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# IDENTIFICATION BY PCR OF SCHISTOSOME CERCARIAE TRAPPED IN NATURE

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## Executive Summary:

Detection of schistosome cercariae in potential transmission foci is necessary to define the risk factors of infection by Bilharzia parasites (*Schistosoma* species). This is critical for formulating and evaluating interventions. However, existing methods provide sporadic or unreliable results, and cercariae recovered by any one method cannot be identified by species. This is a problem in transmission sites in Africa where *Schistosoma mansoni* (causing an intestinal and hepato-splenic disease) can cohabit natural water bodies with *S. haematobium* (causing an urinary disease) and with animal schistosomes. In order to detect cercariae we proposed a new approach. Namely, combining a sensitive and specific identification of cercarial DNA by the polymerase chain reaction (PCR) (Hamburger et al. 1998) with a method for trapping cercariae onto glass slides suitably coated with linoleic acid, which induces cercarial penetration and cause their adherence to the slides (Shiff and Chandiwana). The feasibility of the proposed approach, and the importance of the trilateral collaboration were demonstrated by the following accomplishments: (1) At the Johns Hopkins Univ. (JHU)- Collection of cercariae and shipment to the Hebrew Univ. (HU). (2) At the HU- Optimization of PCR conditions for detetcting *S. haematobium* with the corresponding primers. (3) At the JHU and HU- Preparation of slides with trapped cercariae from *S .mansoni* and *S. haematobium*. (3) At the HU- Development of conditions for preparing cercariae trapped on slides for detection by PCR (Detection by PCR of cercariae recovered from slides was demonstrated). (4) At Blair Research Laboratories (BL)-collection of worms and cercariae of *S. mattheei* (the most prevalent animal schistosome in Zimbabwe), in order to start identifying and cloning specific DNA sequences suitable for their differential identification. (5) At BL- Preparations for maintaining the life cycle of *S. haematobium* for harvesting cercariae for studies at semi-field conditions. (6) Training in Africa of a BL scientist on PCR, and preparing his joining the team at HU for a joint development of the new tools.

**A) Research Objectives:** The general aim of the project was to develop a realistic method for the detection and identification of schistosome cercariae in natural waters for the purpose of studying risk factors of transmission of schistosomiasis in ponds and streams.

The specific objectives of the project were as follows:

A) To use PCR techniques for detecting schistosome cercariae or remains of cercariae collected on a cercarial trap and to tests the limits of sensitivity.

B) To develop primers which will differentiate between cercariae of different species, especially *S. haematobium, S. mansoni and S. matthaei.*

C) To investigate dispersion of schistosome cercariae in pond and natural water systems, both flowing and stagnant diurnally and seasonally.

## B) Research Accomplishments:

**AT The Hebrew University**

1) For amplification of DNA from *S. mansoni* cercariae we have carried out DNA preparation from cercariae as follows: Cercariae were mixed with 300µl lysis solution containing 1M NaOH and 1% triton x-100. The mixture was heated for 1hr at 65°C, the neutralized with HCl, heated to 100°C for 10min., cooled on ice and centrifuged at 10,000 for 15 min. to remove derbies. The supernatant was treated with cold ethanol to precipitate the DNA then the pellet was washed twice withcold 70% ethanol and dissolved in 50µl TE buffer (10mM Tris.Cl pH 8.0 and 1mM Ethylenedimethyl tetraacetic acid-EDTA).

Alternatively fresh or dried cercariae or cercariae preserved in ethyl alcohol (EtOH) were placed in the PCR reaction tube directly without prior preparation of DNA. We also placed cercariae on glass slides covered with nail varnish and linoleic acid (cercarial trap). For PCR we employed the following primers derived from a highly repeated tandemly arranged sequence previously described by one of us (**ref**):

Primer Sm1: 5' GAAAATCGTTGTATCTCCG 3' (19 mer)

Primer Sm 2: 5' CATAAAAATCCGTCCAGTGG 3'(20 mer).

The PCR conditions employed for amplifying *S. mansoni* DNA are slightly modified from those previously published by Hamburger et al. (**ref**). They were as follows: The total reaction volume of 50 µl contained buffer (10mM tris HCl pH 8.3, 3.5 mM $MgCl_2$, 75 mM KCl), 0.2 mM (each)

of dGTP, dCTP, dATP and dTTP, 0.25 Units of taq polymerase, and 20pmol of each of the primers. Temperature cycling involved an initial denaturation at 95°C, then 35 cycles of 95oC for 60sec., annealing at 55°C for 60sec and elongation at 72oC for 40 sec. A final 10 min. elongation step was also included. Results of PCR with *S. mansoni* cercariae are summarized in table 1.

