Establishing the production of male schistosoma mansoni cercariae for a controlled human infection model

Janse, J.J.; Langenberg, M.C.C.; Kos-Van Oosterhoud, J.; Ozir-Fazalalikhan, A.; Brienen, E.A.T.; Winkel, B.M.F.; ... ; Roestenberg, M.

Establishing the production of male *Schistosoma mansoni* cercariae for a controlled human infection model

Jacqueline J. Janse[1], Marijke C.C. Langenberg[1], Janneke Kos-Van Oosterhoud[1], Arifa Ozir-Fazalalikhan[1], Eric A.T. Brienen[1], Béatrice M.F. Winkel[1], Marianne A.A. Erkens[2], Martha T. van der Beek[2], Lisette van Lieshout[1], Hermelijn H. Smits[1], Bonnie L. Webster[3], Maarten L. Zandvliet[4#], Richard Verbeek[4], Inge M. Westra[4], Pauline Meij[4], Leo G. Visser[5], Angela van Diepen[1], Cornelis H. Hokke[1], Maria Yazdanbakhsh[1], Meta Roestenberg[1,5]

[1]Department of Parasitology, Leiden University Medical Center, Leiden, the Netherlands; [2]Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands; [3]Wolfson Wellcome Biomedical Laboratories, Department of Zoology, The Natural History Museum, London, UK; [4]Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, The Netherlands; [5]Department of Infectious Diseases, Leiden University Medical Centre, Leiden, the Netherlands; [#]Present: Gadeta BV, Utrecht, The Netherlands

Running title

Male cercariae for human infection

Word count

Abstract: 100

Text: 1999

Abstract

To accelerate the development of novel vaccines for schistosomiasis, we set out to develop a human model for *Schistosoma mansoni* infection in healthy volunteers. During natural infections female schistosomes produce eggs that give rise to morbidity. Therefore, we produced single sex, male *Schistosoma mansoni* cercariae for human infection without egg production and associated pathology. Cercariae were produced in their intermediate snail hosts in accordance with the principles of Good Manufacturing Practice. The application of GMP principles to an unconventional production process is a showcase for the controlled production of complex live challenge material in the EU or under FDA guidance.

Key words (3-10)

Biomphalaria glabrata; cercariae; controlled human infection model; human challenge model; *Schistosoma mansoni*; schistosomiasis; vaccine

Article's main point

To accelerate the development of novel vaccines and drugs, a controlled human infection model was established for *Schistosoma mansoni* with the application of GMP principles to the unconventional production process for cercariae.

Background

Schistosomiasis ranks amongst the leading neglected tropical diseases in terms of disability–adjusted life years with roughly 252 million people infected worldwide[1]. *Schistosoma mansoni* and *Schistosoma haematobium* are responsible for the majority of cases[2]. Morbidity in schistosomiasis is caused by the inflammatory response to tissue-deposited eggs, which induce granulomas and fibrosis, that can lead to portal hypertension or bladder cancer[2].

Current schistosomiasis control programs rely on the mass administration of praziquantel, but transmission of schistosomes persists primarily due to high reinfection rates[3]. Alternative tools to break transmission and eliminate schistosomiasis are urgently needed. The development of a highly efficacious vaccine would be a major asset to schistosomiasis control programs.

Vaccination studies in mice and non-human primates prove that immunity to schistosomes can be induced by repeated exposure to radiation-attenuated cercariae[4, 5]. Based on such landmark studies, stage-specific antigens have been identified which reduce worm burden by >40%[6]. Currently there are four vaccine candidates in clinical stage of development (Sh28GST, Sm-TSP-2, Sm14 and Smp80)[6, 7]. Typically, these vaccine candidates undergo phase 1 testing for safety after which efficacy needs to be demonstrated in large-scale field trials in endemic areas. To obtain an estimate of the vaccine induced protective efficacy, trials of long duration and/or large population size are necessary.

