New Insights Into the Kinetics and Variability of Egg Excretion in Controlled Human Hookworm Infections

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Four healthy volunteers were infected with 50 Necator americanus infective larvae (L3) in a controlled human hookworm infection trial and followed for 52 weeks. The kinetics of fecal egg counts in volunteers was assessed with Bayesian multilevel analysis, which revealed an increase between weeks 7 and 13, followed by an egg density plateau of about 1000 eggs/g of feces. Variation in egg counts was minimal between same-day measurements but varied considerably between days, particularly during the plateau phase. These analyses pave the way for the controlled human hookworm model to accelerate drug and vaccine efficacy studies.

Keywords. hookworm; controlled human infection; Bayesian statistics; vaccine development.

Hookworms affect almost 500 million people worldwide, predominantly in developing countries. Pathology is caused by blood and protein loss at the site of intestinal attachment, particularly in individuals with low iron and protein stores, such as children or women of childbearing age [1].

Currently, hookworm elimination rates fall behind those of other soil-transmitted helminths [2], especially in high-prevalence areas. High reinfection rates and the exclusion of adult populations in mass drug administration programs targeting school-age children hamper the goal to interrupt transmission [3]. A vaccine would be a crucial tool to aid current hookworm-control programs. Two vaccine candidates are in phase 1 clinical testing in volunteers in Gabon, Brazil, and the United States [4]. However, testing of efficacy in field trials is a large-scale, costly endeavor, slowing down vaccine development [5].

Efficacy in vaccine trials can be estimated using a binary outcome, but quantitative measurements are preferable. This relies on fecal egg counting, a widely used way of measuring infection intensity in humans [6]. However, in field settings, fecal egg counts are highly variable, owing to differences in the host immune response, dietary intake, episodes of diarrhea, transport and storage of samples, laboratory conditions, availability of highly trained technicians, and other technical factors, thereby limiting the power of field trials to detect vaccine efficacy [7].

The development and clinical implementation of hookworm vaccines could be accelerated by the implementation of controlled human hookworm infection (CHHI) models within the product pipeline, as has been shown for malaria and influenza vaccines [8]. The CHHI model has been used for immunomodulatory purposes, such as investigations involving celiac disease [9], using doses of 10 or 20 L3 larvae. A recent trial by Diemert et al, aimed at developing a model for vaccine testing, showed that infection with 50 L3 larvae resulted in infection in 9 of 10 volunteers and was well tolerated [10]. However, egg counts were lower than typically seen in field studies [11].

To obtain a better comparison to the field, higher egg counts would be preferable. Furthermore, an improved understanding of the kinetics and variability of egg excretion over a prolonged period could help dissect factors underlying the variability in egg output. As a result, the most-reliable time points with the lowest variability can be identified, and the power of vaccine trials can be improved.

In this study, we investigated the kinetics of egg excretion over an extended period in a CHHI model, using an infective dose of 50 L3 larvae. Patterns and variability in egg excretion were quantified using Bayesian multilevel analysis. Our findings can be used to create an improved CHHI model that could be of great value in accelerating the testing of novel vaccines or medicine.

METHOD

Necator americanus L3 larvae were produced according to the principles of good manufacturing practice (GMP) but not in a GMP-licensed cleanroom. Material for culture was obtained from a chronically infected donor from James Cook University [9] who was carrying a N. americanus strain originating from Madang, Papua New Guinea, that was previously maintained...
at the University of Nottingham. The donor was confirmed to be negative for blood-borne infections (ie, human immunodeficiency virus [HIV], hepatitis B virus, and hepatitis C virus infections), and a fecal specimen containing *N. americanus* eggs was cultured for 7 days, after which larvae were harvested [12]. Water containing the infectious *N. americanus* L3 larvae was cultured for pathogenic bacteria, and the identity of the infectious larvae was confirmed by polymerase chain reaction (PCR) analysis. Viability of the larvae, as measured by a visual count of moving larvae, varied between 88% and 92% for different batches. Larvae were used for infection within 10 days of harvesting. A detailed production process of the larvae is described in the Supplementary Materials.

