Building bridges: a multidisciplinary approach to controlled human hookworm infection

Hoogerwerf, M.

Chapter 3

New insights into the kinetics and variability of egg excretion in controlled human hookworm infections

Marie-Astrid Hoogerwerf*, Luc E. Coffeng*, Eric A.T. Brienen, Jacqueline J. Janse, Marijke C.C. Langenberg, Yvonne C.M. Kruize, Chelsea Gootjes, Mikhael D. Manurung, Mark Dekker, Luke Becker, Marianne A.A. Erkens, Martha T. van der Beek, Munisha S. Ganesh, Carola Feijt, Beatrice M.F. Winkel, Inge M. Westra, Pauline Meij, Alex Loukas, Leo G. Visser, Sake J. de Vlas, Maria Yazdanbakhsh, Lisette van Lieshout, Meta Roestenberg.

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Abstract

Four healthy volunteers were infected with 50 *Necator americanus* infective larvae (L3) in a controlled human hookworm infection trial and followed for 52 weeks. Kinetics of fecal egg counts in volunteers was assessed with Bayesian multi-level analysis, revealing an increase between weeks 7 and 13 followed by a plateau at about 1000 eggs per gram 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.

Introduction

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 re-infection rates and the exclusion of adult populations in mass drug administration (MDA) programmes targeting school-age children, hamper the goal to interrupt transmission.[3] A vaccine would be a crucial tool to aid current hookworm control programmes. 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 endeavour, slowing down vaccine development. [5]

Efficacy in vaccine trials can be estimated using a binary outcome, however quantitative measures 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 due to differences in the host immune response, dietary intake, episodes of diarrhoea, 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 applied for immunomodulatory purposes in for example 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 patent infection in 9 out 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 with the field, higher egg counts would be preferable. Furthermore, an improved understanding of the kinetics and variability of egg excretion over a prolonged period of time could help dissect factors underlying variability of egg output. As a result, the most reliable time points with 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 time period in a CHHI model using an infective dose of 50 L3 larvae. Patterns and variability in egg excretion were quantified using Bayesian multi-level analysis, based on which recommendations for the improved use of CHHI for testing of novel vaccines or medicine are proposed.

Methods

Necator americanus (Na) L3 larvae were produced according to the principles of Good Manufacturing Practice (GMP) guidelines, but not in a GMP-licensed cleanroom. Material for culture was obtained from a chronically infected donor from James Cook University, [9] carrying a *Na* strain originating from Madang, Papua New Guinea previously maintained at the University of Nottingham. The donor was confirmed negative for blood borne infections (HIV, Hepatitis B and C) and feces was screened for any viral, bacterial and parasitic pathogens. The feces containing *Na* eggs was cultured for 7 days after which larvae were harvested. [12] Water containing the infectious Na L3 larvae was cultured for pathogenic bacteria, identity of the infectious larvae was confirmed by PCR. Viability of the larvae, as measured by a visual count of moving larvae varied between 88-92% for different batches. Larvae were used for infection within ten days of harvesting. A detailed production process of the larvae is described in supplementary material A.

Healthy male and female volunteers aged 18-45 years old were recruited in April 2017 and provided written informed consent. Exclusion criteria were body mass index (BMI) <18.0 or >30.0 kg/m^2, iron deficiency anaemia, positive PCR on feces for *Na* or *Ancylostoma duodenale* hookworm or *Strongyloides*, positive serology for Hepatitis B, C, HIV, contra-indications for the use of albendazole, planned travel to a hookworm-endemic area, incomplete understanding of the study procedures, or any medical condition which 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 highest possible viability. Every dose of 50 *Na* L3 larvae was dispensed within 15 minutes after preparation onto four gauzes, divided in two doses of 10 and two doses of 15 larvae, which were applied to the dorsal side of volunteers' upper arms and calves, respectively, for 60 minutes.

Adverse events, total eosinophil count and haemoglobin were collected weekly during the first 12 weeks and 6 and 12 months after infection. For every adverse event, time and date of onset and end, severity and causality were recorded. Adverse events could be unrelated, unlikely, possibly, probably or definitely related, the latter three 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 onwards and checked for *Na* eggs by Kato-Katz. Slides with 25 milligram of stool were prepared from homogenised stool specimens. [12] Two slides per fecal sample were read by two different slide readers. After 12 weeks of follow-up, volunteers were asked to return at irregular intervals (not a pre-specified pattern) for on-demand feces 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 multi-level 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 three levels to describe the variation of egg counts. Firstly, the level at which egg counts stabilize was allowed to vary between individuals assuming a log-normal distribution. Secondly, variation in daily averages was assumed to follow a gamma distribution. Lastly, the sampling error in repeated egg counts on the same day in the same individual was assumed to follow a Poisson (variance equals mean) or a negative binomial distribution (variance is greater than mean). Parameter values were estimated using the package *rstan* (version 2.16.2) [13] in R (version 3.4.3).