To decrease the risk of downstream efficacy failure, healthy volunteers have been deliberately exposed to infectious agents to test malaria, dengue and influenza vaccines[8]. Such controlled human infection (CHI) trials provided early efficacy estimates which were used to guide further clinical development. The availability of a CHI model for schistosome infection could revolutionize the development of schistosomiasis vaccines.

To ensure the safety of participants in a human schistosome infection trial, a challenge inoculum should be available which complies with regulatory requirements for human use and cannot induce eggs-associated morbidity. We therefore took an important conceptual step to produce single sex,

male, schistosome cercariae in accordance with Good Manufacturing Practice (GMP) principles. The production of live parasites, which comply with the highly demanding regulatory environment in place currently, is a showcase for the production of complex live challenge material in the EU or under FDA guidance.

Methods

Production principles

Manufacturing of single sex cercariae for the purpose of controlled human schistosome infections was performed within the quality control and quality assurance system of the Interdivisional GMP facility of the Leiden University Medical Center (IGFL, LUMC). The product was characterized as an auxiliary medicinal product (AxMP, Regulation EU 536/2014), or previously a non-investigational medicinal product according to the definition of the EU clinical trial directive 2001/20/EC. The principles of GMP as outlined in the EC directive 2003/94/EC were applied in the production process wherever possible. Cercariae were produced in a biosafety level 3 (DM3) laboratory environment with humidity, temperature and pressure control. The production process (figure 1) was standardized in 34 standard operating procedures, which were kept in the institutional document management system. A product dossier was created according to section 2.7 of EC CT-1 guideline (2010/C 82/01) for pharmacological products. All disposables, reagents, solvents, culture media and starting materials were released by the person responsible for Quality Control (QC) and the Qualified Person (QP) specific for advanced cell therapy medicinal products (ATMP). All manufacturing steps and batch numbers of all used materials were entered and logged real time in a GMP compliant digital database using a portable tablet. The use of a tablet facilitated communication between manufacturers and QC officers without breaching hygienic procedures established for containment of the DM3 environment. A separate read-only account was held by the QC officer and the QP. Monitored data was locked for editing by the QP. All QC testing was independently analyzed by the QC, thereafter the product was released by the QP.

Origin of snails and parasites

The *Schistosoma mansoni* (Sm) Puerto Rico strain used for the production was obtained together with their *Biomphalaria glabrata* snail hosts by Prof. C.F.A. Bruijning in 1955[Prof. A.M. Deelder,

personal communication]. This isolate has been maintained in laboratory culture in the LUMC by routine passaging of the schistosomes through laboratory hamsters and/or mice as definitive host and laboratory cultured *Biomphalaria glabrata* snails as intermediate host. Adult worms were genetically characterised to confirm its origin (see Supplement).

Production of cercariae

Sm miracidia were obtained by light-induced hatching of *Sm* eggs isolated from an infected hamster[9]. After single-miracidium infection[10], *Biomphalaria glabrata* snails of the same batch (i.e. infection day) were housed and labelled individually and shed after 5 weeks. Cercariae were counted and viability was determined by calculating percentage moving.

Identity and sex confirmation

Identity and sex of cercariae were determined by a purpose-made multiplex real-time PCR targeting *Schistosoma*-specific ITS2 sequences[11] as well as the Sm W1 repeat[12] (see Supplement). The W1 copy number is much lower in male as compared to female schistosomes[13]. Using DNA of duplicate cercaria samples unequivocally resulted in an ITS2 cycle threshold (Ct) between 15 and 30. Sex was determined by the difference in Ct between W1 and ITS2: >10ΔCt for males, <4ΔCt for females. Sensitivity of the qPCR was confirmed in samples containing only one cercaria (mean ITS2 Ct: 23). To validate the PCR, single sex cercaria samples were sex confirmed by assessment of the morphology of adult worms after hamster infection.

Bioburden testing

Shed waters were tested for bioburden at the ISO-15189 certified clinical medical microbiology laboratory of the LUMC (see Supplement). *Aeromonas spp*, *Staphylococcus aureus*, β-hemolytic streptococci and *Pseudomonas aeruginosa* were defined as pathogenic bacteria potentially

causing skin infections upon transfer with cercariae. Cultures were optimized for detection of these bacteria. To reduce bioburden, snails were kept at 2 mg/L ciprofloxacin and 4 mg/L gentamicin for 24 hours before clinical use.