Healthy male and female volunteers aged 18–45 years were recruited in April 2017 and provided written informed consent. Exclusion criteria were a body mass index (calculated as the weight in kilograms divided by the height in meters squared) of <18.0 or >30.0; iron deficiency anemia; positive results of fecal PCR analysis for *N. americanus*, *Ancylostoma duodenale* hookworm, or *Strongyloides* organisms; positive results of serologic analysis for hepatitis B virus, hepatitis C virus, or human immunodeficiency virus; contraindications for the use of albendazole; planned travel to a hookworm-endemic area; incomplete understanding of the study procedures; or any medical condition that could interfere with participation in the trial.

For the preparation of each dose, individual motile larvae were selected from the prior released batch to ensure the highest possible viability. Every dose of 50 *N. americanus* L3 larvae was dispensed within 15 minutes after preparation onto 4 gauzes, with 2 containing doses of 10 larvae and 2 containing doses of 15 larvae, which were applied to the dorsal side of volunteers’ upper arms and calves, respectively, for 60 minutes.

Adverse events were recorded and total eosinophil count and hemoglobin level were measured weekly during the first 12 weeks and 6 and 12 months after infection. For every adverse event, the time and date of onset and end, severity, and cause were recorded. Adverse events could be unrelated or unlikely, possibly, probably, or definitely related; the latter 3 categories are regarded as “related” in dichotomous analyses. Relatedness was assessed by the clinical trial physician. Fecal samples were collected weekly from week 5 after infection onward and checked for *N. americanus* eggs by the Kato-Katz technique. Slides with 25 mg of stool were prepared from homogenized stool specimens [12]. Two slides per fecal sample were read by 2 different slide readers. After 12 weeks of follow-up, volunteers were asked to return at irregular intervals (not a prespecified pattern) for on-demand fecal specimen donation during the course of a year, depending on the need for samples at the laboratory. These on-demand donations were used for additional egg counts and further culturing of the larvae.

Variability in egg counts between and within individuals over time was analyzed using Bayesian multilevel analysis. We assumed that egg counts initially increase and then stabilize over time, according to a scaled cumulative normal distribution function. We considered the following 3 levels to describe the variation of egg counts. First, the level at which egg counts stabilize was allowed to vary between individuals, assuming a log-normal distribution. Second, variation in daily averages was assumed to follow a gamma distribution. Last, the sampling error among repeated egg counts on the same day in the same individual was assumed to follow a Poisson distribution (in which variance equals the mean value) or a negative binomial distribution (in which variance is greater than the mean value). Parameter values were estimated using the package *rstan* (version 2.16.2) [13] in R (version 3.4.3).

This trial was approved by the local institutional review board (protocol P17.001) and is registered at [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT03126552).

**RESULTS**

Four volunteers were included in the study, of whom 3 were women and 1 was a man, (age range, 19–23 years). Infection with 50 *N. americanus* L3 larvae was well tolerated and resulted in patent hookworm infection in all four volunteers.

All volunteers reported rash at the sites of infection (Figure 1A), lasting for 11, 22, 31, and 32 days, and itching, lasting for 1–2 days. There was no difference in the intensity of rash or itching between the infection sites on arms and those on legs. The most common adverse event was abdominal pain, with 11 episodes among the volunteers; 9 were classified as mild, 1 was classified as moderate, and 1 was classified as severe and lasted 5 hours. Other abdominal events were nausea (in 2 volunteers) and flatulence (in 1 volunteer), all of which were mild (Figure 1B); these events started at week 3, increased in frequency until week 6, and then decreased in frequency through week 9, after which no abdominal complaints were reported. No volunteers reported related adverse events after week 12 (Supplementary Materials).

No volunteers developed anemia. Eosinophil counts increased steeply in all volunteers after infection, peaking at week 6 (range, 2.02 × 10⁹–6.96 × 10⁹ eosinophils/L). Eosinophil counts declined afterward but remained slightly elevated at week 12 in all volunteers (range, 0.82 × 10⁹–1.77 × 10⁹ eosinophils/L). Counts were still elevated in 2 volunteers at week 52 (1.18 × 10⁹ and 0.72 × 10⁹ eosinophils/L), with one having had an elevated count at baseline (0.74 × 10⁹ and 0.34 × 10⁹ eosinophils/L, respectively).

