Building bridges: a multidisciplinary approach to controlled human hookworm infection

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Chapter 4

A randomized controlled trial to investigate safety and variability of egg excretion after repeated controlled human hookworm infection

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Abstract

Background

Controlled human hookworm infections could significantly contribute to the development of a hookworm vaccine. However, current models are hampered by low and unstable egg output, reducing generalizability and increasing sample sizes. This study aims to investigate the safety, tolerability and egg output of repeated exposure to hookworm larvae.

Methods

Twenty-four healthy volunteers were randomized double blind to one, two or three doses of 50 *Necator americanus* L3 larvae at 2-week intervals. Volunteers were monitored weekly and were treated with albendazole at week 20.

Results

There was no association between larval dose and number or severity of adverse events. Geomean egg loads stabilized at 697, 1668 and 1914 eggs per gram feces for the 1x50L3, 2x50L3 and 3x50L3 group respectively. Bayesian statistical modelling showed that egg count variability relative to the mean was reduced with a second infectious dose, however the third dose did not increase egg load or decrease variability. We therefore suggest 2x50L3 as an improved challenge dose. Model-based simulations indicates increased frequency of stool sampling optimizes power of hypothetical vaccine trials.

Discussion

Repeated infection with hookworm larvae increased egg counts to levels comparable to the field and reduced relative variability in egg output without aggravating adverse events.

Background

Hookworm infects around 230 million people worldwide.[1] Chronic infection causes iron-deficiency anaemia and protein loss among the world's poorest,[2] may impede children's cognitive and physical development[3] and adversely affect pregnancy outcomes.[4] This results in high losses in annual productivity (estimated between $7.5 and $138.9 billion),[5] perpetuating the poverty cycle.[6] The impact of periodical deworming, the cornerstone of current hookworm control programs, is impeded by high reinfection rates[7,8] and the looming threat of anthelmintic resistance.[9] A safe and efficacious hookworm vaccine is therefore needed to aid hookworm control,[10] however development is hampered by a lack of preclinical models since animal models do not reflect natural infection in humans.

Experimental infection of volunteers, so-called controlled human hookworm infection (CHHI) trials could be a method to screen novel products instead.[11] In other diseases, controlled human infection models have shown their usefulness in testing vaccine efficacy.[12,13] However, their pertinence hinges on safety of the model, generalizability of results to natural infection and adequately powered sample sizes.

Previous CHHI trials have shown that infections with 50 *Necator americanus (Na)* infectious larvae (L3) are well tolerated with mild abdominal adverse events and that skin eruptions can be alleviated by spreading the infectious dose over four sites.[14,15] Resulting egg counts however have varied, with some trials reaching 100% infectivity and egg counts comparable to field settings,[15] whereas other studies reached lower egg output.[14,16] In addition, microscopic egg counts, the gold standard endpoint of CHHI trials, are highly variable,[17] decreasing the power of hookworm infection studies to detect vaccine efficacy. In a previous trial we used Bayesian statistical modelling to describe the long-term kinetics of hookworm egg excretion investigated[15] and found that one dose of 50L3 larvae resulted in egg counts plateauing around 13 weeks after infection at a level comparable to low-endemic field settings. Although homogenizing the feces decreased the variability of egg counts in the same individual on the same day, considerable inter- and intra-individual variation remained.[15] We hypothesized that repeated infection could increase egg outputs and potentially reduce variability relative to the mean without increasing adverse events, enhancing the power of CHHI trials.

Here we report the outcome of a CHHI trial in which volunteers were exposed to multiple doses of *Na* L3. Based on these data we developed a Bayesian model for the in-depth analysis of the variation in egg counts by dose group and performed hypothetical sample size calculations to determine the power of CHHI models in vaccine trials.

Methods

This study was a randomized, double-blind, placebo-controlled trial investigating the safety and tolerability of cumulative doses of *Na* larvae up to 3x50 L3 and the variability in egg output after repeated infection with *Na* L3.
The trial was approved by the LUMC institutional review board (NL59186.058.17) and registered at clinicaltrials.gov (NCT03257072).

Study subjects
Healthy male and female volunteers aged 18-45 years were recruited from the Leiden area in January and February 2018. All volunteers provided written informed consent before inclusion. In- and exclusion criteria are described in supplement A. Participants were all confirmed hookworm-negative by PCR before inclusion.

Na L3 larvae were cultured from feces of chronically infected hookworm donors according to the principles of Good Manufacturing Practice following a procedure previously described.[15 18]

Study procedures
Volunteers were randomized 1:1:1 to receive one, two or three doses of 50 *Na* L3 at two-week intervals, totaling a cumulative dose of 50, 100 or 150 L3 (Figure 1). Volunteers in the 50 or 2x50L3 group first received respectively two or one dose of placebo. Randomization was performed through a randomization list made by an independent researcher. Larval doses were suspended in sterile water and divided over four gauzes which were applied to both upper arms (doses of 10 L3) and calves (doses of 15 L3) and left for one hour. As placebo, gauzes with sterile water were used. Investigators and participants were blinded to dose allocation.

Volunteers were followed weekly for twenty weeks. At each visit, adverse events (AEs) were collected and blood drawn for eosinophils and hemoglobin. Hemoglobin and eosinophils were measured on the Sysmex DI-60 automated cell morphology analyzer. Skin-related AEs, cough, sore throat, fever and abdominal AEs were solicited at each visit. For every AE timing, severity and causality were recorded. AEs were scored as unrelated, unlikely, possibly, probably or definitely related to hookworm infection, and mild (no interference with daily life), moderate (discomfort interfering with daily life) or severe (causing inability to perform usual daily activity). Two independent, blinded physicians divided the volunteers into two equal groups based on the safety data, one with “high” abdominal AEs signifying the half of volunteers with most abdominal AEs and one with “low” abdominal AEs, the half with the least abdominal symptoms. Division in groups was based on severity, duration and number of abdominal AEs, consensus was reached for each volunteer.

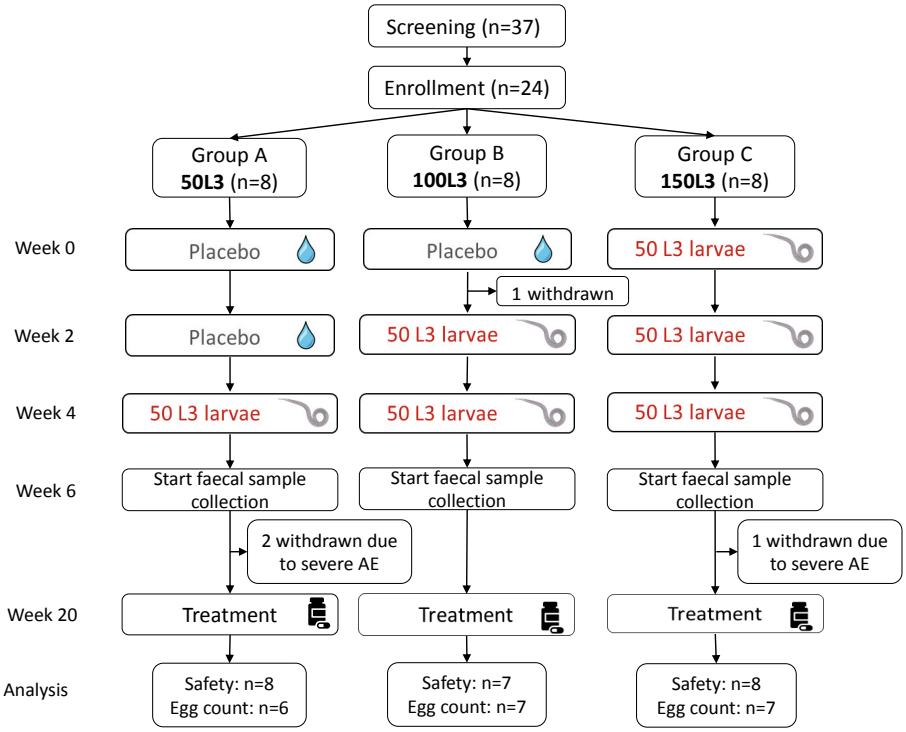


Figure 1. Trial set-up and flow chart. Abbreviation: AE, adverse event.