One week before use, bioburden of shed water was assessed before and after antibiotic treatment. After shedding for clinical use, cercariae were counted, viability was recorded and doses were prepared by two persons separately.

Results

Preclinical production data

In preparation for the production of clinical trial material, 24 preclinical batches of single-miracidium infected snails were produced, averaging 12 snails per batch. Of those 294 snails, 246 (84%) were still alive at week 5. In total 47 snails (19%) shed cercariae, of which 25 (53%) were male (figure 2A). Viability of cercariae was always very high (>90% in all batches), but the number of cercariae per shed varied considerably per snail and over time. At week 5 snails shed an approximate number of 3500 cercariae (geometric mean (GM) 3416, 95%CI 2386-4889), decreasing to 1261 at week 7 (95%CI 704-2259, Mann-Whitney P<0.0001, figure 2B). The number of cercariae per shed was not affected by the cercarial sex (Mann-Whitney P=0.6, figure 2C). Based on these data, cercariae of shed 2 were preferably selected for clinical use.

Sex identification by qPCR

Sex and identity were unequivocally confirmed in all cercarial samples (figure 2D). Samples obtained from snails shedding mixed male and female cercariae always showed ΔCt similar to female only cercariae (ΔCt W1-ITS2 min 2.83, max 4.88 for mixed, ΔCt W1-ITS2 min 2.24, max 3.11 for females). Samples containing only male cercariae always showed different ΔCt (min 11.83, max undetectable). Thus, the >6ΔCt difference between male and female cercarial samples ensured that male sex could always be confirmed with certainty.

Bioburden

All samples tested for bioburden were found to contain *Aeromonas veronii* biovar sobria sensitive for ciprofloxacine, gentamicin and co-trimoxazole. No other pathogenic bacteria were cultured. Because *Aeromonas* spp. are incidentally reported as causative agents of skin infections, production snails were pre-treated with antibiotics before clinical use of cercariae. After this treatment all

samples were free of *Aeromonas* spp. After antibiotic treatment waterborne bacteria such as Chryseobacterium and S*tenotrophomonas maltophilia* persisted, which are not known to cause infections in healthy volunteers.

In-process testing and release

Sm miracidia and hamsters were defined as starting materials and were released by the QC. Based on the critical steps in the production process, three in-process tests were defined: 1) confirmation of single-miracidium infection of snails by a second person, 2) daily examination of snail viability, 3) number and viability of cercariae after first shed (i.e. >100 and >90%).

A two-step release procedure was established with a conditional release procedure immediately after dose preparation, followed by a final release procedure when bioburden tests were completed three days later. Criteria for conditional release were based on 1) absence of potentially pathogenic bacteria after antibiotic treatment of two snails from the same batch; 2) identity confirmation of cercarial species and male sex by qPCR, 3) absence of potentially pathogenic bacteria resistant to ciprofloxacine and gentamicin, 4) presence of >100 cercariae with >90% viability.

Shelf life of the product at 28°C was determined at 2 hours, when 96% (95%CI 91-100%) of cercariae were confirmed viable by microscopy. At 4 hours viability was more variable (95%CI 81-100%, mean 96%) and at 24 hours viability had dropped to 14% (95%CI 4-24%).

Figure legends

Figure 1: Schematic representation of the production process

Figure 2: Percentages of uninfected, dead and infected *Biomphalaria glabrata* snails with either male or female cercariae after single-miracidium infection (A), number of cercariae produced by each snail for each weekly shed starting from week 5 after infection (B) and number of male or female cercariae from shed 1 or 2 (C). Results from sex-specific PCR, shown as the difference in Ct-value between the ITS2 gene and W1 repeat, in 47 cercarial samples from preclinical production (D).

