ml per worm per day, with *A. duodenale* and *N. americanus* infecting adults for 30–120 days and 125–180 days respectively [

---

**Citation:** Jonker FAM, Calis JCJ, Phiri K, Brienen EAT, Khoffi H, et al. (2012) Real-time PCR Demonstrates *Ancylostoma duodenale* Is a Key Factor in the Etiology of Severe Anemia and Iron Deficiency in Malawian Pre-school Children. PLoS Negl Trop Dis 6(3): e1555. doi:10.1371/journal.pntd.0001555

**Editor:** Paul J. Brindley, George Washington University Medical Center, United States of America

**Received:** October 6, 2011; **Accepted:** January 19, 2012; **Published:** March 6, 2012

**Copyright:** © 2012 Jonker et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This study was supported by a grant (064722) from the Wellcome Trust and by independent grants from the Nutricia Research Foundation, the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences, the Dr. P. C. Flu Foundation, and JANIVO Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: femkje.jonker@live.nl
**Author Summary**

Hookworm infections are a major cause of childhood anemia and iron deficiency. Two hookworm species exist of which *Ancylostoma duodenale* is the less common, yet causing more blood loss than *Necator americanus*. Although species differentiation and quantification are both of clinical importance, these are often not performed as the technique is complex and laborious using microscopy. Multiplex real-time PCR is a novel diagnostic tool which allows hookworm species differentiation and infection quantification. We applied this test in 830 stool samples of Malawian children with and without severe anemia. The prevalence of hookworm infections was high. *A. duodenale* was unexpectedly more prevalent than *N. americanus*. *A. duodenale* infections were associated with increased risk for severe anemia and iron deficiency, both of which increased with infection load. The study identifies the need for the quantitative screening of species-specific hookworm infections, which readily can be achieved by real-time-PCR. *A. duodenale* was independently associated with severe anemia and iron deficiency in our study population.

*duodenale* causing 2 to 10 times more blood loss per worm than *N. americanus* [12,13]. This is consistent with a few detailed clinical studies which indicated a stronger association of *A. duodenale* infection with anemia than *N. americanus* [10,15,17]. Due to the complexity of conventional diagnostic procedures (microscopy by a skilled technician after 7 days of stool culture), differentiation of hookworm species rarely has been done in population-based surveys [18,19]. This has been justified on the basis that prevalence of *A. duodenale* infection was limited and therefore the contribution to global disease burden was low, despite causing higher blood loss [15,20]. Furthermore, as both species respond to the same anthelmintic treatment, differentiation is not considered essential for treatment. Yet post-treatment data is scarce and extremely little is known about the species-specific effects of mass drug administration [10,21].

An alternative diagnostic procedure is multiplex real-time PCR which allows species-specific identification, as well as quantification of parasite DNA in human stool samples; based on selected targets and tested on a panel of well-defined DNA and stool samples, these assays were found to be 100% specific and substantially more sensitive compared with microscopy [22–25]. Through the use of different fluorescent labels in a closed system, multiple targets can be detected simultaneously within a single reaction tube with a low risk of contamination, which is ideal for high-throughput analysis. These assays offer significant potential for large scale population based surveys.

The objective of this study was, using a species-specific multiplex real-time PCR, to determine prevalence of the two hookworm species, infection load and species association with severe anemia and bone marrow iron deficiency in pre-school Malawian children.

**Methods**

**Study design and study population**

The study was part of a large case-control study on severe anemia in pre-school Malawian children (Figure S1). A detailed description of the study has been previously published elsewhere [26,27]. In brief three groups of children were recruited between 2002 and 2004 in an urban and rural setting in Southern Malawi. Cases were children (aged 6–60 months) presenting with severe anemia (hemoglobin<5.0 gram per decilitre). For each case, two control children were enrolled, a community control living within 100 to 1000 meters of the case, and a hospital control, presenting at the same hospital or outpatient facility as the case. Controls were eligible for recruitment if aged 6–60 months and if their hemoglobin level was at least 3.0 gram per decilitre.

**Ethics statement**

The study was approved by the Ethics Committees of the College of Medicine, University of Malawi, and the Liverpool School of Tropical Medicine, United Kingdom. Written informed consent was obtained from the parent or guardian.

**Laboratory investigations**

Hemoglobin (Hb) concentrations measured by HemoCue B-Hemoglobin analyzer (HemoCue, Angelholm, Sweden) were used to assess eligibility for the study; results of full blood count analyses by Coulter counter analyzer (Beckman Coulter, Durban, South Africa) were used for statistical analyses. In cases only, a bone marrow sample was collected, and slides were stained with Hematagnost Fe (Merck, Darmstadt, Germany) and graded for iron content [29]. Bone marrow examination was performed as part of the main study to investigate etiology of severe anemia. Bone marrow smears were assessed by a histological grading method which classifies iron status into six grades, iron deficiency was defined as a bone marrow smear score of 0 or 1 [29]. In all study participants a stool sample was collected for parasitological examination. In order not to delay required treatment in case of hookworm infection, stool samples were examined for ova by microscopy of direct smears and a single 25 mg Kato-slide [30,31]. The slides were read within 30 minutes by well trained microscopists and the number of hookworm eggs and presence of other helminth infections were recorded. An aliquot (approximately 0.5 gram) was stored at −20°C for later DNA isolation which was performed in a separate room at the research laboratory in Malawi. PCR was performed in Leiden University, the Netherlands. To prevent contamination with PCR products every step of the procedure was performed in separate rooms.