At week 20 of the trial all volunteers were treated with 400mg albendazole for three days. Two volunteers gave separate informed consent to postpone albendazole treatment and remain as donors for future studies. All other volunteers returned to the trial centre on weeks 1, 2, 3, and 8 after treatment to collect adverse events and ensure complete cure of the infection.

Immunological assays

Serum for analysis of antibodies was collected at weeks 0, 4, 8, 12, 16, 20, 23 and 28 of the trial. Hookworm-specific IgE, IgG, IgG1 and IgG4 were measured by ELISA. Procedures are described in supplement B. Specific antibody levels are expressed in AU/ml. Data was normalised with baseline set at 0 and other measurements presented as AU/ml above baseline, with peak value defined as the highest value measured from baseline.

Seroconversion was defined as any peak AU/ml value of 4x above standard deviation (SD). SD was determined in the week 4 samples of the 50L3 group, as baseline samples were normalised to 0 and at week 4 the 50L3 group had not yet been exposed therefore enabling its use as a control group.

Parasitological assays

Fecal samples were collected weekly from week 6 until week 20 of the trial and at one and three weeks after treatment. Kato-Katz slides were prepared fresh with 25 milligrams of stool, homogenized before preparation. [18] Two Kato-Katz slides per fecal sample were prepared, each read by a separate microscopist. The total number of eggs counted was multiplied by 20 and expressed as eggs per gram feces (epg) per collected stool. The method for detection of *Na* DNA in stool by PCR is described in supplement C.

Statistical analysis

Similar to other proof-of-concept vaccine efficacy studies, groups of 8 subjects were chosen. This sample size would give 80% power to detect a 50% relative reduction in egg counts.

Primary endpoint was the frequency and severity of adverse events per group, assessed in the intention to treat population. Differences between groups were analyzed using a Kruskall-Wallis or Mann-Whitney U test.

Secondary endpoint was the difference in egg load between groups. The egg load was defined as the excretion of eggs in faeces between week 16 and 20 of the trial by Kato-Katz. Mean egg counts per individual were calculated, log-transformed values were compared using a one-way ANOVA. Differences between groups for eosinophilic response and antibodies were evaluated using a Kruskall-Wallis test. The correlation between Kato-Katz and PCR was assessed by Spearman's rho.

To compare level and variability of egg counts between groups in more detail and perform power calculations for hypothetical vaccine trials, we developed a Bayesian non-linear regression model describing egg count time series of each individual. The development of this model is described in supplement D.

After establishment of the model and generation of the model parameters, power calculations were performed, primarily assuming a reduction in egg loads of 50% in a vaccine group as compared to the control group with additional calculations for a 30-70% effect size. Power was calculated with the aim to detect a significant difference defined as a p-value < 0.05 based on Wilcoxon signed rank test for the difference in individual average egg counts (averaged over days and Kato-Katz slides) between trials arms.

Results

Study flow

In February 2018, 24 volunteers were included in the trial. Trial flow is shown in figure 1, participant characteristics in table 1. Despite randomization more females than males were included in the 50L3 group. One volunteer withdrew informed consent after one week for reasons unrelated to the trial. Two volunteers (randomized to the 50L3 group) withdrew 6

weeks after first exposure, the third volunteer (from the 3x50L3 group) withdrew at 9 weeks due to severe abdominal AEs. All symptoms resolved with albendazole treatment. Safety data, eosinophils and Ig-results available until withdrawal for these volunteers has been included in the intention to treat analysis, however volunteers withdrew before detection of eggs and therefore are not included in the per protocol analysis of egg excretion.

Table 1. Participant characteristics and number and duration of adverse events for each study group with p-values (Kruskall-Wallis test)

	Total (n=23)	**50 L3 (n=8)**	**2x50 L3 (n=7)**	**3x50 L3 (n=8)**	**P-value**
Sex **Female** **Male**	15 8	7 1	4 3	4 4	
Age in years, median (range)	22 (19-41)	27.5 (19-38)	21 (19-33)	23 (19-41)	
History of travel to hookworm-endemic area	11	4	4	3	
History of gastro-intestinal disease	1	1*	0	0	
Median number of AEs per volunteer (min-max)	16 (8-31)	17 (11-24)	17 (9-31)	16 (8-27)	P=0.902
Median duration of rash	34 (0-77)	18.5 (0-49)	37 (15-50)	41.5 (21-77)	P=0.091
Total number of related abdominal AEs (% of total)	99 (100%)	32 (32)	39 (39)	28 (28)	P=0.681
Median number of related abdominal AEs per volunteer (min-max)	4 (0-10)	4 (0-6)	4 (1-10)	4 (0-7)	
Maximum severity of abdominal AEs, N= None **Mild** **Moderate** **Severe**		1 2 1 4	0 2 1 4	1 3 1 3	
Median duration of abdominal AEs in days, (range)	22 (0-125)	21.5 (0-38)	29 (4-125)	23.5 (0-110)	p=0.638
Number of volunteers with "high" abdominal AEs	12	4	4	4	

AE: Adverse events
*: gastric reflux

Clinical data

No serious adverse events occurred. Most common related AEs were pruritus and rash during the first weeks after exposure (n=23) and abdominal AEs from week 2 after first infection (n=21). Two volunteers reported no abdominal symptoms, five reported only mild

abdominal bloating or flatulence for less than 5 days. Other gastro-intestinal symptoms started from week 2 after exposure, peaked at week 4 and 5 and resolved 8 weeks after first infection in the majority of volunteers (13/16). Symptoms in the remaining three volunteers resolved with albendazole treatment. Based on the combination of severity and duration of AEs, 12 volunteers were classified as “high” abdominal AEs and 11 volunteers as “low”.

There were no significant differences in number and severity of AEs between study groups (table 1), the number of volunteers experiencing grade 3 AEs or any abdominal AE were similar across study groups.

Hemoglobin stayed stable throughout the trial (figure 2A). Eosinophil counts increased in all groups, starting at three weeks after first exposure, with a peak at week six after initial infection (figure 2B). There was no difference in mean peak eosinophil count between groups (p=0.4).

