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Supplementary appendix

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Supplementary material

Vaccination of human subjects with attenuated *Necator americanus* hookworm larvae: a randomised, placebo controlled phase 1 trial

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Participant eligibility criteria

Inclusion criteria

- Participants will be males and non-pregnant, non-lactating females aged between 18 and 65 years.
- Participants must have BMI within the range of 18-35 kg/m2 and weigh more than 50 kg.
- Participants must understand the procedures involved and agree to participate in the study by giving fully informed, written consent prior to any study assessment.
- Participants must be contactable and available for the duration of the clinical trial and be available for up to 2 months following completion of the trial.
- Heterosexually active females, unless surgically sterile, or at least 1 year post-menopausal, who decide to participate in the study must use adequate birth control methods. These must be in place for the duration of the study. Adequate contraception is defined as:
    - Stable hormonal contraception (with an approved oral, transdermal or depot regimen) for at least 3 months prior to screening i.e. oral contraceptives, either combined or progestogen alone, hormonal implantable contraception, vaginal ring, contraceptive patches.
    - Intrauterine (IUD) device or system in place for at least 3 months prior to screening.
    - Male partner sterilization prior to the female participant's entry into the study, and this male is the sole partner for that participant.
    - Abstinent female participants must agree to start a double contraception method if they start a sexual relationship during the study.

Adequate contraception does not apply to participants of child bearing potential with same sex partners (abstinence from penile-vaginal intercourse), when this is their preferred and usual lifestyle. These participants must not be planning *in vitro* fertilisation within the required contraception period.

Exclusion criteria

- History of Helminth infection (other than *E. vermicularis*) or travel to and residence (greater than 2 weeks) in areas of endemic transmission of these parasites.
    - Areas of endemic transmission include most tropical and subtropical countries where poverty and lack of sanitation co-exist.
- History of atopy or severe allergic reaction, anaphylaxis or convulsions following any vaccination or infusion.
- History of allergic reaction, anaphylaxis or otherwise to iodine, amphotericin, gentamicin, albendazole, chilli or other peppers, or Tabasco sauce.
- Treatment with immune-modulating or cytotoxic medications in the last 6 months.
    - Not including short courses (less than 5 days) of prednisone.
- Immunosuppression:
    - Congenital or acquired immune deficiency states.
    - Haematological malignancies.
    - Significant auto-immune diseases.
- Anaemia (Hb less than 130g/L in males, less than 120g/L in females).
- Any vaccination in the last 30 days.
- Heavily tattooed forearms.
- Participation in any other clinical trial/investigation while participating in this trial.

N. americanus larvae production

Faeces was supplied by a donor who was experimentally infected with *N. americanus* and screened for blood-borne viruses and faecal pathogens, as previously described.[1] The faeces was cultured using a modified Harada-Mori method.[2] Larvae were harvested when they were 7 to 10 days old, washed in sterile water and incubated in 1% povidone-iodine solution (Betadine®, Mundipharma BV, Netherlands) for 10 minutes before repeated washing in sterile water.

For each batch, larval viability was estimated on harvest day day+1 as well as day+14 using a thermally induced motility assay.[3] Approximately 25 L3 suspended in 100 µL of sterile water were placed in 5 wells of two 96-well plates, one of which was stored at laboratory temperature (22–25°C) in the dark for 14 days. Motility was observed at 40× magnification after the addition of 50 µL of water heated to 40°C. Day+1 viability (%) was calculated as *number of motile larvae/total number of larvae x 100*. The relative reduction in viability by day+14 was calculated as *(day+1 control motility% - day+14 control motility%) / day+1 control motility%*. For vaccine and challenge inoculum, larvae were loaded into 96 well plates (25±3 larvae per well for vaccine inoculum, 15±3 larvae per well for challenge inoculum) and sealed prior to transport to the clinical trial site.

***N. americanus* larvae attenuation**

The effect of UVC exposure on larval viability was characterised *in vitro* by placing 96 well plates (without a lid) containing larvae suspended in 100µL of sterile water into a pre-warmed UVC crosslinker (Hoefer, San Francisco, USA) set to deliver 350, 700 and 1400 µJ UVC. Thermally induced motility was assessed pre-UVC exposure at day+1 and at day+14 (Figure S1) and the relative reduction in motility was calculated as follows: (*day+1 control larvae motility% – day+14 attenuated larvae motility%) / day+1 control larvae motility%*.