DNA was isolated using feces suspensions of 200 μl (±0.5 gram of stool per ml PBS containing 2% poly-vinyl-poly-pyrolidone (Sigma, Steinheim, Germany)) and heated for 10 minutes at 100°C. After sodium-dodecyl-sulphate-proteinase K treatment (overnight at 55°C), DNA was isolated with QIAamp Tissue Kit spin columns (QIAGen, Hilden, Germany) [32]. Extracted DNA samples were transported for multiplex real-time PCR assessment in the Netherlands. For detection of inhibition of the amplification process, DNA of Phocin Herpes Virus 1 (PhHV-1) was added to each PCR mixture as an internal control and detected by PhHV-1 specific primers and probe [33]. *A. duodenale* and *N. americanus* multiplex real-time PCR was performed as described previously; targeting the Internal Transcribed Spacer 2 (ITS-2) sequence of each species and using minor groove binding detection probes [25]. The ITS-2 is a conserved DNA target part of the whole genome present in all life stages. In brief, amplification reactions were performed in a volume of 25 μl with PCR buffer (HotstarTaq master mix, QIAGen, Germany), 5 mM MgCl2, 2.5 μg/μl Bovine Serum Albumin (Roche Diagnostics Nederland B.V., Almere, the Netherlands), 1.5 pmol of each *A. duodenale*-specific primer, 2.5 pmol of NED-labeled *A. duodenale*-specific MGB-Taqman probe (Applied Biosystems, Warrington, U.K.), 5 pmol of each *N. americanus*-specific primer, 2.5 pmol of FAM-labeled *N. americanus*-specific MGB-Taqman probe (Applied Biosystems, Warrington, U.K.), 3.75 pmol of each PhHV-1-specific primer, and 10 μM of each NED-labeled and FAM-labeled specific primer (Applied Biosystems, Warrington, U.K.). The reaction mixture also contained HotstarTaq DNA polymerase (Qiagen) 0.5 unit, and PhHV-1 specific primer and probe (Applied Biosystems, Warrington, U.K.). The reaction was performed in a volume of 25 μl with PCR buffer (HotstarTaq master mix, QIAGen, Germany), 5 mM MgCl2, 2.5 μg/μl Bovine Serum Albumin (Roche Diagnostics Nederland B.V., Almere, the Netherlands), 1.5 pmol of each *A. duodenale*-specific primer, 2.5 pmol of NED-labeled *A. duodenale*-specific MGB-Taqman probe (Applied Biosystems, Warrington, U.K.), 5 pmol of each *N. americanus*-specific primer, 2.5 pmol of FAM-labeled *N. americanus*-specific MGB-Taqman probe (Applied Biosystems, Warrington, U.K.), 3.75 pmol of each PhHV-1-specific primer and probe (Applied Biosystems, Warrington, U.K.).
specific primer, and 2.5 pmol of PhHV-1-specific Cy5-double-labeled probe (Biolegio, Nijmegen, The Netherlands) and 5 μl of the DNA sample. Amplification consisted of 15 min at 95°C followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. Negative and positive controls were included in each amplification-run. Amplification, detection and data analysis was performed with the AB7500 real-time PCR system (Applied Biosystems, Warrington, U.K.). The cycle threshold (Ct), meaning the amplification cycle in which the level of fluorescent signal exceeds background fluorescence, was used as the PCR output, reflecting parasite intensity categories were defined as following: low (Ct≤35.0) and positive controls were included in each amplification-run. None of the PCR analyzed samples were excluded due to inhibition as all runs showed a Ct-value for the internal PhHV-1 control within the expected range.