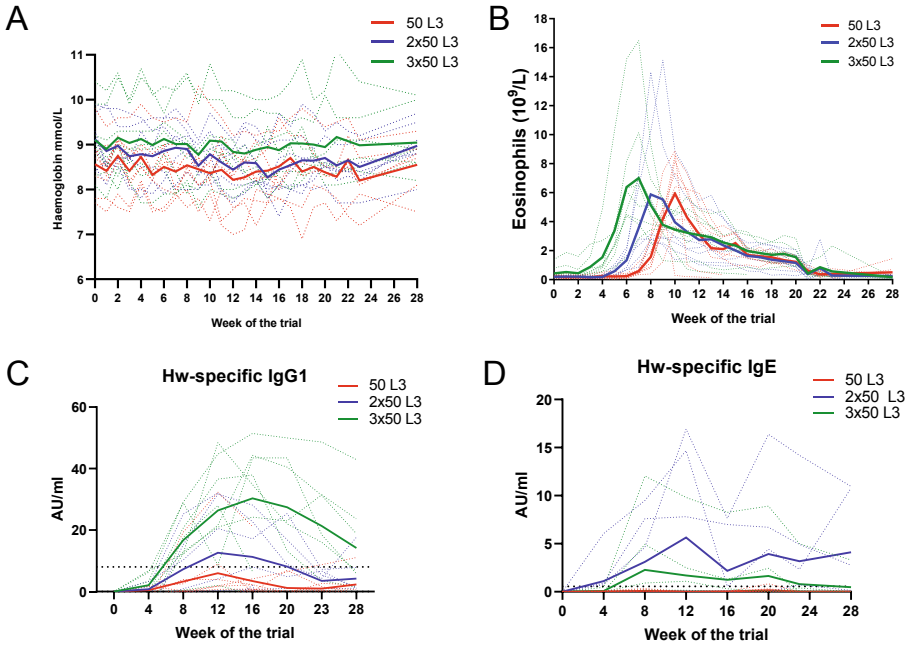


Figure 2. A, B, Hemoglobin levels (A) and eosinophil counts (B) for individual volunteers (dotted lines) and mean for each study group (continuous lines). C, D, Immunoglobulin (Ig) G1 antibody (C) and IgE (D) responses for individual volunteers (dotted lines) and mean for each study group (continuous lines); horizontal dotted line represents threshold for seroconversion (increase from baseline to 4 times the standard deviation). Abbreviation: AU, arbitrary units.

Hookworm-specific antibodies increased with additional doses and peaked at week 12 or 16 of the trial, although there was a large variation between individuals. Rise in IgG1 was significantly different between dose groups (p=0.013 for peak IgG1 value between groups)

(figure 2C). IgG1 seroconversion was reached in respectively 3, 4 and 7 volunteers for the 50 L3, 2x50 L3 and 3x50 L3 groups which showed a weak statistical significance (Chi-square test p=0.12). For IgE, seroconversion was observed in three volunteers in the 2x50 L3 group and two in the 3x50 L3 group. (figure 2D). IgG4 responses were less pronounced than for IgG1, did not show clear differences between dose groups and reached seroconversion in 2, 3 and 2 volunteers respectively (supplementary figure 1A). Total IgG is displayed in supplementary figure 1B.

Parasitological analysis

Volunteers in the 50 L3 group secreted hookworm eggs by Kato-Katz at a median of 7 weeks after first infection (range 5-8), the 2x50 L3 and 3x50 L3 group at 8 weeks (range 7-9) (p=0.676).

Egg loads differed significantly between groups: geomean of the mean counts per individual were 697 epg for the 50 L3 group (95%-CI 228-2131), 1668 (95%-CI 979-2840) for 2x50L3 and 1914 (95%-CI 1455-2517) for 3x50 L3 (one-way ANOVA: p=0.04) (table 2). Post-hoc testing for between group difference showed weak differences between the 50 L3 group and the other two groups (p=0.1 for 50 L3 vs 2x50L3, p=0.05 for 50 L3 vs 3x50 L3) and no difference between the 2x50 and 3x50 L3 groups (p=0.6) (figure 3).

Infection intensity as determined by PCR mirrored these findings (table 2, p=0.02) and were strongly correlated with Kato-Katz counts (p=<0.0001, rho=-0.79) (supplementary fig 2).

PCR and Kato-Katz were all negative three weeks after treatment.

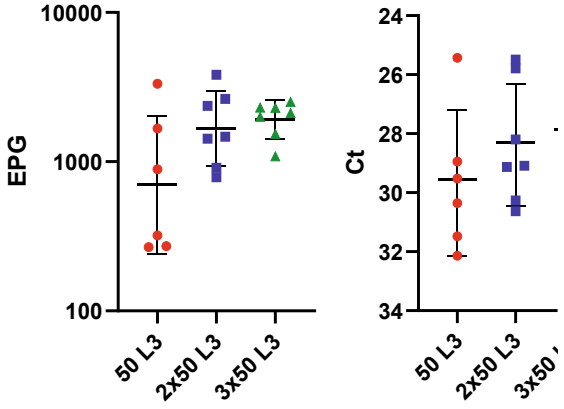


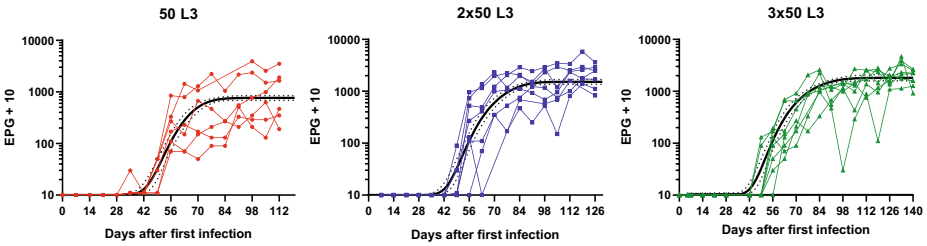
Figure 3. Individual mean Kato-Katz slide measurements (left) and cycle threshold values (right) at weeks 16–20 of the trial. Red circles represent the 50L3 group; blue squares, 2 × 50L3 group; green triangles, 3 × 50L3 group; lines, geometric means; and error bars geometric standard deviations. Abbreviation: epg, eggs per gram feces.

Bayesian statistical modelling

Using Bayesian statistical modelling we first assessed the relative contributions of each L3 dose to the overall egg load. Allowing dose contributions to vary freely between all groups confirmed an equal contribution of the first and second dose of L3 and a lesser contribution of the third dose (estimated at factor 0.4 relative to a single dose, 95%-Bayesian credible interval (BCI): 0.01–1.0). Next, when we allowed the level of temporal overdispersion (i.e. daily variation relative to the mean) to vary freely between all three groups, the level of overdispersion was a factor 1.9 higher (95%-BCI: 1.0–3.2) in the single-dose group compared to the two-dose group; there was no significant difference in temporal overdispersion (and thus relative variation) between the two- and three-dose groups(difference of factor 1.1, 95%-BCI: 0.5–1.9). We therefore further simplified the model assuming that temporal overdispersion differed only between the single dose group (k = 3.2, 95%-BCI: 2.0–4.9) compared to the other two dose groups (k = 5.6, 95%-BCI: 4.1–7.5; i.e. a difference of factor 1.9, 95%-BCI: 1.01–3.05). We verified that random effects for the plateau level and timing of rise in egg counts within each individual were uncorrelated (supplementary figure 3).

We estimated that one, two, or three doses of 50 L3 resulted in a posterior mean egg load at plateau level of 760 (95%-BCI: 640-840) to 1520 (95%-BCI: 1360-1680) and 1800 (95%-BCI: 1560-2080) epg, respectively, corresponding with the descriptive statistics of egg loads previously described (figure 4). The rise in egg counts due to each single dose reached 50% of its maximum level at 67 days (~10 weeks) after exposure (95%-BCI: 63–70) and 97.5% of its maximum after another 18 days (95%-BCI: 15–22). Inter-individual variation in the timing of the initial rising phase was minimal with a standard deviation on the logarithmic scale of 0.08 (95%-BCI: 0.05–0.11).

A summary of the final model estimated parameters is provided in supplementary table 1, and estimated and measured egg counts per individual in supplementary figure 4.