For the dose finding study (part 1) and the clinical trial (part 2), UVC attenuation was performed at the clinical trial site, in a closed room, immediately prior to inoculation. Each participant was allocated a separate 96 well plate containing 25±3L3 per well, which was placed into the UVC crosslinker programmed to deliver the desired dose of UVC. Following attenuation each well was inspected at x40 magnification to ensure L3 motility. Larvae from two wells were used for the inoculum (50 L3 total) with the remainder stored as attenuation controls. Thermally induced motility was estimated at day 14 (Figure S1), and relative reduction in motility was calculated as above.

Results of in vitro motility assays to determine larval viability

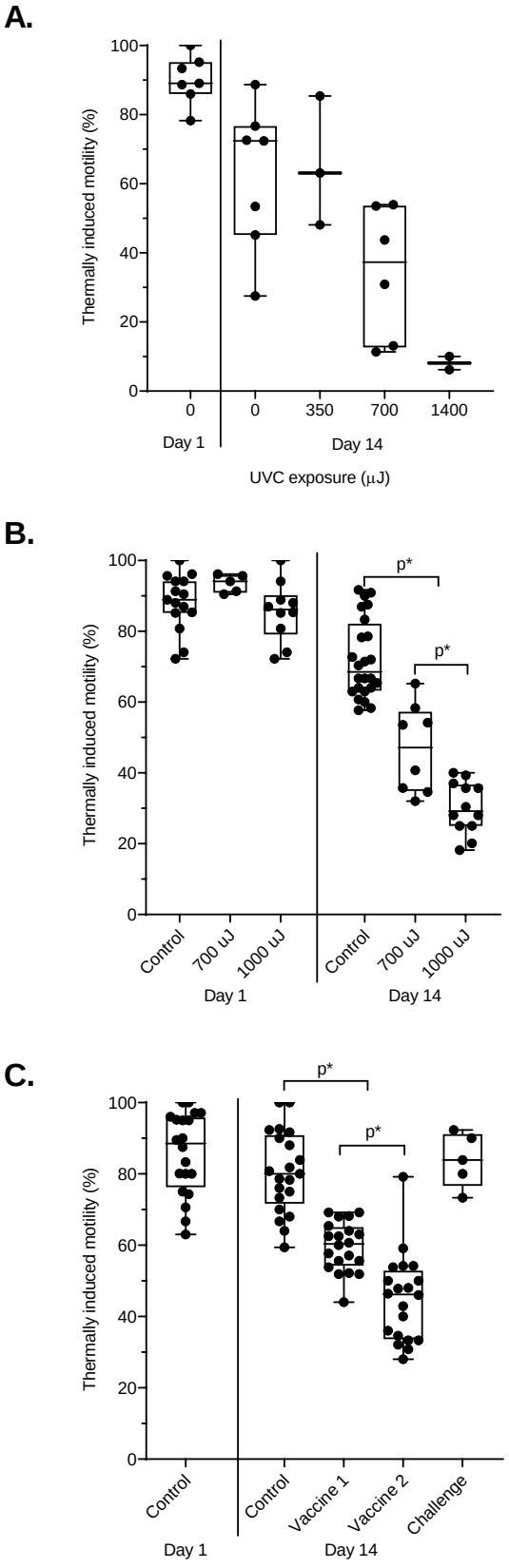

Figure S1. Results of in vitro thermally induced motility assays. Motility of larvae following exposure to different levels of UVC (A), motility of larvae used in the dose finding study (B), motility of larvae used in the challenge study (C). Boxes represent the median with interquartile range and error bars represent the minimum and maximum values. p*: $p<0.05$.

Administration of vaccine, placebo and challenge larvae

Larvae were administered within 48 hours of harvest and production. Larvae from 2 wells were gently transferred by pipette from the 96 well plates on to a single adhesive dressing (vaccine) or to a pair of adhesive dressings (challenge). The wells and the pipette tip were then visually inspected and where required a sterile water wash was used to ensure all larvae were collected and applied to the dressing(s). The placebo inoculum of 200 µL of Tabasco sauce was placed on identical dressings to those used for L3 application. Dressings were applied to each participant's forearm and kept in place for one hour before disposal.