Statistical methods

Data were analyzed with the use of STATA 9 (Stata Corporation, TX) and PASW Statistics 18 (SPSS, Chicago, Illinois) statistical computer packages. Cross-sectional analyses were completed to assess the correlation of severe anemia and iron deficiency with PCR detected hookworm load per species. Using Chi-square test hookworm prevalence per species was compared individually across severely anemic and non-severely anemic as well across iron deficient and iron replete study groups. Spearman’s rank correlation was used for calculation of concordance between Ct-values with hemoglobin and number of iron fragments in the bone marrow. The combined association of characteristics related to risk of severe anemia and iron deficiency was examined by two multivariate logistic regression models, correcting for potential confounding factors. Potential etiologies of severe anemia were entered in the model if they were associated with severe anemia in univariate analysis (p<0.10). The model was adjusted for subjects’ baseline characteristics (age, sex) and other potential confounders: recent hematinic or anti-malarial treatment, history of transfusions and death of a parent [22]. With this model we compared all cases with the two control groups combined, stratified by study location. To explore the possibility that different patient characteristics were important in the two control groups, separate analyses were performed with the community and hospital control groups. To specify the association between iron deficiency (dependent variable) and hookworm (independent variable) we adjusted for subjects’ baseline characteristics (age, sex, study location) and other potential confounders (HIV infection and wasting defined as a Z-score of weight for height <−2 [34]). Stepwise backward multiple logistic regression analyses were performed; p values less than 0.05 were considered as statistically significant. The latter analyses only included case patients with bone marrow aspirate results. For both analyses attributable-risks were calculated using adjusted odds ratios [35]. When both hookworm species were analyzed together, the lowest Ct-value was counted. Reported p-values are two-sided.

Results

Stool samples of 830 (72.9%) of the 1138 children enrolled in the severe anemia study were stored for real-time PCR analysis (Figure S1). Table 1 summarizes mean hemoglobin and the general characteristics per study group (see also table S2). The mean age was lower in the case group; for which we adjusted in the multivariate model. Other baseline characteristics were not statistically different. Bone marrow iron examination was performed in a subgroup of 160 severely anemic children (cases) (Table 2, S1). Baseline characteristics were not significantly different between children with or without available stool sample or bone marrow sample (data not shown).

Hookworm

Microscopy was done on 780 (94.0%) of the 830 stool samples. Failure was due to small sample stool volume or constitution (too watery for Kato-slide examination). Hookworm eggs were identified in 44 of 780 (5.6%) samples, of which 18 (37.5%) showed a high-load infection, defined by more than 1,000 eggs per gram feces (epg). In the severely anemic children (cases), significantly more high-load hookworm infections were detected with microscopy compared to the controls; 5.9% (14/236) vs. 0.7% (4/544), p<0.001. Real-time PCR identified 242 (29.2%) hookworm infections in the 830 children. *A. duodenale* and *N. americanus* DNA was detected in 217 (26.1%) and 41 (4.9%) children, respectively. Six children were infected with both species. Within the healthy study population (community controls) 73

| Table 1. Baseline characteristics of 830 hookworm-PCR tested children stratified per study group. |
|---------------------------------|-----------------|-----------------|-----------------|
| Characteristic                  | Cases           | Hospital Controls | Community Controls |
|                                | Hb≤5.0 g/dL (N = 252) | Hb>5.0 g/dL (N = 291) | Hb>5.0 g/dL (N = 287) |
| Living in an urban area         | 134 (53.2%)     | 150 (51.5%)     | 146 (50.9%)     |
| Male                            | 119/252 (47.2%) | 147/291 (50.5%) | 139/287 (48.4%) |
| Age in months (mean ± SD)       | 19.9±12.6       | 22.7±12.0       | 25.6±13.6       |
| Hemoglobin* in g/dL (mean ± SD) | 3.6±0.8         | 9.6±2.2         | 9.9±1.9         |

*n = 826.

doi:10.1371/journal.pntd.0001555.t001

| Table 2. Baseline characteristics of sub population (severely anemic cases) stratified per iron status. |
|---------------------------------|---------------|---------------|
| Characteristic                  | Iron deficient† | Iron replete‡ |
|                                | (n = 68)      | (n = 92)      |
| Living in an urban area         | 45 (66.2%)    | 52 (56.5%)    |
| Male                            | 25 (36.8%)    | 49 (53.2%)    |
| Age in months (mean ± SD)       | 18.1±10.4     | 21.6±13.8     |
| Iron fragments (mean ± SD)      | 0.3±0.5       | 3.8±1.0       |

†Iron deficiency was defined as a bone marrow iron grade of none (grade 0) or very slight (grade 1).
‡Iron replete means sufficient iron (≥grade 2).

doi:10.1371/journal.pntd.0001555.t002
hookworm infections were detected (25.4%) of which the larger part was caused by *A. duodenale* (Table 3). In 182 (83.5%) of the 218 PCR positive samples tested parasitologically, microscopy did not identify the hookworm infection, 130 (71.4%) of these samples showed low quantities of hookworm DNA (Ct >35). Furthermore in 8 (18.2%) of the 44 microscopy positive samples the presence of hookworm was not confirmed with real-time PCR. These 8 samples had a median of 220 epg per gram.