	50 L3	2x50 L3	3x50 L3
Geomean eggs per gram of mean egg count per individual week 16-20 of the trial (95%-CI)	697 (228-2131)	1668 (979-2840)	1914 (1455-2517)
Geomean of mean Ct-value per individual wk 16-20 of the trial (95%-CI)	29.6 (27.1-32.3)	28.3 (26.5-30.3)	27.8 (27.0-28.7)
Posterior mean in Bayesian model (95% BCI)	760 (640-840)	1520 (1360-1680)	1800 (1560-2080)
Temporal overdispersion (shape parameter k, higher values indicate less variability relative to mean) (95% BCI)	3.2 (2.0–4.9)	5.6 (4.1-7.5)	5.6 (4.1-7.5)

Figure 4. Egg counts as observed and predicted by a bayesian nonlinear regression model, for 50L3 (left), 2 × 50L3 (middle), and 3 × 50L3 (right) groups. Solid line in each panel represents the group average; dashed lines, upper and lower limits for the 95% bayesian credible interval (BCI) of the point estimate. Table below graphs shows mean values for Kato-Katz slides and polymerase chain reaction, bayesian posterior means, and shape parameter k for temporal overdispersion; for the latter, higher values indicate less variability relative to the mean. Abbreviations: BCI, bayesian credible interval; CI, confidence interval; Ct, cycle threshold; epg, eggs per gram feces.

Correlation between adverse events, egg counts and antibody response

Volunteers with a higher egg output had higher mean hookworm-specific IgG1 (R=0.537, p=0.015 for egg load, R=-0.586, p=0.007 for Ct value) but not more IgE, IgG4 or eosinophils. Volunteers with more abdominal adverse events had higher levels of eosinophils (mean 9.1x10^9 vs 4.4x10^9 in “high” versus “low” counts, p=0.003) but an equal number of eggs (geomean 914 epg vs 1086 epg, p=0.6) (figure 5). Antibody levels did not correlate with adverse events or eosinophils.

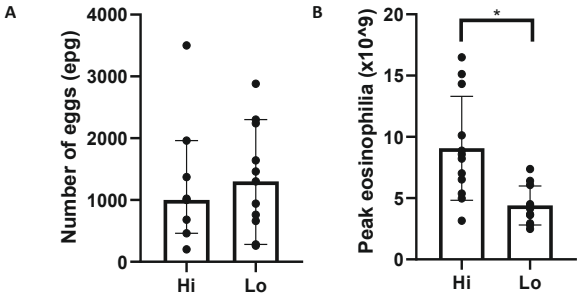


Figure 5. Comparison of volunteers in the upper ("high") and lower ("low") groupings according to the severity, duration, and number of abdominal adverse events, for both egg load (A; shown as median with 95% CI) and eosinophilia (B; shown as mean with standard deviation). *P = .003. Abbreviations: CI, confidence interval; epg, eggs per gram feces.

Power calculations

Using the Bayesian non-linear regression model, power calculations were performed by repeatedly simulating synthetic data for a two-armed trial assuming the use of a vaccine that reduces egg counts by 50% compared to placebo. Sampling of stools was assumed to start during the stable phase in week 15 after first infection. Although multiple doses of L3 increase study power somewhat (due to higher egg counts in the control group and lower random daily variation within individuals), power is mostly driven by the number of repeated samples that are collected at weekly intervals (figure 6). A power of >80% can be reached in groups as small as six volunteers if samples are taken for five weeks with two doses of 50 L3. In contrast, a power of only 60% is reached with a single sample in groups of 15 volunteers with three infectious doses. Assessing multiple Kato-Katz slides per sample does not improve power compared to a single slide. The power calculation was re-run with an expected vaccine efficacy of 30-70%. This showed that with sufficiently frequent sampling even at only 30% efficacy groups of 15 participants can reach 80% power (supplementary figure 5).

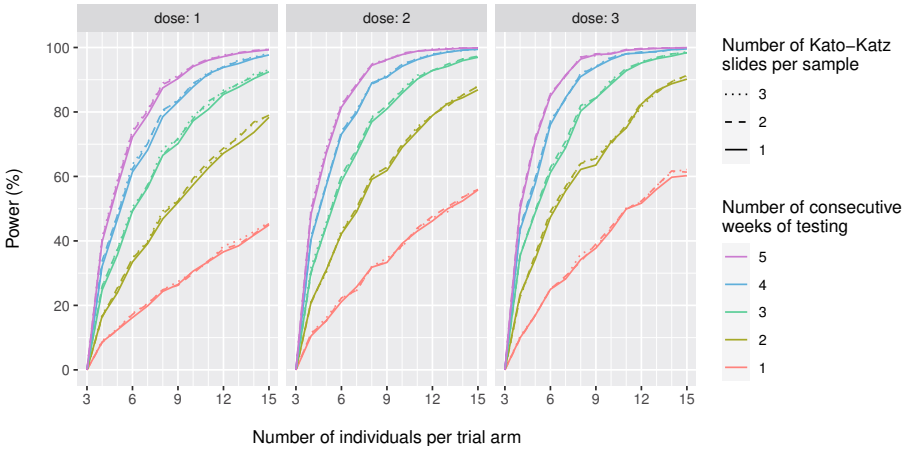


Figure 6. Power calculation using bayesian modeling of egg counts showing the number of individuals per trial arm in a vaccine trial, assuming 50% reduction in egg counts in the vaccine versus the placebo group, after 1 (left), 2 (middle), or 3 (right) doses of 50L3. Colors represent the numbers of weekly samples obtained with the top line in each graph representing 5 samples and each subsequent lower line one sampling less, the lowest line represents 1 sample.

Discussion

This dose escalation study showed that higher cumulative doses of hookworm L3 did not enhance adverse events, which displayed high inter-individual variability and a reasonable tolerability profile. Repeated infectious doses resulted in an increase in total egg counts and decreased relative variability in egg excretion. Power calculations based on a Bayesian non-linear regression model showed that repeated sampling is the most important parameter determining the power of the controlled human hookworm infection model to detect potential vaccine efficacy.

Volunteers reported a considerable number of abdominal adverse events, with three volunteers receiving rescue treatment early due to severe abdominal AEs. However, these events do not seem to be related to L3 dose, as two of the rescued volunteers were in the lowest dose group. Within each dose group we found considerable inter-individual variation in number and severity of adverse events. Severity of adverse events correlated with eosinophilic response, which both peak around week 6 after infection. We hypothesize that this may reflect symptomatic eosinophilic enteritis, which has previously been associated with hookworm attrition. [19] We however could not establish a relationship between egg load and eosinophil counts.

Larval dose and eosinophilic response were not related, however there was a clear relationship between dose and humoral responses to the larvae. Remarkably, we found

IgG1 to be the most pronounced responding Ig subtype in this acute infection, contrasting epidemiological studies where IgE and IgG4 are hallmarks of active chronic infection.[20] IgE has previously been observed to develop only after repeated exposure[20] and may confer protection.[21] We did observe an IgE response in some volunteers after repeated exposure however given the limited response in only a few volunteers, we were not able to confirm any protective effect.

PCR results showed a similar pattern of increased infectious loads with higher cumulative infectious doses and showed good correlation with Kato-Katz outcomes. However, the high SD of between 2.0 and 0.9 Ct, corresponding to a variation of double or quadruple the amount of DNA indicate high variability in PCR outcomes. This may be caused by both variability in the egg excretion itself and by differences in the number of DNA-copies per egg, as a Na egg may be in a 2-16 cell stage, increasing possible sources for variation.[22] In addition to the more difficult field applicability of PCR-techniques and supplementary variability of Ct-values between labs,[22] this underscores that as yet microscopic techniques remain the cornerstone of hookworm diagnosis and highlights the necessity to improve power and accuracy of diagnostic outcome measures.