Discussion

In summary, we were able to establish a rigorous production process for male *Schistosoma mansoni* cercaria, complying with current regulatory standards for human use. Despite the complex, unconventional production process that includes a snail host, production was in accordance with the principles of GMP with clearly defined release criteria which were supervised by a QC and QP ATMP.

CHI trials are increasingly used for the down selection of novel drugs or vaccines[8]. However, specific guidelines regarding the production of challenge material are often lacking. Whereas the FDA states stage-appropriate GMP is required, EMA guidelines for auxiliary medicinal products state that full GMP may not be required but deviations must be justified (Clinical trials, Directive 2001/20/EC, NIMPs)[14]. In this specific case, the production process in snails is exceptional and requires thorough risk analysis, intense collaboration of pharmacists, clinicians and technicians. Paramount to the safety of volunteers in a controlled human schistosome trial is the ability to determine cercaria sex, so egg-induced pathology cannot occur. To this end, we have developed technologies and procedures to ensure unique identification and tracking of individual snails.

In order to confirm identity and sex of cercariae, we established a sensitive and specific multiplex real-time PCR. We confirmed high variability in the copy numbers of the female-specific W1 repeat in male cercaria, corroborating previous data[13]. Nevertheless male cercariae could be confidently distinguished from female or “mixed” cercarial samples, based on a large difference in Ct between male and female/mixed samples. Should the production of female cercariae be necessary in the future, multiple individual cercariae from one snail should be tested to confirm sex.

We have performed full characterization of the bioburden associated with the snail microbiome. We focused on excluding microbiological contaminants which might cause a risk of adverse events when applied to the cercaria-penetrated skin. We believe that the current remaining non-pathogenic bioburden does not pose any risk for volunteers. In addition, the rearing of sterile snails has not been reported previously and might be biologically challenging given the increasing evidence that the microbiome is essential for the healthy being of particularly non-vertebrates[15].

Both the *Biomphalaria glabrata* snails as well as the Sm parasites have been kept in the laboratory for more than 60 years. Although the origin of the isolates lacked a full historical paper trail, the main advantage of such an old and highly established laboratory culture is the naivety to praziquantel, excluding possible resistance. Nevertheless, for clinical use, extensive follow-up is needed to ensure full cure after praziquantel administration. We performed genotyping to confirm the parasite strain as *S. mansoni* from Puerto Rico. Future efforts will focus on establishing a master and working bank of parasites, although cryopreservation of schistosome cercariae, miracidia or eggs has not been previously performed and may be challenging.

In conclusion, through an interdisciplinary approach, we were able to achieve highly controlled production of viable male *Schistosoma mansoni* cercariae. Following these efforts, the next steps will be the use of cercariae for infection of healthy volunteers in a proof-of-concept clinical trial, aiming to find a safe and infectious dose. Establishing a controlled human infection model for schistosomiasis will be a game-changing step to accelerate the development of novel vaccines and drugs for this devastating disease.

Notes

Conflicts of interest statement

The authors declare no conflict of interest and funding. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

Funding statement

MR was supported by a VENI grant from ZONMW and a Gisela Thier fellowship from the LUMC.

JJ was supported by a grant from Dioraphte foundation.

Meeting(s) where the information has previously been presented

2017 ASTMH, Baltimore, Maryland, USA

2017 IABS meeting Rockville, Maryland, USA

2017 CHIMS for Schistosomiasis stakeholders' meeting, Entebbe, Uganda

Corresponding author

Dr. M. Roestenberg

Department of Parasitology

Leiden University Medical Center

Albinusdreef 2

2333 ZA Leiden

The Netherlands

Tel.: +31 71 5264400

Fax: +31 71 5266907

E-mail: m.roestenberg@lumc.nl

References

1. Hotez PJ, Alvarado M, Basanez MG, et al. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. PLoS neglected tropical diseases **2014**; 8:e2865.