N. americanus L3 Antigen specific IgG assay

Serum samples were collected by venepuncture from participants on study days 42, 84, 112, 126 and 161 and IgG antibody responses were quantified by indirect ELISA. *N. americanus* L3 were collected from multiple donations of faeces provided by a single volunteer known to be infected with *N. americanus*. A modified Harada-mori culture was setup less than 24 hours after each donation of faeces had been produced. Eggs were hatched and progressed from L1 through to L3 before migrating out of the culture to the edge of the petri dish. Larvae were collected with a transfer pipette, washed 5 times in water before being resuspended in PBS and stored at -80°C. Somatic extract was prepared by adding 500 µl of lysis buffer (3 M urea, 0.2% SDS, 1% Triton X-100, 50 mM Tris-HCl) to approximately 75,000 L3 before repeated freeze/thaw cycles followed by homogenisation with a hand held tissue grinder on crushed ice. After homogenising, L3 extract was sonicated at 4°C using a Sonica Q55 sonicator at 70% amplitude with a 3.2 mm probe. Samples were pulsed for 10 seconds three times with 30 second intervals for cooling on ice. Sonicated material was then centrifuged at 10,000 *g* at 4°C for 10 min and the supernatant was collected and passed through a 0.22 µm filter before determining protein concentration with a BCA assay (Pierce).

ELISA microtitre plates (96 well, flat bottom, high binding, Greiner) were coated with *N. americanus* L3 PBS-soluble somatic extract at a final coating concentration of 5 µg/mL overnight at 4°C. Plates were blocked with PBST/5% BSA for 2 hours at RT and washed with PBS/0·05% Tween-20 (PBST) three times then coated with subject sera diluted 1:400 in PBST/1% BSA for 1 hour at 37°C. Plates were washed as above then exposed to goat anti-human IgG (Fc specific)-horseradish peroxidase (Sigma) diluted 1:5,000 in PBST/1% BSA, and incubated for 1 hour at 37°C. Plates were then washed as above and TMB developing reagent (Invitrogen) was added at RT for 20 mins. The reaction was stopped with an equal volume of 1N HCl, and colourimetric change read in a spectrophotometer at 450 nm using a Fluostar Omega spectrophotometer (BMG Labtech). Data was plotted as the change in absorbance units above baseline optical density from day+1 for each participant. Data was plotted using GraphPad Prism version 8.4.3.

Human Peripheral Blood Mononuclear cell (PBMC) isolation and cytokine assay

Human PBMCs were isolated by Ficoll-gradient separation from 10 ml of blood collected in Lithium Heparin tubes (BD Biosciences, San Jose, CA, USA), as previously described.[4] Briefly, blood tubes were inverted gently several times prior to centrifugation at 784 x g for 10 minutes at room temperature. Plasma (1-2 ml) was removed and stored at -20°C. All remaining plasma was removed and 25ml of sterile phosphate buffered saline (PBS; QIMR Berghofer, Brisbane, QLD, Australia) was added and tubes inverted several times. Ficoll-Paque™ PLUS (13 ml; GE Healthcare, Silverwater, NSW, Australia) was deposited under the samples and centrifuged (392 x g for 30 minutes at room temperature without brake). After centrifugation, the buffy coat containing PBMCs was removed and washed with 50 ml of PBS, centrifuged at 392 x g for 10 minutes at 4°C. The supernatant was discarded and PBMC were re-suspended in 4 ml complete media (sterile Roswell Park Memorial Institute Medium 1640 (RPMI1640; Gibco™, Life Technologies, Carlsbad CA, USA), (10% (v/v) FCS, 100 µg/ml penicillin–streptomycin (Gibco™), 1x GlutaMAX™ (Gibco™), 1x non-essential amino acids (Gibco™), 110 mg/L (1 mM) Sodium Pyruvate (Gibco™), 5 mM HEPES (Gibco™), and 0.05 mM 2-mercaptoethanol (Sigma, St Louis, MO)). Cells were diluted in Dulbecco's Phosphate Buffered Saline (DPBS) (1x) (Gibco™) and Trypan Blue Stain (Invitrogen™), counted using Countess™ Cell Counting Chamber Slides on the Countess II FL (both from Invitrogen™), as per manufacturer's protocol, and cryopreserved in liquid nitrogen at 5 x 10^6 - 10^7 cell/ml.