Repeated dosing resulted in egg counts that are more representative of infection levels in endemic areas, with mean epg only slightly below the WHO threshold for moderate infection (2000 epg).[23] These egg counts are higher than previously reported using a single inoculation,[14 15] enabling a better comparison with natural infection. However, the third dose does not seem to add significantly to the total egg output compared to two doses. Possibly, repeated exposure induces some immunity against the larvae of the subsequent infection resulting in less surviving worms, or competition for nutrients and feeding sites occurs as the number of larvae establishing in the intestine simultaneously is higher than in a natural, trickled infection.

The high variability of egg secretion complicates the use of the CHHI model for vaccine testing. We therefore used Bayesian statistical modelling to more accurately estimate individual and population egg outputs and to better describe the variability in egg counts. Use of Bayesian modelling carries the advantage of a robust inference and the possibility to carry uncertainties of parameter estimates into power calculations, providing more accurate power estimates. This confirmed that the third infectious dose has a very limited contribution to the egg count plateau and to reduction of relative variability compared to two doses. Consequently, the use of the 2x50L3 dose seems the most rational option for future use in CHHI, as it leads to egg counts comparable to endemic areas with reduced relative variability compared to a single dose without aggravating adverse events. The power calculation based on the Bayesian statistical model underscores the importance of repeated sampling over time, which has higher impact on study power than the number of infections. More importantly, if we assume that variation between sampling on consecutive days is similar as variation within weekly samples and therefore take five consecutive samples

during the first weeks of the plateau phase (from week 13 after infection), CHHI trial duration may be substantially shortened from our current 20 weeks follow-up to for example 15 weeks. Naturally, sample sizes in future vaccine studies may be further increased to also detect differences in AEs or SAEs.

Due to the staggered design timing of first infection varied between different dose groups. However, egg load was analyzed at plateau level for all groups, ensuring comparability of outcomes. The study was underpowered to detect a difference in adverse events. Given the remarkably equal distribution of adverse events in the groups, significant differences seem unlikely. Although study set-up cannot mimic natural repeated infection with very frequent exposure to small inoculae and group sizes were diminished due to withdrawal of four volunteers, this remains the largest study to date to investigate any kind of repeated exposure to hookworm infection.

In conclusion, this trial has further advanced the controlled human hookworm infection model by introducing repeated challenge in the model and underscoring the importance of repeated sampling after development of the plateau phase. These findings are an important step in the applicability of the CHHI model for future vaccine- and drug research.

Notes

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References

1. Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet (London, England)* 2018;392(10159):1789-858.
2. Loukas A, Hotez PJ, Diemert D, et al. Hookworm infection. *Nature reviews Disease primers* 2016;2:16088.
3. Brooker S, Bethony J, Hotez PJ. Human Hookworm Infection in the 21(st) Century. *Advances in parasitology* 2004;58:197-288.
4. Brooker S, Hotez PJ, Bundy DA. Hookworm-related anaemia among pregnant women: a systematic review. *PLoS Negl Trop Dis* 2008;2(9):e291.
5. Bartsch SM, Hotez PJ, Asti L, et al. The Global Economic and Health Burden of Human Hookworm Infection. *PLoS neglected tropical diseases* 2016;10(9):e0004922.
6. Hotez PJ, Fenwick A, Savioli L, et al. Rescuing the bottom billion through control of neglected tropical diseases. *Lancet* 2009;373(9674):1570-5.
7. Speich B, Moser W, Ali SM, et al. Efficacy and reinfection with soil-transmitted helminths 18-weeks post-treatment with albendazole-ivermectin, albendazole-mebendazole, albendazole-oxantel pamoate and mebendazole. *Parasites & vectors* 2016;9:123.
8. Supali T, Djuardi Y, Bradley M, et al. Impact of six rounds of mass drug administration on Brugian filariasis and soil-transmitted helminth infections in eastern Indonesia. *PLoS neglected tropical diseases* 2013;7(12):e2586.
9. Soukhathammavong PA, Sayasone S, Phongluxa K, et al. Low Efficacy of Single-Dose Albendazole and Mebendazole against Hookworm and Effect on Concomitant Helminth Infection in Lao PDR. *PLoS Neglected Tropical Diseases* 2012;6(1):e1417.
10. Hotez PJ, Diemert D, Bacon KM, et al. The Human Hookworm Vaccine. *Vaccine* 2013;31 Suppl 2:B227-32.
11. Gaze S, Bethony JM, Periago MV. Immunology of experimental and natural human hookworm infection. *Parasite immunology* 2014;36(8):358-66.
12. Roestenberg M, Hoogerwerf MA, Ferreira DM, et al. Experimental infection of human volunteers. *The Lancet Infectious diseases* 2018;18(10):e312-e22.
13. Darton TC, Blohmke CJ, Moorthy VS, et al. Design, recruitment, and microbiological considerations in human challenge studies. *The Lancet Infectious diseases* 2015;15(7):840-51.
14. Diemert D, Campbell D, Brelsford J, et al. Controlled Human Hookworm Infection: Accelerating Human Hookworm Vaccine Development. *Open forum infectious diseases* 2018;5(5):ofy083.
15. Hoogerwerf MA, Coffeng LE, Brienen EAT, et al. New Insights Into the Kinetics and Variability of Egg Excretion in Controlled Human Hookworm Infections. *The Journal of infectious diseases* 2019;220(6):1044-48.
16. Mortimer K, Brown A, Feary J, et al. Dose-ranging study for trials of therapeutic infection with Necator americanus in humans. *The American journal of tropical medicine and hygiene* 2006;75(5):914-20.
17. Werkman M, Wright JE, Truscott JE, et al. Testing for soil-transmitted helminth transmission elimination: Analysing the impact of the sensitivity of different diagnostic tools. *PLoS neglected tropical diseases* 2018;12(1):e0006114.
18. Polderman AM, Eberhard M, Baeta S, et al. The Rise and Fall of Human Oesophagostomiasis2010.

19. Croese J, Wood MJ, Melrose W, et al. Allergy controls the population density of Necator americanus in the small intestine. *Gastroenterology* 2006;131(2):402-9.

20. McSorley HJ, Loukas A. The immunology of human hookworm infections. *Parasite immunology* 2010;32(8):549-59.

21. Pritchard DI, Quinnell RJ, Walsh EA. Immunity in humans to Necator americanus: IgE, parasite weight and fecundity. *Parasite immunology* 1995;17(2):71-5.

22. Papaiakovou M, Gasser RB, Littlewood DTJ. Quantitative PCR-Based Diagnosis of Soil-Transmitted Helminth Infections: Faecal or Fickle? *Trends in parasitology* 2019;35(7):491-500

23. Committee WHOE. Prevention and control of schistosomiasis and soil-transmitted helminthiasis. *World Health Organization technical report series* 2002;912:i-vi, 1-57, back cover.

Supplementary material

Supplement A: In- and exclusion criteria

Inclusion-criteria:

1. Subject is aged ≥ 18 and ≤ 45 years and in good health.
2. Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3. Subject is able to communicate well with the investigator, is available to attend all study visits.
4. Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period.
5. For female subjects: subject agrees to use adequate contraception and not to breastfeed for the duration of study.
6. Subject has signed informed consent.

Exclusion-criteria:

A potential subject who meets any of the following criteria will be excluded from participation in this study:

1. Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, haematological, infectious, immune-deficient, psychiatric and other disorders, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:

- Body Mass Index (BMI) <18.0 or >30.0 kg/m2 at screening;
- positive HIV, HBV or HCV screening tests;
- the use of immune modifying drugs within three months prior to study onset (inhaled and topical corticosteroids and oral anti-histamines exempted) or expected use of such during the study period;
- having one of the following laboratory abnormalities: ferritine <10 ug/L, transferrine <2.04 g/L or Hb <7.0 mmol/L for females or <8.0 mmol/L for males;
- history of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years;
- any history of treatment for severe psychiatric disease by a psychiatrist in the past year;
- history of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset.