2. Colley DG, Bustinduy AL, Secor WE, King CH. Human schistosomiasis. Lancet (London, England) **2014**; 383:2253-64.

3. Deribew A, Kebede B, Tessema GA, et al. Mortality and Disability-Adjusted Life-Years (Dalys) for Common Neglected Tropical Diseases in Ethiopia, 1990-2015: Evidence from the Global Burden of Disease Study 2015. Ethiopian medical journal **2017**; 55:3-14.

4. Richter D, Harn DA, Matuschka FR. The irradiated cercariae vaccine model: looking on the bright side of radiation. Parasitology today (Personal ed) **1995**; 11:288-93.

5. Kariuki TM, Van Dam GJ, Deelder AM, et al. Previous or ongoing schistosome infections do not compromise the efficacy of the attenuated cercaria vaccine. Infection and immunity **2006**; 74:3979-86.

6. Status of Vaccine Research and Development of Vaccines for Schistosomiasis. Prepared for WHO PD-VAC. **2014**.

7. Siddiqui AA, Siddiqui SZ. Sm-p80-Based Schistosomiasis Vaccine: Preparation for Human Clinical Trials. Trends in parasitology **2017**; 33:194-201.

8. Darton TC, Blohmke CJ, Moorthy VS, et al. Design, recruitment, and microbiological considerations in human challenge studies. The Lancet Infectious diseases **2015**; 15:840-51.

9. Peterson NA, Hokke CH, Deelder AM, Yoshino TP. Glycotope analysis in miracidia and primary sporocysts of Schistosoma mansoni: differential expression during the miracidium-to-sporocyst transformation. International journal for parasitology **2009**; 39:1331-44.

10. Vogel H. The influence of the sexual partner on growth and development of Bilharzia mansoni and in cross-matings between different species of Bilharzia. Zentralbl Bakteriol **1942**; 148:78-9.

11. Obeng BB, Aryeetey YA, de Dood CJ, et al. Application of a circulating-cathodic-antigen (CCA) strip test and real-time PCR, in comparison with microscopy, for the detection of Schistosoma haematobium in urine samples from Ghana. Annals of tropical medicine and parasitology **2008**; 102:625-33.

12. Webster P, Mansour TE, Bieber D. Isolation of a female-specific, highly repeated Schistosoma mansoni DNA probe and its use in an assay of cercarial sex. Molecular and biochemical parasitology **1989**; 36:217-22.

13. Quack T, Doenhoff M, Kunz W, Grevelding CG. Schistosoma mansoni: the varying occurrence of repetitive elements in different strains shows sex-specific polymorphisms. Experimental parasitology **1998**; 89:222-7.

14. The rules governing medicinal products in the European Union; Volume 10 - Guidance documents applying to clinical trials; Guidance on investigational medicinal products (IMPs) and 'non investigational medicinal products' (NIMPs). **2011**.

15. Bahrndorff S, Alemu T, Alemneh T, Lund Nielsen J. The Microbiome of Animals: Implications for Conservation Biology. Int J Genomics **2016**; 5304028.