Kato-Katz slides were all negative for eggs at weeks 5 and 6 after infection and became positive for eggs in 1 volunteer at week 7 after infection. At week 9, all volunteers were excreting...
eggs, based on analysis of Kato-Katz slides. The median egg density at the end of the first follow-up period (ie, at week 12) was 560 eggs/g of stool (range, 160–1680 eggs/g of stool). After the initial follow-up, volunteers donated fecal specimens at irregular intervals, enabling data collection up to week 52.

Bayesian multilevel analysis showed that, by week 10, egg counts had risen to about half the maximum level and reached their maximum level around week 13 (Figure 2A). Thereafter, egg excretion remained relatively stable, with considerable variation among daily averages within individuals. The estimated population-level plateau in the egg count was 25.8 eggs/slide, corresponding to around 1000 eggs/g of feces. Assumption of negative binomial variation instead of Poisson distribution among repeated egg counts on the same day did not improve the model fit (Figure 2B).

**DISCUSSION**

This study shows that experimental infection with 50 L3 *N. americanus* larvae divided over 4 application sites is well tolerated and leads to patent infection, with eosinophilia and fecal egg excretion in all 4 volunteers. Long-term follow-up showed that egg counts increased from week 7 to week 13 after infection and then reached a stable level. The peak of adverse events, primarily abdominal complaints, occurred around 6 weeks after the infection, coinciding with peak eosinophilia. This time point is thought to mark the establishment of the larvae in the intestine.

To alleviate dermal symptoms, we divided the infectious larval dose over 4 extremities. Although the median duration of rash was similar, the intensity of local events was decreased as compared to observations by Diemert et al [10]. The levels of egg excretion, however, were much higher than in any previous report [9, 10] and were not accompanied by more severe abdominal adverse events [10]. This enhanced infectivity could be related to the viability of larvae, which was >88%, and all were used within 10 days of harvesting. Alternatively, the application of larvae over several sites may have enhanced infectivity. The plateaus in egg counts observed in this study are below the World Health Organization–defined cutoff for a light infection (ie, 2000 eggs/g of stool) [6]. Comparable average levels of excreted eggs are widely observed in areas of endemicity [14].

Although volunteers with experimental hookworm infection have undergone long follow-up before [15], this is the first study to describe the kinetics of *N. americanus* egg excretion over a prolonged period in multiple volunteers. We found low variability between egg counts on the same day within the same individuals, allowing for a Poisson distribution. This is remarkable because variation in egg counts in field studies is generally

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**Figure 1.** Overview of adverse events. **A,** Representative rash at week 1 after infection. (left: arm, 10 L3 *Necator americanus* larvae; right: leg, 15 L3 *N. americanus* larvae). **B,** All reported adverse events considered to be related to the study.
much higher and can only be described by a negative binomial distribution [7]. It is possible that the highly standardized method of slide preparation and feces homogenization in this trial and the lack of reinfection reduced the variability in egg output. However, more data are needed to further quantify the day-to-day variation within and between individuals. Although findings of PCR analysis may potentially be less variable, microscopy findings are still the preferred primary end points in phase 2 vaccine or drug trials. Bayesian multilevel analysis is helpful to accurately assess the stabilizing levels of egg excretion from 13 weeks onward despite several levels of variability and, as such, will provide key information for the future design of controlled human hookworm trials testing novel medicines or vaccines. Vaccine efficacy can be detected through a lower plateau level, a later or slower increase in egg counts, or a combination of these factors. The timing and level of the plateau in egg counts could be a reliable end point for clinical trials, assuming that follow-up is extended beyond 13 weeks. Despite the small sample size, these promising findings add to the existing arguments for using CHHI models for early vaccine efficacy studies.

In conclusion, we found that controlled human infection with 50 L3 *N. americanus* larvae was well tolerated, resulting in infection of all volunteers and unparalleled high egg counts. Although the number of subjects in this study was limited, our results show great promise in developing a sustainable human hookworm infection model that, aided by Bayesian statistical analysis of egg kinetics, could be of great value in accelerating clinical testing of novel vaccines and medicines.

**SUPPLEMENTARY DATA**

Supplementary materials are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

**Notes**

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