2. Known hypersensitivity to or contra-indications for use of albendazole, including co-medication known to interact with albendazole metabolism (e.g. carbamazepine, phenobarbital, phenytoin, cimetidine, theophylline, dexamethasone)
3. Known allergy to amphotericin B or gentamicin
4. For female subjects: positive urine pregnancy test at screening
5. Positive fecal qPCR or Kato-Katz for hookworm at screening, any known history of hookworm infection or treatment for hookworm infection or possible exposure to hookworm in the past
6. Being an employee or student of the department of Parasitology of the LUMC
7. Current or past scars, tattoos, or other disruptions of skin integrity at the intended site of larval application
8. Subjects with planned travel to hookworm endemic areas during this trial
9. Receipt of a vaccine within 4 weeks prior to the study initiation
10. Known food allergy

Supplement B: ELISA Procedure
Hookworm antigen: *Necator americanus* L3 extract.

Cultured Hookworm L3 larvae were collected in sterile water and stored in 50ml tubes at -80°C after which they were freeze dried and stored at -80°C again. After thawing, the larvae were suspended in PBS, transferred to a glass homogenizer and kept on ice whilst washing 4 times. The solution in the homogenizer was crushed for 15 minutes and left for one hour on ice. Crushing and resting was repeated three times. Crushed hookworm larvae were transferred to a glass tube, and the homogenizer was washed once with PBS. The crushed hookworm larvae were sonified (Branson Sonifier) 6 times for 30 seconds with an interval of 20 seconds. The samples were kept overnight at -80°C. The frozen samples were thawed and centrifuged for 25 minutes, 13.000 rpm at 4^{o}C. The supernatant as well as the resuspended pellet were centrifuged again, after which supernatants from both tubes were collected and pooled. Antigen concentration was determined using a BCA kit (ThermoFisher 23225) following manufacturers instruction.

IgG, IgG1 and IgG4 ELISA: High-binding C96-wells maxisorp plates (Nunc-Immuno™ 430341) were coated with 5ug/ml hookworm antigen in 0.1M Na-carbonate pH 9.6. After overnight incubation at 4^{o}C, plates were washed 4 times with 0.05% Tween 20 (Sigma-Aldrich, 27, 434-8) in PBS wash buffer. Plates were blocked with 5% BSA/PBS for one hour at 37^{o}C. After washing, a mix of positive serum was used in a serial dilution of 1:2 as positive controls, samples were at least 1:2 diluted in PBS 0.05% Tween 20 assay buffer. Plates were incubated overnight at 4^{o}C for IgG1 and IgG4, and 1 hour at 37^{o}C for total IgG. For IgG1 detection, conjugated monoclonal antibody of HRP-labelled anti human IgG1 (Fc) (clone MH161-1, HP6188, Sanquin; cat no: M1328) was added and for IgG4 detection HRP-labelled anti human IgG4 (CH3) (clone MH164-1, HP6196, Sanquin; cat no: M1331) was added, both at a

concentration of 1.8uL diluted in 5.5 ml PBS 0.05% tween and both 50 uL/well. For total IgG detection, 50 uL/well 1.8 uL alkaline phosphate conjugated anti-human IgG (Sigma A9544, 4^{o}C) in 5.5 ml PBS 0.05%Tween-4% BSA was added. Plates were incubated at 37^{o}C (one hour for IgG, four hours for IgG1 and IgG4). After washing, TMB substrate was added (TMB Microwel substrate system(KPL, 50-76-00) for IgG1 and IgG4, 6mg p p-nitrophenylphosphate (p-NPP) in 6 ml diethylanolamine (DEA) buffer for IgG)) and development was stopped with 18M H_2SO_4 in water, after which plates were read at by 450 nm at the ELISA plate reader. Total IgG plates were incubated in the dark for one hour at room temperature and then read at wavelength 405 nm.

IgE ELISA: Polysorp F96-wells plates (Nunc-Immuno™ 475094) were coated with 5ug/ml hookworm antigen in 0.1 M Na-carbonate buffer, pH 9.6. After overnight incubation at 4^{o}C wells plates were washed 4 times with 0.05% Tween 20 (Sigma-Aldrich, 27, 434-8) in PBS wash buffer. Blocking with 5% BSA/PBS was performed for one hour at room temperature. After washing four times, a mix of positive sera were added in a serial dilution of 1:2 in PBS 0.05% Tween 20 assay buffer and serum samples were diluted at least 1:2 in assay buffer. Plates were incubated overnight at 4^{o}C. After five washings 100uL/well of 11uL biotinylated goat anti human IgE (Vector BA3040, 0.5 mg/ml) diluted in 11 ml PBS 0.05%Tween was added and plates were incubated for three hours at 37^{o}C. Samples were flicked off and washed five times after which 100uL/well of 3,6 uL of Streptavidin alkaline phosphatase (strep-AP-conjugate, Roche 11.089.161.001, 4ºC) diluted in 11 ml PBS 0.05%Tween was added and incubated for three hours at 37^{o}C. After flicking the conjugate and washing 5 times 100uL/well p p-nitrophenylphosphate was added at a concentration of 1 mg/ml in diethylanolamine buffer. Plates were incubated for two hours in the dark and then read at 405nm at the Elisa plate reader.

All Ig analyses:
OD values are converted to AU/ml using a standard curve with unknown concentration, enabling comparison of a rise or fall in serum concentration within the same subject.

Supplement C: PCR procedure
For PCR, DNA was extracted following a previously described method[1], including the addition of Phocin Herpes Virus (PhHV-1) to the lysis buffer as an internal control. Amplification reactions were performed in white PCR plates in a volume of 25 µl with PCR buffer (HotstarTaq master mix, QIAgen, Germany), 5 mM MgCl2, 2.5 µg Bovine Serum Albumin (Roche Diagnostics Nederland B.V., Almere, the Netherlands), 5 pmol of each Na-specific primer, 1.25 pmol of Na-specific XS-probe and 5ul of the DNA sample[2] 3. Primers and probe for the detection of Na are described in supplementary table 1. Amplification, detection and analysis were performed using the Bio-Rad CFX96™ realtime detection system, resulting in Cycle threshold (Ct)-value as the output, reflecting the parasite-specific DNA load in the tested sample. All samples were analysed in the same PCR-run.

Primers and Probes for Na PCR[2 3]

Oligonucleotide name	Oligonucleotide sequence	Genbank accession no of target sequence
Necator americanus		
Na58F	5'-CTGTTTGTCGAACGGTACTTGC-3'	AJ001599
Na158R	5'-ATAACAGCGTGCACATGTTGC-3'	AJ001599
Na81t_XS_FAM	FAM-5'-CTGTACTACGCATTGTATAC-3'-BHQ1	AJ001599

Supplement D: Development of Bayesian model

The initial rise in egg counts in each individual was described with an S-shaped function, using a cumulative normal distribution function, which was scaled to the plateau level within each individual. The model captures differences between groups in terms of the level of stabilization (the plateau level) and daily variation in egg counts (fixed effects) and inter-individual variation in the timing of the initial rise and the level of stabilization (random effects). The duration of the initial rise in egg counts after a single dose was assumed to be the same for all individuals. Multiple doses of L3 over time were assumed to result in a plateau which reflects the sum of multiple (shifted) S-curves (figure 1). Repeated doses of L3 larvae were allowed to result in diminishing, equal or increased effect contribution to the final plateau egg level. Random effects for timing of the rise and the level at which egg counts stabilized within individuals were assumed to each follow an independent log-normal distribution. To make the model identifiable, the random effect for variation in stabilization level was constrained to sum to zero (on the logarithmic scale) within each dose group. Average fecal egg densities within individuals were assumed to vary randomly between days following a gamma distribution, where the level of overdispersion (i.e. daily variation relative to the mean egg count, determined by the shape parameter k of the distribution with lower values for k signifying higher variation) was allowed to differ between the three dose groups. Repeated egg counts on the same day (i.e. performed on a single homogenized stool sample) were assumed to follow a Poisson distribution; a negative binomial distribution did not provide a better fit. This corresponded to the findings in the previously developed model[4], underscoring the importance of feces homogenization.