Table 1: Results of PCR with *S. mansoni* cercariae given different treatments/storage conditions.

| Treatment/ storage of cercariae | Amplification by PCR (with 1 or more cercariae) |
|---|---|
| Fresh cercariae | +ve |
| Died cercariae from cercarial traps | -ve |
| Dried cercariae (directly) | +ve |
| Cercariae stored in Et OH And washed in TE | +ve |
| Cercariae kept frozen in deionized water | +ve |
| Cercariae kept frozen in TE | +ve |
| Cercariae kept frozen in PBS | highly inconsisternt +ve |
| DNA from cercariae kept frozen in PBS | highly inconsistent +ve |
| DNA harvested from fresh cercariae | +ve* |
| DNA from dried cercariae (directly) | +ve* |
| DNA from cercariae kept frozen in deionized water | +ve* |
| DNA from cercariae kept frozen in TE | +ve* |

*+ve: Positivity weaker than with cercariae put directly into the PCR reaction tube

Our results indicate that a single cercaria can easily be detected and that in most cases preparation of DNA is not necessary for obtaining positive amplification by PCR. Also, PBS salts appear to inhibit PCR amplification. While tried cercariae are amplified directly those dried on cercarial traps are not doing so regularly, perhaps because of the inhibitory effects of nail varnish and/or linoleic acid. This is subject to further examination.

2) For amplification of DNA from *S. haematobium* cercariae we employed cercariae sent by Dr. Shiff in ETOH. We carried out DNA preparation from *S. haematobium* cercariae by the same

method described above for *S.mansoni*. Optimization of PCR conditions was done by employing the Optiprime PCR optimization kit of Stratagene.

The following primers derived from a highly repeated tandemly arranged sequence of *S. haematobium* (Hamburger et al. Unpublished):

Sh1: 5' GATCTCACCTATCAGACGAAAC 3' (22mer)
Sh2: 5' TCACAACGATACGACCAAC 3' (19 mer)

The PCR conditions employed for PCR with *S. haematobium* were as follows (Hamburger et al. unpublished): The total reaction volume of 50 µl contained buffer (10mM tris HCl pH 9.2, 3.5 mM $MgCl_2$, 75 mM KCl), 0.2 mM (each) of dGTP, dCTP, dATP and dTTP, 0.25 Units of taq polymerase, and 20pmol of each of the primers. Temperature cycling involved an initial denaturation at 95°C, then 35 cycles of 95oC for 60sec., annealing at 55°C for 60sec and elongation at 72oC for 40 sec. A final 10 min. elongation step was also included.

Positive amplifications were obtained with 1 or more cercariae.

Currently, *S. haematobium* cercariae dried onto glass slides covered with nail varnish and linoleic acid (cercarial trap) are being tested with or without prior preparation of DNA.

The work described above demonstrated the feasibility of combining cercarial trapping by the chemical trap with cercarial identification by PCR. This accomplishment fulfills the basic requirement of the proposed work. Identification of *S. haematobium* cercariae was also made possible. The demonstration that cercariae can be identified by PCR without prior extraction of DNA should present a major advantage for field application. Further improvements of the method and further identification of DNA sequences for primer design (with animal schistosomes ) are still required. Also adaptation of the system for the field studies proposed still lies ahead

**At Blair Laboratories**

As a preamble, it is worth noting that the Blair Research Laboratory has previously experimented with cercarial traps to capture cercariae from natural water bodies. This work has being published in peer reviewed journals and highlighted the major limitation of the traps i.e. the cercariae on the traps could not be identified to species level. For Zimbabwe and other African countries, identification of trapped schistosome cercariae is critical in formulating and evaluating interventions. This is because the human cercariae of *Schistosoma Mansoni and S. Haematobium* often coexists in natural water bodies with those of the bovine schistosome, *Schistosoma mattheei.* From the perspective of Blair researchers collaboration with HUJ and JHU offer the possibility of the development and transfer of a PCR technology that can be applied under field conditions to determine the species of cercariae captured by the traps.

In pursuant of the above goals the Blair research team collected 150 *S. mattheei* worms from a local abattoir. A vetenarian collaborator based at the University of Zimbabwe's Faculty of Medicine assisted in this process. At the advice of DR. CJ Shiff the worms are being kept at 80% ethanol. The material will be shipped to HUJ for primer design. Since it is difficult to obtain adequate worms in this way, we also obtained the eggs of the bovine schistosome from infected bladders. We have infected 50 *Bulinus globosus* host snails. We are maintaining the infected snails and will shed them for cercariae around mid April when they will be patent. Additionally, Blair personnel have carried out the organizational arrangements for collecting *S. haematobium* eggs from urine of infected school children for maintaining life cycles of this parasite along the same lines as with *S. mattheei.* Materials will be shipped to Israel for future work.