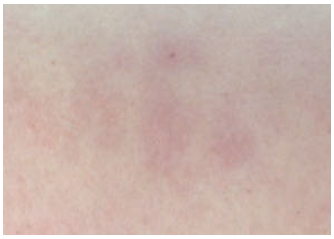
This trial was approved by the local IRB (P17.001) and is registered under NCT03126552 (clinicaltrials.gov).

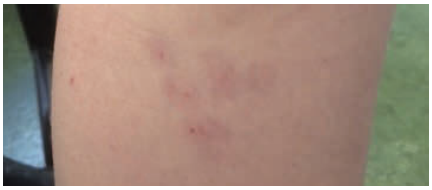
Results

Four volunteers were included in the study, three female, one male, aged 19-23 years. Infection with 50 *Na* 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 respectively, and itching lasting for 1-2 days. There was no difference in intensity of rash or itching between arms and legs. The most common adverse event was 11 episodes of abdominal pain, reported in all volunteers. Nine episodes were classified as mild, one as moderate and one as severe, lasting five hours. Other abdominal events were nausea (2 volunteers) and flatulence (one volunteer), all mild (figure 1B) starting at week three, increasing in frequency until week 6 and then declining until all abdominal complaints disappeared at week 9. None of the volunteers reported related adverse events after week 12. (Supplementary material B)

A





B			Number of volunteers (n=4)	Mild	Moderate	Severe
Solicited	*Local*	Rash	4	4	-	-
		Itching	4	4	-	-
	Systemic	Abdominal pain	4	2	1	1
		Nausea	2	2	-	-
		Flatulence	1	1	-	-
		Cough	2	1	1	-
Unsolicited	*Systemic*	Headache	3	1	1	1
		Sore throath	2	1	1	-

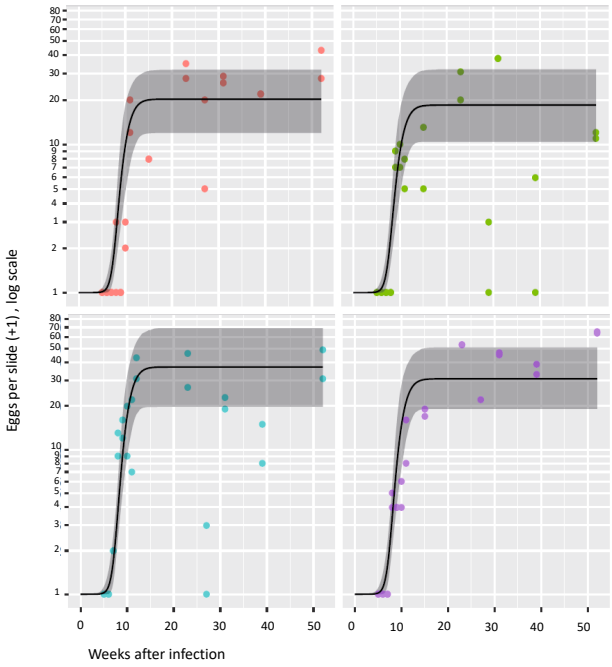
Figure 1. Overview of adverse events.

Representative rash at week 1 after infection. (left: arm, 10 L3 *Na* larvae; right: leg, 15 L3 *Na* larvae). A. All reported adverse events considered related to the study.

None of the volunteers developed anaemia. Eosinophil counts increased steeply in all volunteers after infection, peaking at week 6 (range 2.02-6.96x10^9/L). Eosinophils declined afterwards but remained slightly elevated at week 12 in all volunteers (range 0.82-1.77x10^9/L) and still elevated in two volunteers at week 52, one of which had already elevated eosinophils at baseline (baseline 0.74x10^9/L, week 52 1.18x10^9/L and baseline 0.34x10^9/L , week 52 0.72x10^9/L respectively).

Kato-Katz slides were all negative at week 5 and 6 and became positive in one volunteer at 7 weeks after infection. At week 9 all volunteers showed egg excretion by Kato-Katz. Median number of eggs per gram (epg) at the end of the first follow-up period at week 12 was 560 (range 160-1680). After the initial follow-up volunteers donated feces at irregular intervals, enabling data collection up to week 52.

Bayesian multi-level 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 still considerable variation in daily averages within individuals. The estimated population-level plateau was 25.8 eggs per slide, corresponding to around 1000 epg. Assuming negative binomial variation instead of Poisson distribution in repeated egg counts on the same day did not improve the model fit (figure 2B).



Parameter	Description	Negative binomial variation (log eggs per slide)	Poisson variation (log eggs per slide)
β_{pop}	Population average at plateau level	25.0 (7.2-57.2)	25.8 (10.2 - 57.2)
σ_{β}	Inter-individual variation in stable level on logarithmic scale	0.7 (0.1 - 2.3)	0.7 (0.1 - 2.2)
μ_t	Time when faecal egg density has reached half of its stable level (weeks)	10.2 (8.9 - 11.8)	10.2 (9.0 - 11.7)
σ_t	Parameter determining slope of climb to plateau (97.5% of plateau level is reached at weeks)	1.5 (0.8 - 2.4)	1.6 (0.9 - 2.3)
k_{day}	Shape parameter for deviations of daily average egg counts from expected levels within individuals (lower values indicate higher variation)	1.5 (0.9 - 2.4)	1.5 (0.9 - 2.3)
k_{test}	Shape parameter for variation in repeated egg counts on same day within individuals (lower values indicate higher variation)	38.0 (10.3-99.5)	-

Figure 2. Individual fecal egg counts over time

A. Observed egg counts vs. estimated individual fecal egg density kinetics (solid line with grey band for 95%-Bayesian credible interval), assuming Poisson variation in repeated egg counts.
B. Parameters when assuming negative binomial or Poisson variation, posterior mean with 95%-Bayesian credible interval.