The joint posterior distribution of model parameters was estimated with dynamic Hamiltonian Monte Carlo, using *rstan* version 2.18.2 in *R* version 3.6.0 and *RStudio* version 1.2.1335[5]. Parameter estimates were summarized as posterior means and 95%-Bayesian credible intervals (BCI). The parsimonious structure of the Bayesian model (i.e. the model with the fewest parameters that adequately describes the data) with regard to relative differences between groups (contribution of repeated L3 doses and level of random daily variation in fecal egg density) was identified based on whether 95%-BCIs included the value 1.0 (i.e. no multiplicative effect). To estimate model parameters more accurately, additional data on egg counts from four additional volunteers from a previous pilot study was included in the analysis[4].

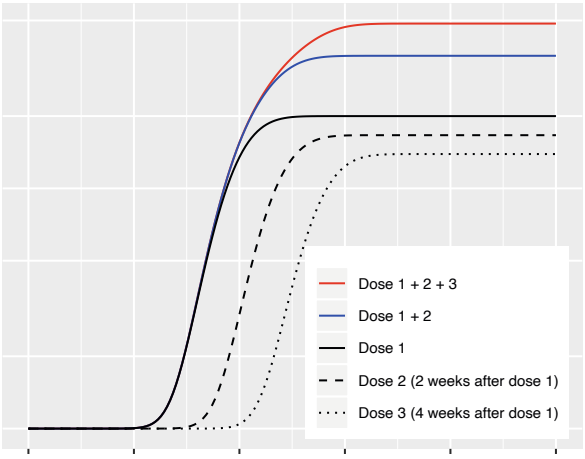
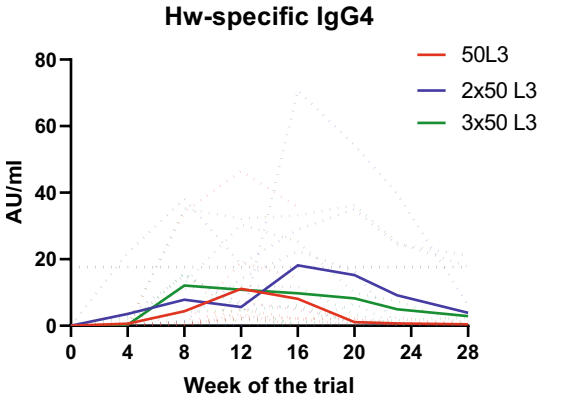


Figure 1. Hypothetical S-shaped curves of repeated doses, assuming that dose 1 leads to on average 19 eggs, dose 2 to 15, and dose 3 to 12.

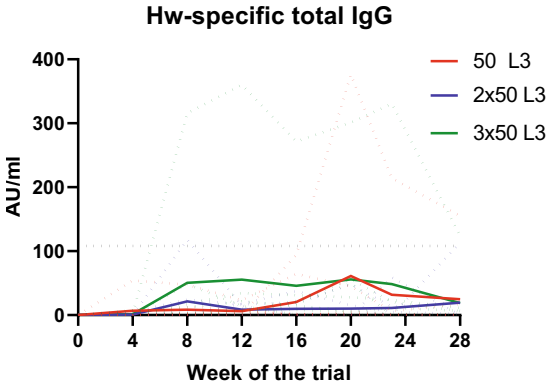
References for supplementary material

1. Verweij JJ, Pit DS, van Lieshout L, et al. Determining the prevalence of Oesophagostomum bifurcum and Necator americanus infections using specific PCR amplification of DNA from faecal samples. Tropical medicine & international health : TM & IH 2001; 6:726-31.
2. Kaisar MMM, Brienen EAT, Djuardi Y, et al. Improved diagnosis of Trichuris trichiura by using a bead-beating procedure on ethanol preserved stool samples prior to DNA isolation and the performance of multiplex real-time PCR for intestinal parasites. Parasitology 2017; 144:965-74.
3. Verweij JJ, Brienen EA, Ziem J, Yelifari L, Polderman AM, Van Lieshout L. Simultaneous detection and quantification of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in fecal samples using multiplex real-time PCR. The American journal of tropical medicine and hygiene 2007; 77:685-90.
4. Hoogerwerf MA, Coffeng LE, Brienen EAT, et al. New Insights Into the Kinetics and Variability of Egg Excretion in Controlled Human Hookworm Infections. The Journal of infectious diseases 2019; 220:1044-8.
5. Stan Development Team (2019). RStan: the R interface to Stan. R package version 2.18.2, http://mc-stan.org/.