**Anemia**

Hookworm infections were detected with PCR in 34.1% of the severely anemic cases and in 27.0% of the non-severely anemic controls (p<0.05). *A. duodenale* was found in 32.1% of the severely anemic cases and in 23.5% of the non-severely anemic controls (p<0.01, Table 3). The prevalence of high intensity infections (Ct<25) of *A. duodenale* infections was different; 8.7% among cases and 0.7% among controls (p<0.001). Additionally, in moderately anemic children (Hb 5–11 g/L) prevalence of *A. duodenale* was higher, 26.0% (105/404), than in non-anemic children (Hb≥11 g/dL), 17.6% (30/170) (p<0.05). There was no difference in prevalence of *N. americanus* infections between moderately anemic and non-anemic children. Within the whole study population (cases and controls combined) hookworm infection load and hemoglobin were negatively correlated (spearman correlation coefficient $r = 0.173$, **p** 0.0001). After correction for other causal factors of severe anemia using multivariate analysis, *A. duodenale* remained a significant risk factor for severe anemia; odds ratios increased with infection load and are shown in figure 1 and table S3.

**Iron deficiency**

Iron deficiency was prevalent in 42.5% (68/160) of severely anemic children who had available bone marrow samples. Of the iron deficient children 60.3% (41/68) had a hookworm infection, compared to 16.3% (15/92) for the iron replete group (p<0.0001). This difference in prevalence was only noted for *A. duodenale* infections and was greater in children with a high infection load (Table 4). Infection load for *A. duodenale*, but not for *N. americanus*, was negatively correlated with the fragmental iron staining score (spearman correlation coefficient $r = 0.336$, p<0.001 and $r = 0.9$ respectively, n = 160). The association between hookworm infection load and iron deficiency remained significant after correcting for other factors using multivariate analysis (Figure 2, table S4).

**Discussion**

Using the multiplex real-time PCR test for hookworm identification we revealed the hidden burden of hookworm in pre-school children in Southern Malawi; hookworm prevalence was much higher than expected. We have shown that hookworm infections, mainly *A. duodenale*, are significantly associated with the development of iron deficiency and severe anemia in pre-school children in Malawi. One of the strengths of this study is the sample size and comprehensive data set. It is the first report assessing the association of hookworm intensity and species differentiation with iron deficiency measured with the reference standard for iron status.

Severe anemia is a leading cause of hospital admissions and death in children living in sub-Saharan Africa, where between 12–29% of all children admitted to hospital are severely anemic and require blood transfusion. This results in high mortality (8–17%) [36–38]. The cause of severe anemia however, often remains unexplained. Using the same dataset, we have previously reported factors associated with severe anemia and identified hookworm infection, diagnosed by microscopy, as a significant correlate of infection [26]. This applied to infections with a high load (>1,000 epg) only. Using real-time PCR we now demonstrate that high-load infections have the greatest impact on disease burden, however also moderate *A. duodenale* infections were associated with significantly increased risk of severe anemia. This indicates that the association of microscopy-detected hookworm with severe anemia is an underestimation of the real impact of hookworm infections [39]. The disease burden of *A. duodenale* infections may be a significant contributor to unexplained severe anemia prevalence in sub-Saharan Africa.

Iron deficiency is often considered the same as anemia, especially in resource poor settings where both are very common. Estimations of prevalence of iron deficiency in Malawi vary from

---

**Table 3. PCR-determined hookworm distribution stratified per study group.**

|                  | Cases Hb≤5.0 (N = 252) | Hospital Controls Hb>5.0 (N = 291) | Community Controls Hb>5.0 (N = 287) |
|------------------|------------------------|----------------------------------|----------------------------------|
| Any hookworm infection | 86 (34.1%) *           | 83 (28.5%)                        | 73 (25.4%)                       |
| A. duodenale positive | 81 (32.1%) **          | 72 (24.7%)                        | 64 (22.3%)                       |
| Infection load low | 32 (12.7%)             | 54 (18.6%)                        | 46 (16.0%)                       |
| moderate          | 27 (10.7%) **          | 15 (5.2%)                         | 17 (5.9%)                        |
| high              | 22 (8.7%) ***          | 3 (1.0%)                          | 1 (0.3%)                         |
| N. americanus positive | 10 (4.0%)            | 19 (6.5%)                         | 12 (4.2%)                        |
| Infection load low | 6 (2.4%)               | 14 (4.8%)                         | 7 (2.4%)                         |
| moderate          | 3 (1.2%)               | 3 (1.0%)                          | 5 (1.7%)                         |
| high              | 1 (0.4%)               | 2 (0.7%)                          | 0 (0%)                           |

Infection load is defined by the following cycle thresholds (Ct): low 35<Ct<50; moderate 25<Ct≤35; high Ct≤25. In case of dual infection the lowest Ct-value was counted. Cases are compared with combined control groups using Chi-square.

**P** <0.05, **P** <0.01, **P** <0.001.

doi:10.1371/journal.pntd.0001555.t003
Yet, iron deficiency without anemia is important to recognize as it may delay cognitive development in pre-school and school-age children [6,7,41]. On the other hand, anemia of inflammation is often anemia without iron deficiency. During inflammation iron supplementation should be avoided, since the absorption will be poorly and may possibly increase the risk of infection [42–46]. Thus instead of presumptive treatment of iron deficiency, prevention of iron deficiency would be rather preferred and should include the use of anthelmintics if prevalence of A. duodenale is as high as in this study.