**At Johns Hopkins University**

1) Procurement of reagents: Shipments of linoleic acid were obtained and tested from Sigma and Aldrich companies. The product supplied from Sigma which has also been procured by Dr Hamburger was defective and did not stimulate schistosome cercariae. Product from Aldrich Chemical Company was then purchased and proved to be satisfactory. Additional supply was purchased and sent to Dr. Hamburger.

2) The major activity allocated to JHU during this phase of the operation was to produce supplies of *S. haematobium* cercariae. Such activity is very time consuming as the parasite is difficult to

maintain in the laboratory but necessary to have alive for testing. A large supply of approx 10 000 preserved cercariae were sent to Dr Hamburger for molecular work. Preparation for this shipment involved considerable effort in time. A small number of cercariae are available alive on an on-going basis for specific experimentation, however due to a short life span, these would be used for specific experiments if and when necessary.

## C) Scientific Impact of Collaboration:

The collaboration during the first year established the feasibility of combining a cercarial trapping by a chemical trap with cercarial identification by PCR. This was accomplished by combining procurement of biological materials at Blair and JHU on the one hand, with the preparation of the relevant technical background at the HU. This technical background is intended for transfer to Blair through appropriate training in order to enable future monitoring of cercariae in natural water bodies in Zimbabwe. The collaborative aspects involved: (1) At the Johns Hopkins Univ. (JHU)- Collection of cercariae and shipment to the Hebrew Univ. (HU). (2) At the HU- Optimization of PCR conditions for detecting *S. haematobium* with the corresponding primers. (3) At the JHU and HU- Preparation of slides with trapped cercariae from *S.mansoni* and *S.haematobium*. (3) At the HU- Development of conditions for preparing cercariae trapped on slides for detection by PCR (Detection by PCR of cercariae recovered from slides was demonstrated). (4) At Blair Research Laboratories (BL)-collection of worms and cercariae of *S. mattheei* (the most prevalent animal schistosome in Zimbabwe), in order to start identifying and cloning specific DNA sequences suitable for their differential identification. (5) At BL- Preparations for maintaining the life cycle of *S. haematobium* for harvesting cercariae for studies at semi-field conditions. (6) Training in Africa of a BL scientist on PCR, and preparing his joining the team at HU for a joint development of the new tools.

## D) Description of Project Impact:

At HU the major impact is the establishment of methods to be used in the field after further refinement. In particular the demonstration that cercariae can be identified by PCR without prior extraction of DNA should present a major advantage for field application. Also, establishing the relevant technologies for enabling training of Blair personnel intended for future testing of the technology in the field. At Blair the major impact is establishment of parasite colonies in the laboratory, and relevant training for future testing of the new technologies in the laboratory, at

semi field conditions and finally in the field. The activities at Blair will also enable further development of molecular tools at HU for differential identification of animal schistosomes and human schistosomes. The work at JHU and its collaboration with the HU enabled the development of the said procedures and the demonstration of the feasibility of the proposed monitoring approach.

## E) Strengthening of Developing Country Institution:

The joint activities at the 3 collaborating institutions established a technological package ready for transfer to Zimbabwe and to other developing countries in Africa, pending a brief technological training. This package will enable further refinement of the new molecular tools on location as well as their adaptation for field surveys. Preparations at Blair will enable absorption of the technologies and their implementation for further studies near the laboratory and in the field.

## F) Future work:

Future work at HU will involve refinement of the monitoring by PCR of materials scrapped off cercarial traps. Being able to do the tests without prior DNA extraction will present a major technological facilitation for field application. Cloning and characterization of repeated sequences of *S. mattheei* will enable development of PCR assays for differentiating animal schistosomes from human schistosomes. At HU we also expect to synthesize stable derivatives of fatty acids (for another project) and send them to JHU for attempting improvement of the cercarial trapping capabilities. At Blair, cercariae of *S. mattheei* will be harvested from *Bulinus globosus* snails which were infected by miracidia from eggs harvested in abattoirs from infected bovine bladder. The cercariae will be employed for optimizing PCR conditions. The cercariae will also be used to infect 100 hamsters and at the expected worm load of 10-20 per animal should yield 1000-2000 *S. matthee* worms at perfusion. These material will be brought to the Hu by a Blair staff member who is intended for acquiring the expertise for detecting cercariae by PCR. Additionally, *S. haematobium* eggs from urine of infected school children will be started (preparation for this operation were already done during the first year). This will enable maintaining life cycles of this parasite along the same lines as with *S. mattheei.* Materials will be shipped to Israel to use in primer design, and will be used locally for cercarial detection in semi-field conditions and in the field.