Figure 1

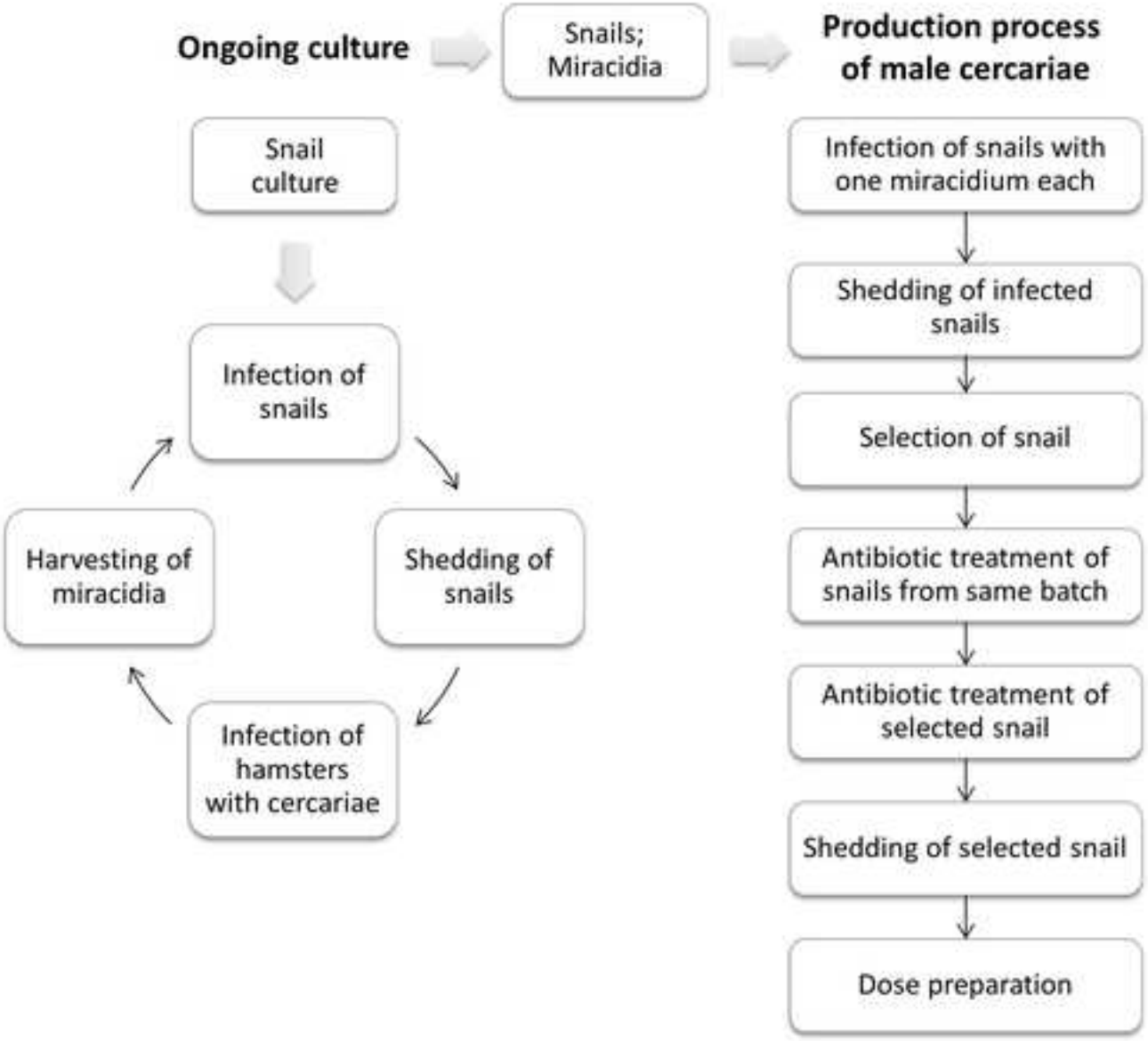
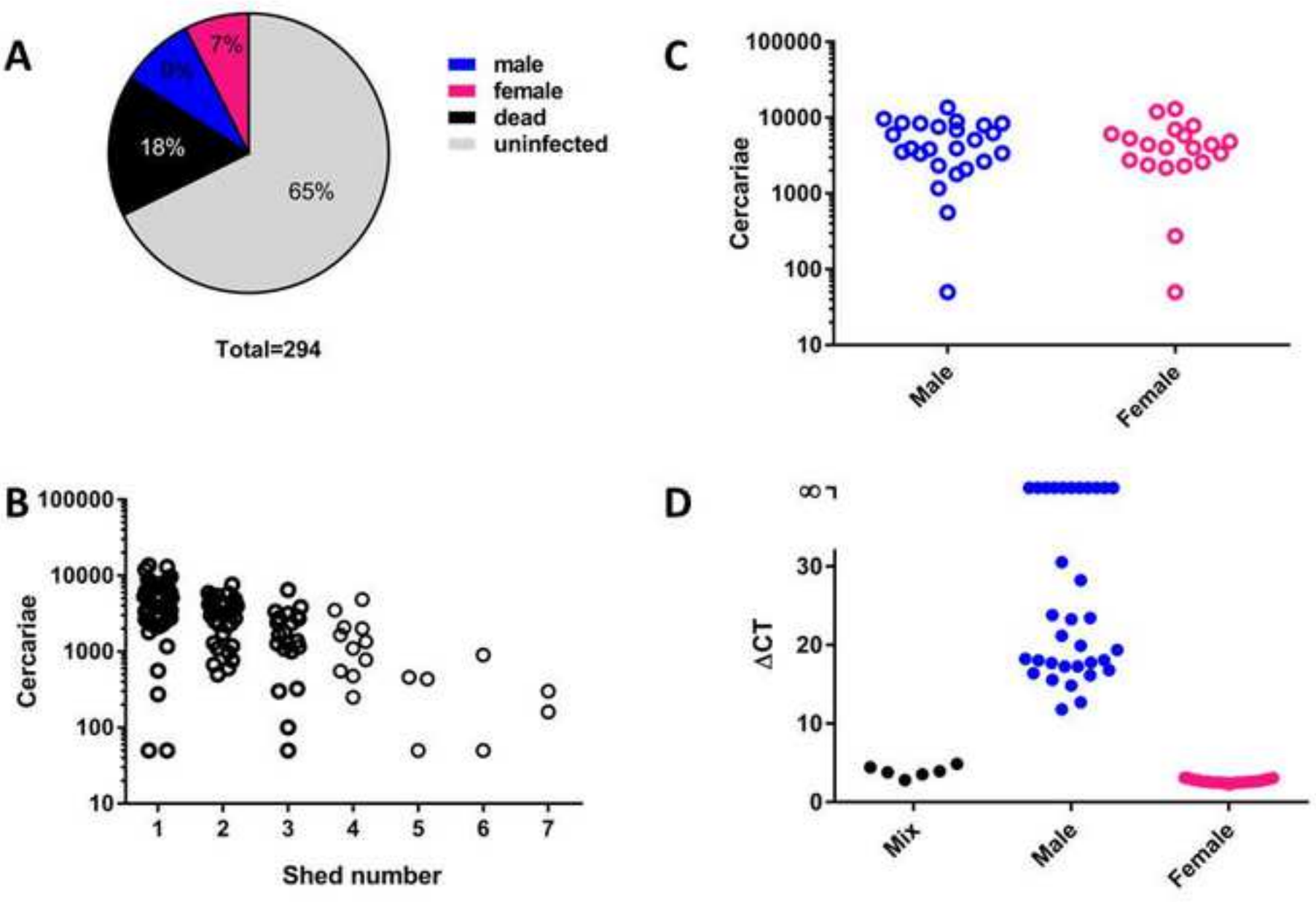