Supplementary figures

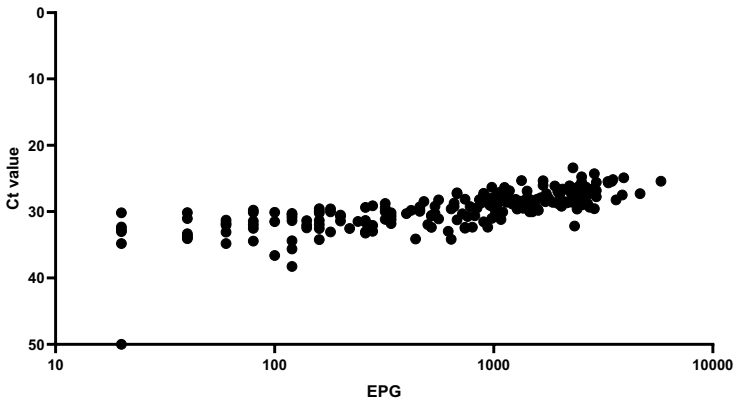


A

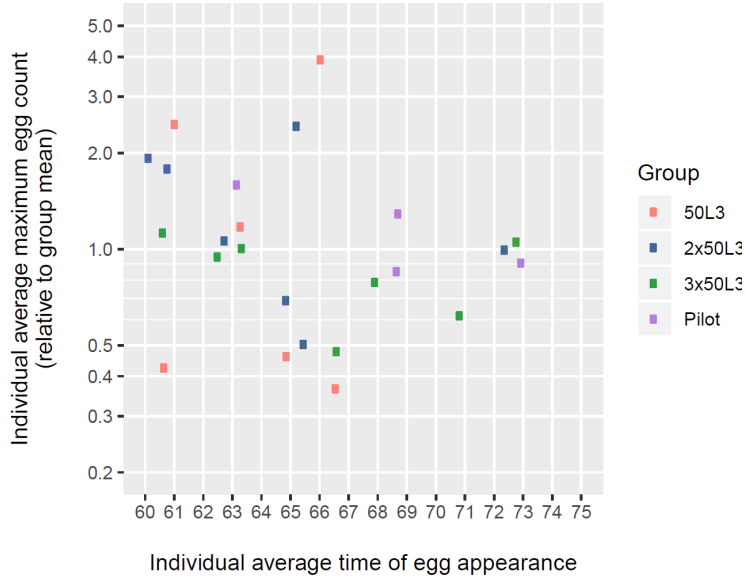


B

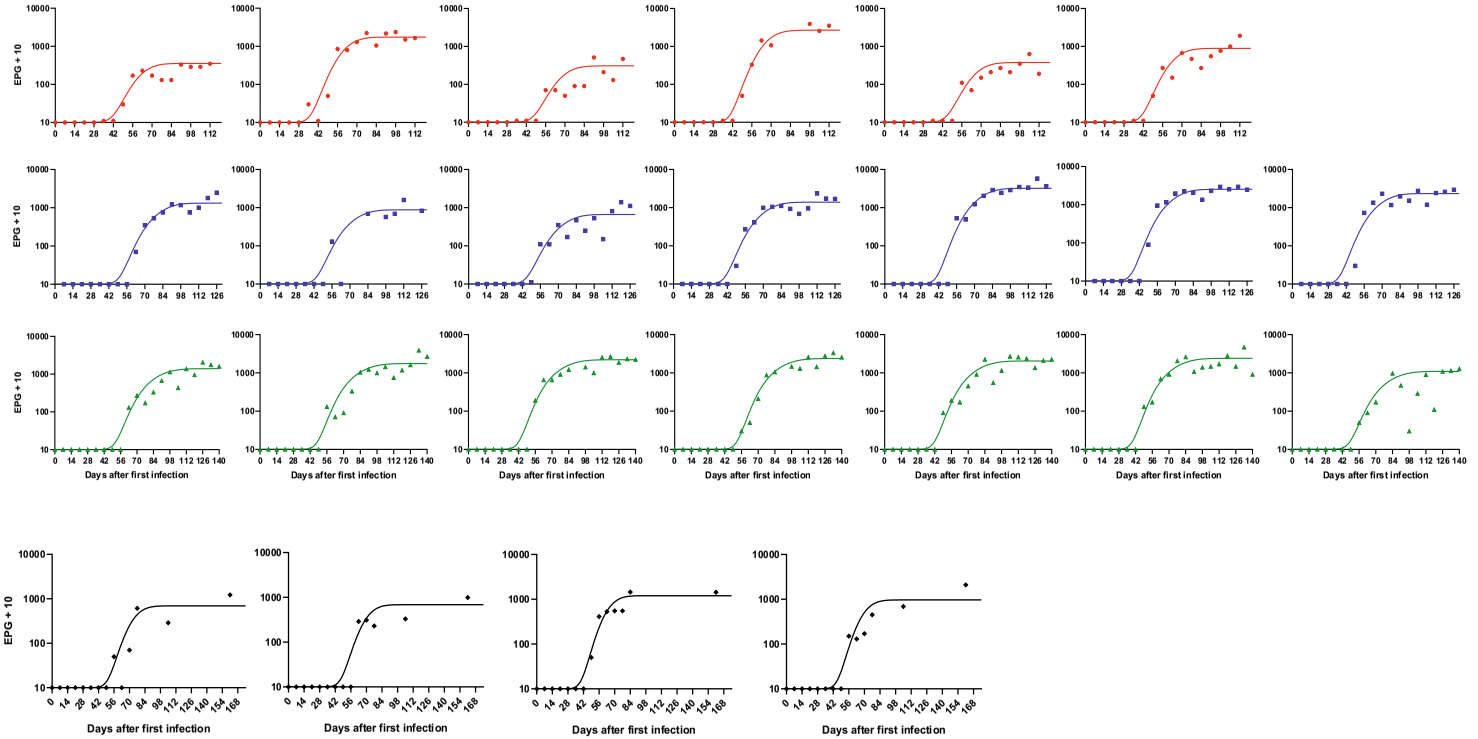
Supplementary figure 1. IgG4 antibody responses (panel A) and total IgG responses (Panel B) during the trial for individual volunteers (dotted lines) and mean per study group (continuous lines) Horizontal dotted line indicates threshold for seroconversion (rise from baseline 4x above SD). (50L3: red lines, 2x50L3: blue lines, 3x50L3: green lines)



Supplementary figure 2. Correlation between Kato-Katz measurements and PCR Ct-values. Spearman's rho: r=-0.79, p<0.001

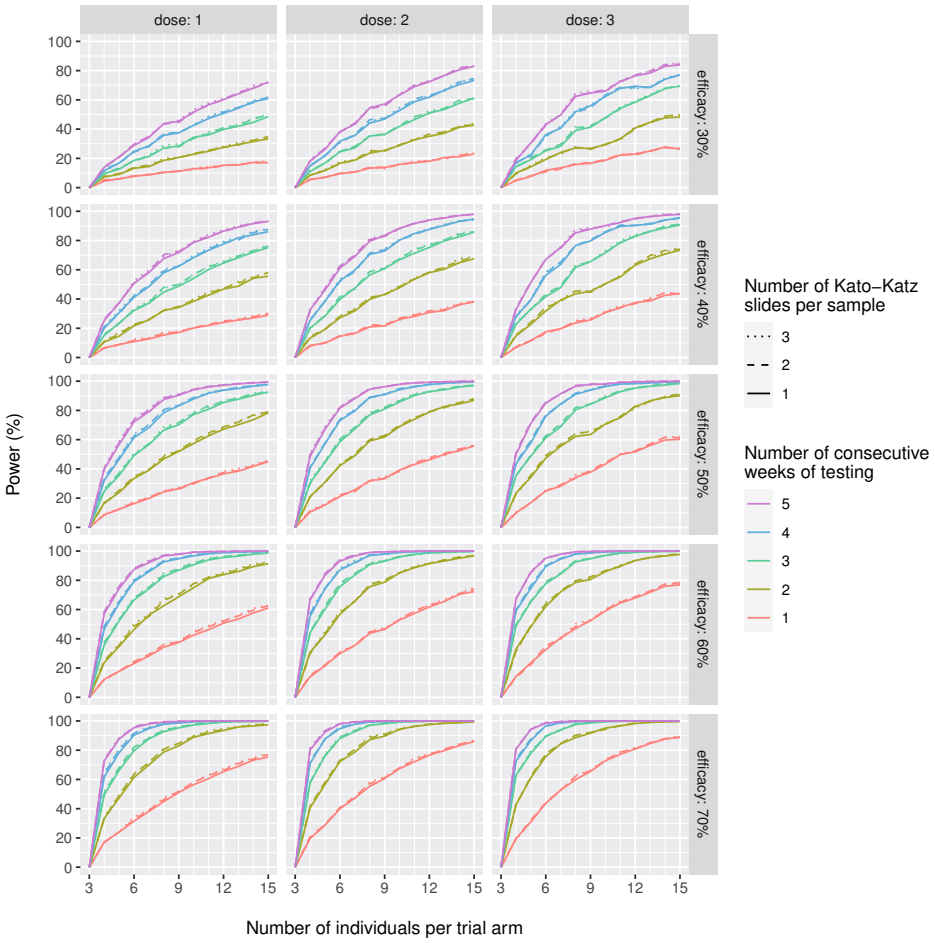


Supplementary figure 3. Relation between appearance of eggs and plateau level. X-axis: individual time of egg appearance in days, plotted against the dispersion from group mean of individual maximum egg count. Pilot study: 4 volunteers included in previous study with infectious dose of 50 L3.



Supplementary figure 4. Individual measured (dots) and estimated (S-shaped curve) egg counts

Red circles: 50L3, blue squares 2x50 L3, green triangles 3x50 L3, bottom panel in black diamonds displays data from pilot study (infectious dose 50L3)



Supplementary figure 5. Power calculation using Bayesian modelling of egg counts showing the number of individuals per trial arm in a vaccine trial assuming 50% reduction in egg counts of the vaccine versus the placebo group, following 1, 2 or 3 doses of 50L3 and assuming vaccine efficacy between 30-70%. The number of slides per KK samples are shown as dotted (3 slides), dashed (2 slides) and solid (1 slide) lines. Different colors represent the number of weekly samples taken.