Controlled human infection models as a tool for malaria and schistosomiasis vaccine research

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II

Development of a controlled human schistosomiasis infection model

CHAPTER 5

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

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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 (GMP). The application of GMP principles to an unconventional production process is a showcase for the controlled production of complex live challenge material in the European Union or under Food and Drug Administration guidance.

Schistosomiasis ranks among 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 because of 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 nonhuman 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 that reduce the worm burden by >40% [6]. Currently there are 4 vaccine candidates in the 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 schistosome-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 that 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 that complies with regulatory requirements for human use and cannot induce egg-associated morbidity. We therefore took an important conceptual step to produce single-sex, male, schistosome cercariae in accordance with good manufacturing practice (GMP) principles. This process for producing live parasites, which complies with the highly demanding regulatory environment in place currently, is a showcase for the production of complex live challenge material in the European Union (EU) or under Food and Drug Administration guidance.

Methods

Production Principles

Manufacture 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 Leiden University Medical Center (LUMC). The product was characterized as an auxiliary medicinal product (EU regulation 536/2014) or previously as a noninvestigational medicinal product according to the definition of EU clinical trial directive 2001/20/EC. The principles of GMP as outlined in European Commission directive 2003/94/ EC were used in the production process wherever possible.

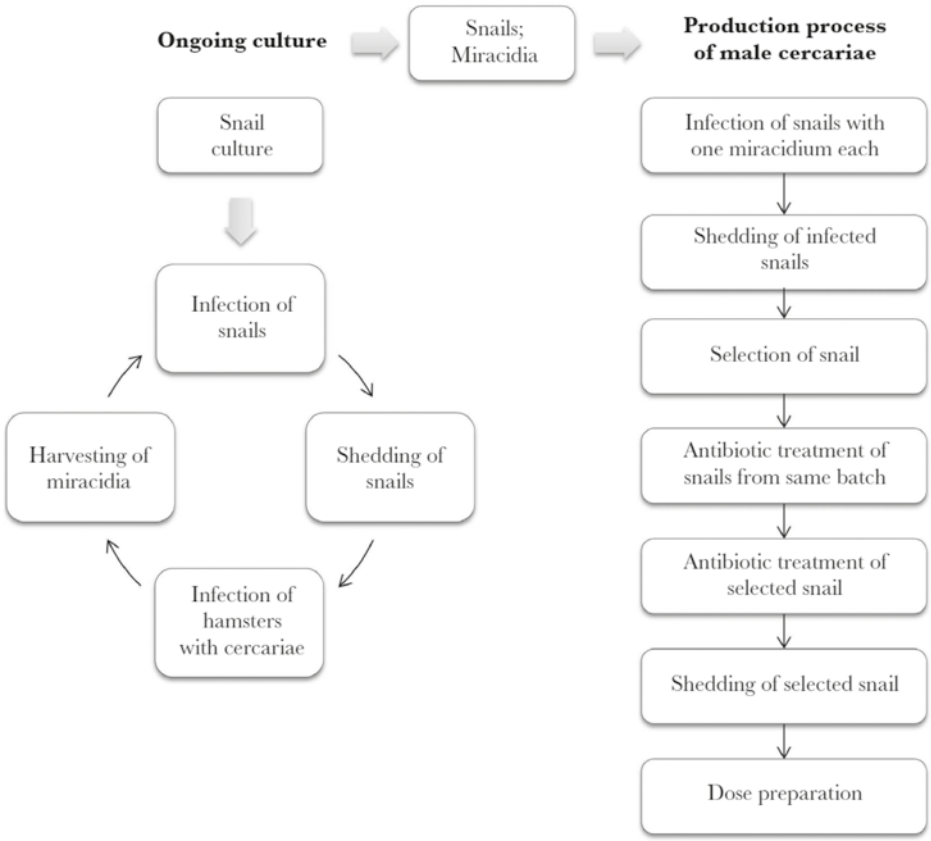

Figure 1. Schematic representation of the production process.

Cercariae were produced in a biosafety level 3 laboratory environment with controlled humidity, temperature, and pressure. 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 for use by the quality control (QC) officer and the qualified person (QP) responsible for advanced cell therapy medicinal products. All manufacturing steps and batch numbers of all used materials were entered and logged in 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 biosafety level 3 environment. A separate read-only account was held by the QC officer and the QP. Monitored data were locked by the QP to prevent editing. All QC testing was independently analyzed by the QC officer, and thereafter the product was released by the QP for use.