Figure 2



Establishing the production of male *Schistosoma mansoni* cercariae for a controlled human infection model

Jacqueline J. Janse[1], Marijke C.C. Langenberg[1], Janneke Kos-Van Oosterhoud[1], Arifa Ozir-Fazalalikhan[1], Eric A.T. Brienen[1], Béatrice M.F. Winkel[1], Marianne A.A. Erkens[2], Martha T. van der Beek[2], Lisette van Lieshout[1], Hermelijn H. Smits[1], Bonnie L. Webster[3], Maarten L. Zandvliet[4#], Richard Verbeek[4], Inge M. Westra[4], Pauline Meij[4], Leo G. Visser[5], Angela van Diepen[1], Cornelis H. Hokke[1], Maria Yazdanbakhsh[1], Meta Roestenberg[1,5]

[1]Department of Parasitology, Leiden University Medical Center, Leiden, the Netherlands; [2]Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands; [3]Wolfson Wellcome Biomedical Laboratories, Department of Zoology, The Natural History Museum, London, UK; [4]Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, The Netherlands; [5]Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands; [#]Present: Gadeta BV, Utrecht, The Netherlands

Supplement

Origin of parasites

Genomic DNA was extracted from pooled worms using the DNeasy tissue kit (QIAgen) according to the manufacturer's protocol. The mitochondrial cox1 region was PCR amplified and sequenced as described in Webster et al., 2013 [1]. The sequences were compared to the *S. mansoni* cox1 database to find the closest identity, which was a 99% match to Genbank Accession: HE601612 (Mitochondrial genome of *S. mansoni* Puerto Rico).