Discussion

This study shows that experimental infection with 50 L3 *Necator americanus* larvae divided over four application sites is well tolerated and leads to patent infection with eosinophilia and fecal egg excretion in all four volunteers. Long-term follow-up showed that egg counts increased from week 7 to week 13 post infection and then reached a stable level. The peak of adverse events, primarily abdominal complaints, occurred around six 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 four extremities. Although median duration of rash was similar, the intensity of local events was decreased as compared to Diemert et al. [10] The levels of egg excretion, however, were much higher than 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 had viability rates of >88% and were all used within ten days of harvesting. Alternatively, the application of larvae over several sites may have enhanced infectivity. The plateaus of egg counts observed in this study are below the WHO defined cut-off for a light infection of 2000 epg. [6] Comparable average egg excretions are widely observed in endemic areas. [14]

Although experimentally infected hookworm volunteers have been subjected to long follow-up before, [15] this is the first study to describe the kinetics of *Na* egg excretion over a prolonged period of time 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 as variation in egg counts in field studies is generally much higher and can only be described by a negative binomial distribution. [7] Possibly, the highly standardised method of slide preparation and feces homogenization in this trial and the lack of reinfection reduces variability in egg output. However, more data are needed to further quantify the day-to-day variation within and between individuals. Although PCR analysis may potentially show less variability, microscopic techniques are still the preferred primary endpoint in phase 2 vaccine or drug trials. Bayesian multi-level analysis is helpful to accurately assess the stabilizing levels of egg excretion from 13 weeks onwards 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, later or slower rise in egg counts or a combination. The timing and level of the plateau in egg counts could be a reliable endpoint for clinical trials, assuming 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 a controlled human infection with 50 L3 *Na* larvae was well tolerated, resulted in patent infection in all volunteers with unparalleled high egg counts.

Despite the fact that 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.

Notes

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Supplementary material A

Description of procedure for larval production

Fecal samples were collected from a healthy donor confirmed negative for HIV, Hepatitis B and Hepatitis C carrying a chronic hookworm infection. Donor feces were confirmed negative for gastro-intestinal pathogens by real-time in-house multiplex PCR for Norovirus, Sapovirus, Astrovirus, Adenovirus, Rotavirus, Salmonella, Shigella, Campylobacter, Yersinia, *Entamoeba histolytica*, *Giardia lamblia* and Cryptosporidium. In addition, fecal samples were cultured for *Staphylococcus aureus* and highly-resistant Gramnegative rods using enrichment broth and subsequent subculture on non-selective and selective culture media for MRSA, ESBL-positive Enterobacteriaciae and ciprofloxacin- and tobramycin-resistant gram negative rods. Eggs were counted by Kato-Katz and hookworm egg morphology was confirmed by microscopy.

For culture of infectious L3 larvae, feces were mixed with gentamicin and amphotericin B and subsequently charcoal which was subsequently cultured in water for 7-10 days. At this time, larvae were collected and washed with betadine solution (100 mg/ml) to reduce bioburden. Larvae were counted, checked for viability and larval species morphology was confirmed by microscopy. A multiplex PCR was performed to distinguish *Necator americanus* from similar larvae (*Strongyloides stercoralis* and *Anylostoma duodenale*). Bacterial culture was performed to confirm absence of potentially pathogenic bacteria including *Staphylococcus aureus*, beta-hemolytic streptococci *Aeromonas* and *Pseudomonas aeruginosa* using general culture media. Upon preparing the correct dose on gauze, larvae were counted and checked for viability again.

Final batch release is performed by the QC and QP of the LUMC pharmacy according to pre-specified criteria.

Supplementary material B

Overview of all related adverse events present per week after infection.

Week	Rash	Itching	Abdominal pain	Nausea	Flatulence	Sore throat	Coughing	Headache
0	3	3	0	0	0	0	0	0
1	4	4	0	0	0	0	0	1
2	4	1	0	0	0	0	0	0
3	3	0	2	1	0	1	0	2
4	3	0	0	0	0	0	1	0
5	1	0	2	2	1	0	0	1
6	0	0	2	0	1	0	1	2
7	0	0	0	0	0	2	0	1
8	0	0	2	0	0	0	0	0
9	0	0	1	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0

All adverse events were grade 1 (mild), except two severe adverse events in week 6 (abdominal pain and headache, both lasting as severe for less than one day) and three moderate adverse events in week 6 (sore throat, coughing and headache).