Cryopreserved PBMCs were thawed at 37°C for 5 minutes in a water bath. Once samples were thawed, sterile RPMI1640 was added drop-wise (1ml), followed by the addition of 8ml of RPMI1640 supplemented with 250 units of Benzonase® Nuclease (Sigma). Cells were centrifuged for 600 x g for 7 minutes at room temperature, counted as described above, and adjusted to a concentration of 1.25 x 10^6 cells/ml, in complete media containing 25 µg/ml polymixin B (Sigma). 2.5 x 10^5 cells were cultured at 37ºC, 5% (v/v) CO_2 in a final volume of 200 µL in 96-well round-bottom plates (Corning Inc., Corning NY, USA), with or without 10 µg/ml *N. americanus* excretory/secretory antigens prepared, as previously described.[5] Supernatants were harvested after 120 hours and stored at -20°C until cytokine levels were measured using the human Th1/Th2 CBA cytokine detection kit II (BD Biosciences, Franklin Lakes, NJ; cat# 551809) and acquired on a BD LSR Fortessa (BD Biosciences). The limit of detection data are presented below.

Limit of detection data

Cytokine	Median fluorescence	Standard deviation	Limit of detection (pg/mL)
IL-2	3.3	0.2	2.6
IL-4	2.3	0.2	2.6
IL-5	2.6	0.2	2.4
IL-10	2.4	0.2	2.8
TNF	2.0	0.2	2.8
IFN-γ	2.1	0.3	7.1

Dermal reaction results in the dose finding study (part one)

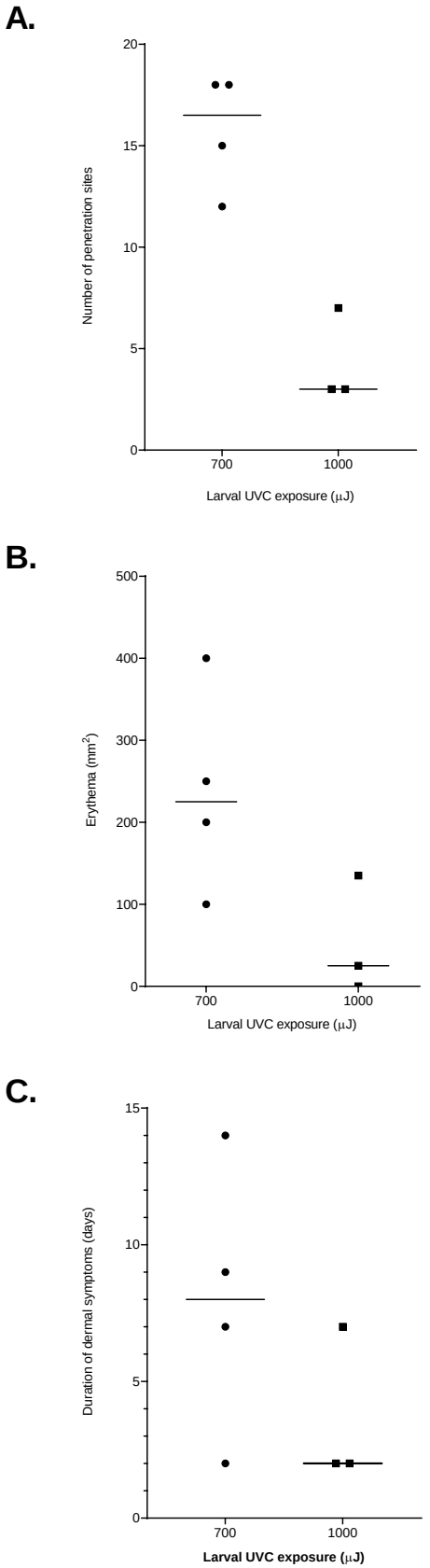

Figure S2. Dermal reaction in the dose finding study. Number of penetration sites identified (A), area of erythema at day 3 (B), and duration of dermal symptoms (C). Horizontal lines represent the median.