Sex-identity *Schistosoma* multiplex qPCR

DNA from at least 50 cercariae was isolated with the QIAamp DNA mini kit spin columns (QIAgen, Hilden, Germany) and diluted 1:100. The multiplex PCR amplified a 77-bp fragment from the *Schistosoma*-specific ITS2 sequences (with primers Ssp48F, Ssp124R and probe Ssp78T) [2] and a 121-basepair fragment from the female specific *Schistosoma mansoni* W1 repeat (with primers SmW1-238F, SmW1-358R and probe SmW1-291T (Biolegio, Netherlands)) [3]. Amplification was performed in 20 µl PCR mix and 5 µl of cercarial DNA. Table 1 shows the concentrations of the PCR mix used with the sequences of all primers and double-labelled probes. The CFX real-time detection system (Bio-Rad laboratories) was used with amplification program of 15 min at 95°C followed by 50 cycles of 15s at 95°C, 30s at 60°C, and 30s at 72°C. Negative and positive (mixed, male and female) control samples were included.

Table 1: PCR mix and primer/probe sequences of the sex-identity Schistosoma multiplex PCR

Components	Concentration	Per sample	Sequence
H_20		2,73	
$MgCl_2$	25 mM	3,50	
BSA	5 mg/ml	0,50	
Primer Ssp48F	25 µM	0,06	5'-GGTCTAGATGACTTGATTGAGATGCT-3'
Primer Ssp124R	25 µM	0,06	5'-TCCCGAGCGTGTATAATGTCATTA-3'
Probe Ssp78T	10 µM	0,13	FAM-5'-TGGGTTGTGCTCGAGTCGTGGC-3'-BHQ1
Primer SmW1-238F	25 µM	0,20	5'-TGTTTGTGGATGCGATGGTG-3'
Primer SmW1-358R	25 µM	0,20	5'-TGTGCACAAGCAACGATTCC-3'
Probe SmW1-291T	10 µM	0,13	YAK-5'-GCGATGATGCATTAGGGTGTGTGGT-3'-BHQ1
HotStarTaq Master Mix		12,50	

Bioburden testing

Shed water was plated on Trypcase Soy Agar with 5% sheep blood, Cystine lactose electrolyte deficient agar, Columbia agar with colistin and nalidixic acid, Xylose Lysine Desoxycholaat Agar (Biomerieux, Marcy-l'Étoile, France) and Cefsulodin Irgasan Novobiocine agar (Media Products, Groningen, The Netherlands) and incubated aerobically for 48 hours at 35°C. Plates were checked daily for the growth of bacteria. Identification was performed by MALDI TOF mass spectrometry (MALDI Biotyper, Bruker Daltonik GmbH, Bremen, Germany) and susceptibility testing in a Vitek 2 instrument (Biomerieux) or, in case of streptococci by disk diffusion (Oxoid, Thermofisher, Landsmeer, The Netherlands).

References

1. Webster BL, Webster JP, Gouvras AN, et al. DNA 'barcoding' of Schistosoma mansoni across sub-Saharan Africa supports substantial within locality diversity and geographical separation of genotypes. Acta tropica **2013**; 128:250-60.
2. Obeng BB, Aryeetey YA, de Dood CJ, et al. Application of a circulating-cathodic-antigen (CCA) strip test and real-time PCR, in comparison with microscopy, for the detection of Schistosoma haematobium in urine samples from Ghana. Annals of tropical medicine and parasitology **2008**; 102:625-33.
3. Webster P, Mansour TE, Bieber D. Isolation of a female-specific, highly repeated Schistosoma mansoni DNA probe and its use in an assay of cercarial sex. Molecular and biochemical parasitology **1989**; 36:217-22.