Unexpected was the high prevalence of A. duodenale whilst we expected N. americanus to be the dominant specie in this area [15,16]. This is important as we showed that A. duodenale was an independent risk factor for both moderate/severe anemia and iron deficiency. Based on the results from ‘healthy’ community controls, this study suggests that A. duodenale was the predominant hookworm species in children below five years of age in this area in southern Malawi. Whether this pattern is similar for all ages needs to be investigated, but PCR based analysis of stool samples from a community-based cross-sectional survey in the nearby Mozambique indicated that the ratio between the two hookworm species changes with age; A. duodenale was the predominant species in children, whereas in adults both species were almost equally present (Van Lieshout, unpublished data). Clearly more studies are needed to determine the species specific distribution and their risk factors in different regions, and how they relate to the burden of infection.

![Figure 1. PCR-detected hookworm infection and its association with severe anemia.](image-url)

**Table 4. PCR-determined hookworm distribution in severely anemic children stratified per iron status.**

| Iron deficient | Iron replete | (n = 68) | (n = 92) |
|---------------|--------------|---------|---------|
| Any hookworm infection | 41 (60.3%)*** | 15 (16.3%) | |
| A. duodenale positive | 39 (57.4%)*** | 12 (13.0%) | |
| Infection load | low | 12 (17.6%) | 8 (8.7%) |
| | moderate | 13 (19.1%)** | 3 (3.3%) |
| | high | 14 (20.6%)*** | 1 (1.1%) |
| N. americanus positive | 5 (7.4%) | 4 (4.3%) | |
| Infection load | low | 0 (0.0%) | 2 (2.1%) |
| | moderate | 1 (1.5%) | 1 (1.1%) |
| | high | 4 (5.9%) | 1 (1.1%) |

Infection load is defined by the following cycle thresholds (Ct): low 35 < Ct < 50; moderate 25 < Ct ≤ 35; high Ct > 25. In case of dual infection the lowest Ct-value was counted. Cases are compared with combined control groups using Chi-square.

**P < 0.05;***P < 0.001.

Iron deficient was defined as a bone marrow iron grade of none (grade 0) or very slight (grade 1).

Iron replete means sufficient iron (≥grade 2).

doi:10.1371/journal.pntd.0001555.g001

20–40% [40]. Yet, iron deficiency without anemia is important to recognize as it may delay cognitive development in pre-school and school-age children [6,7,41]. On the other hand, anemia of inflammation is often anemia without iron deficiency. During inflammation iron supplementation should be avoided, since the absorption will be poorly and may possibly increase the risk of infection [42–46]. Thus instead of presumptive treatment of iron deficiency, prevention of iron deficiency would be rather preferred and should include the use of anthelmintics if prevalence of A. duodenale is as high as in this study.

Unexpected was the high prevalence of A. duodenale whilst we expected N. americanus to be the dominant specie in this area [15,16]. This is important as we showed that A. duodenale was an independent risk factor for both moderate/severe anemia and iron deficiency. Based on the results from ‘healthy’ community controls, this study suggests that A. duodenale was the predominant hookworm species in children below five years of age in this area in southern Malawi. Whether this pattern is similar for all ages needs to be investigated, but PCR based analysis of stool samples from a community-based cross-sectional survey in the nearby Mozambique indicated that the ratio between the two hookworm species changes with age; A. duodenale was the predominant species in children, whereas in adults both species were almost equally present (Van Lieshout, unpublished data). Clearly more studies are needed to determine the species specific distribution and their risk factors in different regions, and how they relate to the burden of infection.

**Table 4. PCR-determined hookworm distribution in severely anemic children stratified per iron status.**

- Iron deficient
- Iron replete

| Iron deficient | Iron replete | (n = 68) | (n = 92) |
|---------------|--------------|---------|---------|
| Any hookworm infection | 41 (60.3%)*** | 15 (16.3%) | |
| A. duodenale positive | 39 (57.4%)*** | 12 (13.0%) | |
| Infection load | low | 12 (17.6%) | 8 (8.7%) |
| | moderate | 13 (19.1%)** | 3 (3.3%) |
| | high | 14 (20.6%)*** | 1 (1.1%) |
| N. americanus positive | 5 (7.4%) | 4 (4.3%) | |
| Infection load | low | 0 (0.0%) | 2 (2.1%) |
| | moderate | 1 (1.5%) | 1 (1.1%) |
| | high | 4 (5.9%) | 1 (1.1%) |

Infection load is defined by the following cycle thresholds (Ct): low 35 < Ct < 50; moderate 25 < Ct ≤ 35; high Ct > 25. In case of dual infection the lowest Ct-value was counted. Cases are compared with combined control groups using Chi-square.

**P < 0.05;***P < 0.001.

Iron deficient was defined as a bone marrow iron grade of none (grade 0) or very slight (grade 1).