Adverse events in the dose finding study (part one)

Table S1. Summary of adverse events recorded in the dose finding study

	Cohorts 1 and 2 (700 µJ UVC) [n=4]		Cohort 3 (1000 µJ UVC) [n=3]	
AE type	**Participants with at least one AE [n (%)]**	**AEs per participant [mean (95% CI)]**	**Participants with at least one AE [n (%)]**	**AEs per participant [mean (95% CI)]**
Any AE	4 (100%)	3.25 (1.48 - 5.02)	3 (100%)	3.00 (1.04 – 4.96)
AE related to study treatment	4 (100%)	2.75 (1.12 - 4.38)	3 (100%)	2.33 (0.60 - 4.06)
Dermal reactions	4 (100%)	2.5 (0.95 - 4.05)	3 (100%)	2.33 (0.60 - 4.06)

AE: adverse event; CI: confidence interval; UVC: ultraviolet C.

Total IgE results in the challenge study (part two)

Table S2. Total IgE (kIU/L) at day 1 and day 112

Study day	Placebo (n=5)	Vaccine (n=10)
	Median (IQR)	
Day 1	18.0 (9.0 - 72.0)	21.0 (17.0 - 50.0)
Day 112	19.0 (10.0-57.0)	40.0 (19.0 - 51.0)

Table S3. Change in total IgE (kIU/L) from day 1 to day 112

Placebo (n=5)	Vaccine (n=10)	Z-statistic	p-value
Median (IQR)			
1.0 (-15.0-1.0)	12.5 (2.0 - 21.0)	-2.335	0.0195

Photographs of dermal reaction observed in the challenge study (part two)

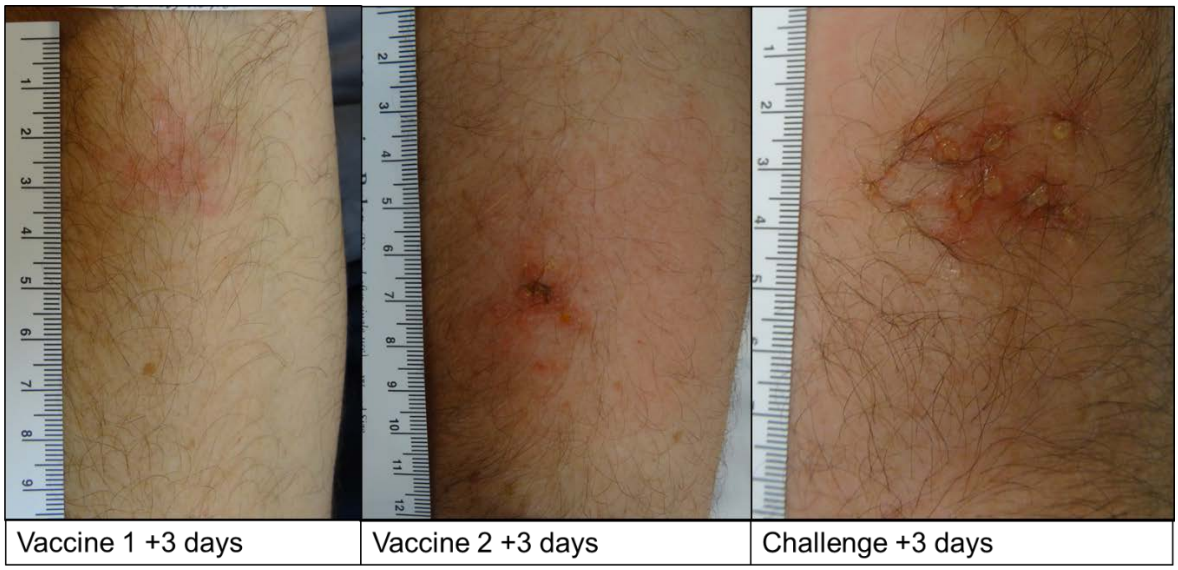

Figure S3. Photographs of dermal reaction observed in the challenge study. Clinical photography was performed 3 days following administration of the attenuated hookworm vaccine and after challenge with unattenuated larvae. Photographs are of one participant (participant R407) and are representative of dermal reactions observed in the vaccinated cohort.

References

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