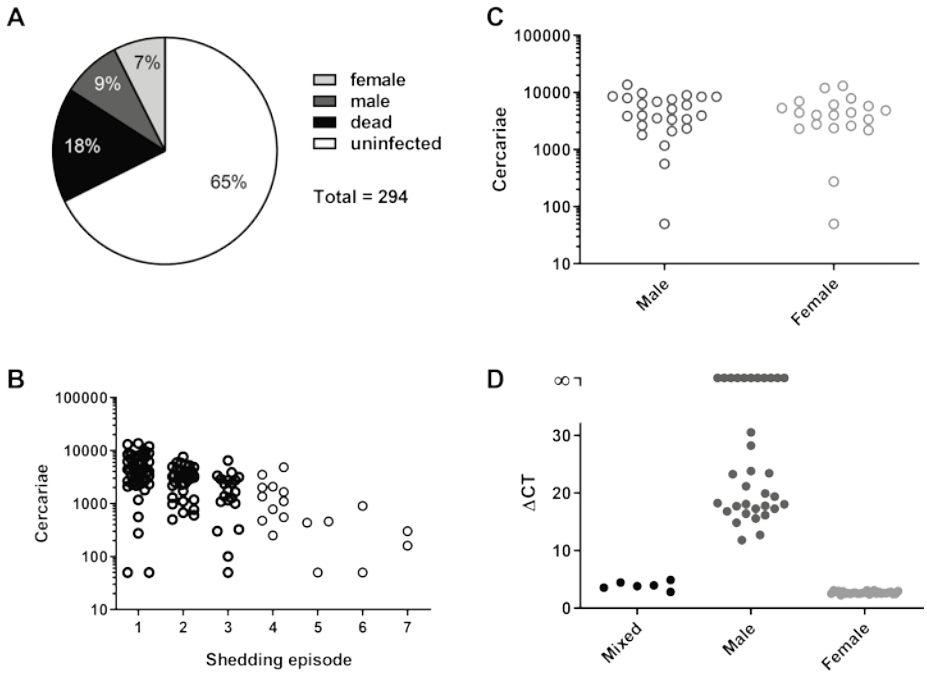

Figure 2. A. Percentages of uninfected, dead, and infected Biomphalaria glabrata snails with either male or female cercariae after single-miracidium infection. **B**. Number of cercariae produced by each snail for each weekly shedding episode, starting from week 5 after infection. **C.** Number of male or female cercariae from shedding episode 1 or 2. **D**. Results from sex-specific polymerase chain reaction analysis, shown as the difference in cycle threshold (ΔCt) between the ITS2 gene and the W1 repeat, in 47 cercarial samples from preclinical production.

Origin of Snails and Parasites

The *S. mansoni* Puerto Rico strain used for production was obtained together with its *Biomphalaria glabrata* snail host 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 the definitive host and laboratory-cultured *B. glabrata* as the intermediate host. Adult worms were genetically characterized to confirm their origin (Supplementary Materials).

Production of Cercariae

S. mansoni miracidia were obtained by light-induced hatching of *S. mansoni* eggs isolated from an infected hamster [9]. After infection by a single miracidium [10], *B. glabrata* snails of the same batch (ie, infection day) were housed and labeled individually, and they shed cercariae after 5 weeks. Cercariae were counted, and their viability was determined by calculating the percentage of the counted larvae that were moving.

Identity and Sex Confirmation

The identity and sex of cercariae were determined by a purpose-made multiplex real-time polymerase chain reaction (PCR) targeting *Schistosoma*-specific ITS2 sequences [11], as well as the *S. mansoni* W1 repeat [12] (Supplementary Materials). 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 on the basis of the difference in Ct between W1 and ITS2, with males having a ΔCt of >10, and females having a ΔCt of <4. Sensitivity of the quantitative PCR was confirmed in samples containing only 1 cercaria (mean ITS2 Ct, 23). To validate the PCR finding, the sex of single-sex cercaria samples was confirmed by assessment of the morphology of adult worms after hamster infection.

Bioburden Testing

The bioburden in shed waters was determined at the ISO-15189–certified clinical medical microbiology laboratory of the LUMC (Supplementary Materials). *Aeromonas* species, *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 the bioburden, snails were kept in an environment containing 2 mg/L ciprofloxacin and 4 mg/L gentamicin for 24 hours before clinical use.