Iron replete means sufficient iron (≥grade 2).
A surprising finding was that severe anemia was less common in children having low-load hookworm infections (35 < Ct < 50) when compared to non-infected children (Figure 1). We considered two possible explanations. Firstly, low-load hookworm infections cause iron deficiency which protects against bacteremia, a cause of severe anemia [26]. Alternatively a low hookworm load is seen in children with an effective immune response which are able to control their hookworm infection and other infections that may cause severe anemia. The proportional benefit of treating low-load hookworm infections requires further study as even these infections may contribute to burden of disease [8].

From a public health perspective the implication of this diagnostic method could be substantial as hookworm prevalence based on conventional microscopy seems largely underestimated, with the consequence that substantial areas may unjustly remain untreated. Screening for hookworm with real-time PCR would lead to more reliable prevalence data which should benefit the efficiency of mass drug administration. In 2007 the World Health Organization stated that in areas with a hookworm prevalence of more than 20%, all pre-school and school-age children should yearly be treated with anthelmintics, and where prevalence is more than 50% they should be treated twice a year [47]. Although large scale deworming has been proven to decrease hookworm prevalence and contribute to an improved health and well-being [10,48], the importance of appropriate interventions still remain neglected in most endemic countries [49] and there is some debate whether administration of anthelmintic drugs results in substantial improvement of hemoglobin concentration [21]. Differential effects of treatment might relate to geographic differences in species-specific distribution and infection load. Monitoring deworming programs using real-time PCR would provide more precise data on the effects of mass treatment. In addition, assessment of differences in treatment effects per species may diminish risk for the development of drug resistance.

A concern was that 18.2% (8/44) of the microscopy positives were PCR negative. This finding may indicate genetic variation of the PCR target gene [50]. On the other hand this was not supported by the fact that almost all PCR-missed infections showed low egg counts. Misidentification during microscopy could be another explanation. Nevertheless, most procedures have a certain chance to miss very light infections, and as examination is based on a small test sample volume only, it is probable that both eggs and free DNA are not completely homogeneously distributed within the stool sample. Hookworm was not detected by microscopy in 83.5% (182/218) of the PCR positive samples, since the specificity of real-time PCR was already proved to be close to 100% [25] this demonstrates the limited sensitivity of microscopy to detect hookworm infections even in an ideal laboratory setting. A limitation is that the association between iron deficiency and hookworm was only assessed in severely anemic children. These children may represent a small group of children with severe disease and interpretation of the associations may be different in the majority of otherwise “healthy children” infested with hookworm.

Although the value of real-time PCR for clinical diagnostics is limited in resource poor settings, this method brings forward new exciting prospects for epidemiology of intestinal parasites in these settings. The collection of stool samples in ethanol allows storage at room temperature and transportation to central research centers with facilities for real-time PCR. Moreover, using the same DNA isolation method simultaneously monitoring of other parasitic infections can be performed. For example *A. duodenale*, *N.


Table S2 Additional baseline characteristics of 830 hookworm-PCR tested children stratified per study location.

| Characteristics | Hookworm Infection (Model I) | Hookworm Infection (Model II) |
|-----------------|-------------------------------|-------------------------------|
| Sex             | Male                          | Female                        |
| Age             | Infant                        | Toddler                       |
| Parity           | Singleton                     | Multiple                      |

Table S3 Unadjusted and adjusted Odds ratios with 95% CI for severe anemia of all variables included in the models for hookworm infection (Model I) and A. duodenale infection (Model II).

| Variable         | Unadjusted OR (95% CI) | Adjusted OR (95% CI) |
|------------------|------------------------|----------------------|
| Age              |                        |                      |
| Parity           |                        |                      |
| Sex              |                        |                      |

Table S4 Unadjusted and adjusted Odds ratios with 95% CI for iron deficiency of all variables included in the models for hookworm infection (Model I) and A. duodenale infection (Model II).

| Variable         | Unadjusted OR (95% CI) | Adjusted OR (95% CI) |
|------------------|------------------------|----------------------|
| Age              |                        |                      |
| Parity           |                        |                      |
| Sex              |                        |                      |

Supporting Information

Figure S1 Flow chart study population. This flowchart presents number of children enrolled in the main study, a case control study investigating etiology of severe anemia (I); number of children enrolled in the sub study investigating association of hookworm infection and severe anemia (II) and number of children enrolled in the sub study investigating association of hookworm infection and iron deficiency (III); HC: Hospital Control; CC: Community Control.