We propose that the selected Blair scientist takes the said biological materials and to Jerusalem for three or so months of attachment in DR. Hamburger's laboratory. This will enable him to gain useful experience to participate in primer development and to learn some of the techniques. Blair intends to acquire the PCR technology and thus the need for a thermocycler to be used in DNA based identification of cercariae by the new technologies from cercariae collected outdoors.

The future activities by Dr. Shiff will include backup for supply of *S. haematobium* cercariae on cercarial chemical traps to enable further improvement of preparing this material for PCR, preferably without prior extraction of DNA. Further preparation of trapped cercariae (on glass slides with cercarial attractants) will be prepared and shipped to the HU for further improvement of the extraction of materials from the traps. Should new fatty acid derivatives be available for this study they will be sent from the HU to JHU for testing their activity on cercaral traps in an attempt to improve cercarial rapping. Parallel experiment will bed one in the laboratory at Blair once the life cycle of *S. haematobium* is established there. We also plan to do similar trapping studies at the HU with *S. mansoni.*

SECTION III

## A) Managerial Issues:

The actual start of the project at the HU was delayed by about 3 months but it started before project funds became available, thanks to HU financial support. Transfer of funds to the collaborating laboratories was enabled only in September 1998. Since the collaborating laboratories are entirely dependent on these funds the start of their work was delayed. The 1st managerial report was omitted because of this delay.

The project had a slow take off at Blair due to a number of administrative requirements and other reasons. The key research assistant, Denford Madhina, who had began work on the project was unexpectedly assigned other duties by the Ministry of Health. He was an ideal candidate for the work as he had participated in the development of the cercarial trap under Dr Shiff and in fact earned a masters degree working on the topic. There was also some delay in notification of transfer of funds. This and the fact that the local Blair PI had a hectic traveling schedule in

October and November somewhat affected the speedy implementation of the project. However as outlined in section 1, the Blair Research team is now fully established and is able to generate the required data for the project. The day to day administration of the project has further being decentralized to avoid any bottlenecks when the PI is away. The project has assisted to establish a reliable e-mail facility at BRL and this should improve communication especially with the coordinating centre at HUJ. Whilst our administration keeps meticulous records of expenditure they cannot produce the statements of expenditures on a quarterly basis due to pressure of other work. The practice of the Institute is to release certified financial reports by the Ministry of Health's finance department that is responsible for expenditures on the project. The department also has an internal audit function. Project funds are not normally invested so no interest is expected to accrue to the account. External auditing is done on a project by project basis and the costs for this have to be borne by the project. At Blair the 20% overhead is to cover the cost of the auditing but where a project provides less than this amount, the project has to pay directly for audit expenses. This applies to P/311 and an audit line item has to be incorporated if this is deemed a requirement by USAID and HUJ. We would also want some clarification on the statement that " at present USAID has obligated 46% of the total grant" due to the BRL.

The identification of a technical assistant with PCR training was delayed by the fact that the incumbent Mr Lincoln Chiimba had to undertake further training in PCR at the Kenya Medical Research Institute (KEMRI) and also here in Zimbabwe. This training took place during August-October 1998 and was supported by the International Atomic Energy Agency (IAEA). This led to some inconvenience as the research assistant, another project personnel was also not available as he had to be assigned to other work by the Ministry of Health.

## B) Budget:

The expenditure timing may be different than initially planned but budget items allocations were not changed

## c) Special concerns:

Not applicable

## D) Collaboration, Travel, Training and Publications:

a) Visit to Zimbabwe March-April 1998. All three participants met to plan the work ahead and determine priorities, identify personnel and determine time-tables and budgetary allocations. [Note this was done prior to available funds from the sponsor]

b) Visit of Dr. Shiff to Israel Aug. 1998 to meet Dr. Hamburger and investigate issues concerning the availability of satisfactory reagents for the work. (travel to Israel was not billed to the project)

c) Visit of Dr. Shiss to Zimbabwe November 1998 (costs covered by other sources) to meet Mr. Makura and Dr. Chandiwana, inspect and procure additional materials for the snail breeding facilities, determine that the transfer of funds to the Blair Research Trust Account had actually occurred. Project at Blair was allocated a number and account so that activities could now commence. Discussed methods of communication for the three senior.

## E) Request for A.I.D. Actions:

Transfer of funds without delay is requested because it is essential for continuation of the work, especially in the collaborating laboratories.

The laboratory at Blair will require a thermocycler for carrying out PCR work on location. We would like the AID to Help Identify a source for funding the purchase of a thermocycler. If a purchase of a thermocycler will be realized, the laboratory at HU will undertake the supply of Taq polymerase, nucleotides and special micro-pipette tips required for the PCR.