One week before use, the bioburden in shed water was assessed before and after antibiotic treatment. After shedding for clinical use, cercariae were counted, their viability was recorded, and doses were prepared by 2 persons separately.

Results

Preclinical Production Data

In preparation for the production of clinical trial material, 24 preclinical batches of 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 approximately 3500 cercariae (geometric mean [GM], 3416 cercariae; 95% confidence interval [CI], 2386–4889), decreasing to 1261 cercariae (95% CI, 704–2259) at week 7 ($P < .0001$, by the Mann-Whitney U test; Figure 2B). The number of cercariae per shed was not affected by the cercarial sex ($P = .6$, by the Mann- Whitney U test; Figure 2C). On the basis of these data, cercariae from shedding episode 2 were preferably selected for clinical use.

Sex Identification by Quantitative PCR

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 values similar to those containing females only (ΔCt W1-ITS2 range, 2.83–4.88 for mixed-sex cercariae and 2.24–3.11 for female cercariae). Samples containing only male cercariae always showed different ΔCt values (range, 11.83 to undetectable). Thus, the ΔCt difference of >6 between male and female cercarial samples ensured that male sex could always be confirmed with certainty.

Bioburden

All samples that underwent bioburden testing were found to contain *Aeromonas veronii* biovar sobria susceptible to ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole. No other pathogenic bacteria were cultured. Because *Aeromonas* species are incidentally reported as causative agents of skin infections, production snails were pretreated with antibiotics before clinical use of cercariae. After this treatment, all samples were free of *Aeromonas* species. After antibiotic treatment, waterborne bacteria such as *Chryseobacterium* species and *Stenotrophomonas maltophilia*, which are not known to cause infections in healthy volunteers, persisted.

In-Process Testing and Release

S. mansoni miracidia and hamsters were defined as starting materials and were released by the QC officer. Based on the critical steps in the production process, 3 in-process tests were defined: (1) confirmation of single-miracidium infection of snails by a second person, (2) daily examination

of snail viability, and (3) determination of the number and viability of cercariae after the first shedding episode (ie, >100 and >90%).

A 2-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 3 days later. Criteria for conditional release were based on (1) absence of potentially pathogenic bacteria after antibiotic treatment of 2 snails from the same batch, (2) identity confirmation of cercarial species and male sex by quantitative PCR, (3) absence of potentially pathogenic bacteria resistant to ciprofloxacin and gentamicin, and (4) presence of >100 cercariae with >90% viability.

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

Discussion

In summary, we were able to establish a rigorous production process for male *S. mansoni* cercaria that complied 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 officer and a QP responsible for advanced cell therapy medicinal products.

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 Food and Drug Administration states that stage-appropriate GMP is required, European Medicines Agency guidelines for auxiliary medicinal products state that full GMP may not be required, but deviations must be justified [14]. In this specific case, the production process in snails is exceptional and requires thorough risk analysis and 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 that egg-induced pathology cannot occur. To this end, we have developed technologies and procedures to ensure unique identification and tracking of individual snails.

To confirm the 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 cercariae, corroborating previous data [13]. Nevertheless male cercariae could be confidently distinguished from female or mixed-sex cercarial samples, based on a large difference in Ct between male and female/mixed-sex samples. Should the production of female cercariae be necessary in the future, multiple individual cercariae from 1 snail should be tested to confirm cercarial sex.

We fully characterized the bioburden associated with the snail microbiome. We focused on excluding microbiological contaminants that might confer a risk of adverse events when applied to cercaria-penetrated skin. We believe that the current remaining nonpathogenic 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, owing to increasing evidence that the microbiome is particularly essential for the well-being of nonvertebrates [15].

Both the *B. glabrata* snails and the *S. mansoni* parasites have been kept in the laboratory for >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 this drug. 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 performed and may be challenging.

In conclusion, through an interdisciplinary approach, we were able to achieve highly controlled production of viable male *S. 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 to find a safe and infectious dose. Establishing a CHI model for schistosomiasis will be a game-changing step to accelerate the development of novel vaccines and drugs for this devastating disease.

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Supplementary

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'-GGTCTAGATGACTTGATTGA GATGCT-3'
Primer Ssp124R	25 µM	0,06	5'-TCCCGAGCGTGTATAATGTC ATTA-3'
Probe Ssp78T	10 µM	0,13	FAM-5'-TGGGTTGTGCTCGAGT CGTGGC-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'-GCGATGATGCATTAGG GTGTGTGGT-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).

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