References

1. Brooker S, Clements AC, Bundy DA (2006) Global epidemiology, ecology and control of soil-transmitted helminth infections. Adv Parasitol 62: 221–261.
2. Feasey N, Wansbrough-Jones M, Malhey DC, Solomon AW (2010) Neglected tropical diseases. Br Med Bull 93: 179–200.
3. Hotta PJ (2008) Neglected infections of poverty in the United States of America. PLoS Negl Trop Dis 2: e256.
4. Stoltzufus RJ (2001) Iron-deficiency anaemia: reexamining the nature and magnitude of the public health problem. Summary: implications for research and programs. J Nutr 131: 697S–708S.
5. Brooker S (2010) Estimating the global distribution and disease burden of intestinal nematode infections: adding up the numbers—a review. Int J Parasitol 40: 1137–1144.
6. Tolentino K, Friedman JF (2007) An update on anemia in less developed countries. Am J Trop Med Hyg 77: 44–51.
7. World Health Organization (2001) Iron deficiency anaemia: assessment, prevention and control. Available: http://www.who.int/nutrition/publications/micronutrients/anemia_iron_deficiency/WHO_NHD_01_03/en/index.html.
8. Knopp S, Mohammad KA, Stothard JR, Khanam IS, Rollinson D, Marti H (2010) Patterns and risk factors of helminthiasis and anaemia in a rural and a peri-urban community in Zanzibar, in the context of helminth control programs. PLoS Negl Trop Dis 4: e681.
9. Olsen A, Magnusson P, Ouma JH, Andreassen J, Friis H (1998) The outcome of severe anaemia in Malawian children. PLoS One 3: e2903.
10. Smith JL, Brooker S (2010) Impact of hookworm infection and deworming on iron status of children and programs. J Nutr 131: 697S–700S.
11. Albonico M, Stoltzfus RJ, Savidio L, Tielich JM, Chwaya HM (1998) Epidemiological evidence for a differential effect of hookworm species, Ancylostoma duodenele or Necator americanus, on iron status of children. Int J Epidemiol 27: 530–537.
12. WHO G, Pawlowski ZS SGSG (1991) Hookworm infection: approaches to Prevention and Control. Available: http://www.who.int/wormcontrol/documents/who_docs/en/.
13. Nikhoma E, Van Hensbroek PB, Van Lieshout L, Van Hensbroek MB (2005) Severe anaemia in an 11-month-old girl. Lancet 365: 1202.
14. Knopp S, Magens AF, Khamis BS, Steunmann P, Stothard JR (2008) Diagnosis of soil-transmitted helminths in the era of preventive chemotherapy: effect of multiple stool sampling and use of different diagnostic techniques. PLoS Negl Trop Dis 2: e231.
15. Polderman AM, Eherhardt M, Baeta S, Gasser RB, Van Lieshout L (2010) The rise and fall of human oesophagostomiasis. Adv Parasitol 71: 96–153.
16. Polderman AM, Eherhardt M, Baeta S, Gasser RB, Van Lieshout L (2010) The rise and fall of human oesophagostomiasis. Adv Parasitol 71: 96–153.
17. Verweij JJ, Blange RA, Templeton K, Schinkel J, Brienen EA (2004) Simultaneous detection of Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum in fecal samples by using multiplex real-time PCR. J Clin Microbiol 42: 1220–1223.
18. Verweij JJ, Brienen EA, Ziem J, Yeliari L, Polderman AM (2007) Simultaneous detection and quantification of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in fecal samples using multiplex real-time PCR. Am J Trop Med Hyg 77: 695–699.
19. Calis JC, Phiri KS, Faraghe EB, Brahin BJ, Bates I (2008) Severe anaemia in Malawian children. N Engl J Med 358: 888–899.
20. Phiri KS, Calis JC, Faraghe B, Nikhoma E, Ngoma K (2008) Long term outcome of severe anaemia in Malawian children. PLoS One 3: e2903.

Acknowledgments

We thank the parents and guardians of the children admitted in the study; the SevAna study team; and the staffs of the Queen Elizabeth Central Hospital and Chikwawa District Hospital.

Author Contributions

Conceived and designed the experiments: MBvH LvL. Performed the experiments: JJC KP EATB HK BJB JJV MBvH LvL. Analyzed the data: FAMJ JCJC LVL. Contributed reagents/materials/analysis tools: JJC KP EATB HK BJB JJV MBvH LvL. Wrote the paper: FAMJ JCJC LVL. Contributed reagents/materials/analysis tools: FAMJ JCJC LVL. Performed the experiments: MBvH LvL. Contributed reagents/materials/analysis tools: MBvH LvL. Conceived and designed the experiments: MBvH LvL. Performed the experiments: MBvH LvL. Contributed reagents/materials/analysis tools: MBvH LvL. Wrote the paper: FAMJ JCJC LVL. Contributed reagents/materials/analysis tools: FAMJ JCJC LVL. Performed the experiments: FAMJ JCJC LVL. Contributed reagents/materials/analysis tools: FAMJ JCJC LVL. Conceived and designed the experiments: MBvH LvL. Contributed reagents/materials/analysis tools: MBvH LvL. Wrote the paper: FAMJ JCJC LVL. Contributed reagents/materials/analysis tools: FAMJ JCJC LVL.
28. Gale E, Torrance J, Bothwell T (1963) The quantitative estimation of total iron stores in human bone marrow. J Clin Invest 42: 1076–1082.
29. Rath CE, Finch CA (1948) Sternal marrow hemosiderin; a method for the determination of available iron stores in man. J Lab Clin Med 33: 81–86.
30. Katz N, Chaves A, Pellegrino J (1972) A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni. Rev Inst Med trop Sao Paulo 14: 397–400.
31. Kreppel HP, van der Velde EA, Baeta S, Forderman AM (1995) Quantitative interpretation of coprocultures in a population infected with *Osphagostomum bifurcum*. Trop Geogr Med 47: 157–159.
32. Verweij JJ, Pit DS, Van Lieshout L, Baeta SM, Dery GD, Gasser RB (2001) Determining the prevalence of *Osphagostomum bifurcum* and *Necator americanus* infections using specific PCR amplification of DNA from faecal samples. Trop Med Int Health 6: 726–731.
33. Niesters HG (2002) Clinical virology in real time. J Clin Virol 25 Suppl 3: 83–12.
34. WHO (1986) Use and interpretation of anthropometric indicators of nutritional status. WHO Working Group. Bull World Health Organ 64: 929–941.
35. Bruzzi P, Green SB, Byar DP, Brinton LA, Schairer C (1985) Estimating the population attributable risk for multiple risk factors using case-control data. Am J Epidemiol 122: 904–914.
36. English M, Ahmed M, Ngando C, Berkley J, Ross A (2002) Blood transfusion for severe anaemia in children in a Kenyan hospital. Lancet 359: 494–495.
37. Lackritz EM, Campbell CC, Ruebush TK, Hightower AW, Wakube W (1992) Effect of blood transfusion on survival among children in a Kenyan hospital. Lancet 340: 524–528.
38. Newton CR, Warn PA, Winstanley PA, Peshu N, Snow RW (1997) Severe anaemia in children living in a malaria endemic area of Kenya. Trop Med Int Health 2: 165–178.
39. Green HK, Sousa-Figueiredo JC, Basanez MG, Betson M, Kabatereine NB (2011) Anaemia in Ugandan preschool-aged children: the relative contribution of intestinal parasites and malaria. Parasitology. pp 1–12.
40. WHO (2005) Worldwide prevalence of anaemia 1993–2005. Available: www.who.int/entity/wormcontrol/documents/joint_statements/en/ppc_unicef_finalreport.pdf.
41. Iannotti LL, Tielsch JM, Black MM, Black RE (2006) Iron supplementation in early childhood: health benefits and risks. Am J Clin Nutr 84: 1261–1276.
42. Cercamondi CI, Egli IM, Ahouandjinou E, Dossa R, Zeder C (2010) Aëble Plasmodium falciparum parasitemia decreases absorption of fortification iron but does not affect systemic iron utilization: a double stable-isotope study in young Beninese women. Am J Clin Nutr 92: 1385–1392.
43. Clark TD, Sembra RD (2001) Iron supplementation during human immunodeficiency virus infection: a double-edged sword? Med Hypotheses 57: 476–479.
44. Doherty CP, Cox SE, Fulford AJ, Austin S, Hiltmers DC (2008) Iron incorporation and post-malaria anaemia. PLoS One 3: e2133. 10.1371/journal.pone.0002133 [doi].
45. Murray MJ, Murray AB, Murray CJ (1978) The adverse effect of iron repletion on the course of certain infections. Br Med J 2: 1113–1115.
46. Sazawal S, Black RE, Ramirez M, Chowaya HM, Stoltzfus RJ (2006) Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet 367: 137–143.
47. WHO Prevention and control of schistosomiasis and soil-transmitted helminthiasis. WHO Technical Report Series No. 912. Available: www.who.int/entity/wormcontrol/documents/joint_statements/en/ppc_unicef_finalreport.pdf.
48. Guyatt HL, Brooker S, Khamia CM, Hall A (2000) Evaluation of efficacy of school-based anthelmintic treatments against anaemia in children in the United Republic of Tanzania. Bull World Health Organ 79: 695–703.
49. World Health Organization (2010) Working to overcome the global impact of neglected tropical diseases. Available: www.who.int/entity/neglected_diseases/2010report/en/.
50. Traub RJ, Inpankaew T, Suthikornchai C, Sukithana Y, Thompson RC (2008) PCR-based coprodiagnostic tools reveal dogs as reservoirs of zoonotic ancylostomiasis caused by *Ancylostoma ceylanicum* in temple communities in Bangkok. Vet Parasitol 153: 67–73.
51. Basuni M, Imani J, Othman N, Verweij JJ, Ahmad M (2011) A pentaplex real-time polymerase chain reaction assay for detection of four species of soil-transmitted helminths. Am J Trop Med Hyg 84: 330–343.
52. Supali T, Verweij JJ, Wiria AE, Djurardi Y, Hamid F (2010) Polyparasitism and its impact on the immune system. Int J Parasitol 40: 1171–1176.
53. Wiria AE, Prasetyiana MA, Hamid F, Wamunes LJ, Leil B (2010) Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmuNoSPIN Study). BMC Infect Dis 10: 77.