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IDENTIFICATION BY PCR OF SCHISTOSOME CERCARIAE TRAPPED IN NATURE

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Executive Summary:

Detection of schistosome cercariae is required for defining the risk factors of infection. Available methods for trapping cercariae need to be improved and tools for species identification of the cercariae recovered have to be developed. In Zimbabwe *S.chistosoma mattheei* , the main animal schistosome species develops in snail hosts of the human schistosome *S. haematobium*, and cohabits the same transmission sites. We aimed at combining a cercarial trap based on chemical attraction of cercariae by linolenic acid (Schiff et al.[1]), with the development of new molecular tools for differentiating *S. mattheei* from *S. haematobium.* and *S. mansoni.* The outcome of the proposed approach, and the importance of the trilateral collaboration were as follows: (1) At the HU- 1a- Attempts in Jerusalem at chemical trapping of cercariae of *S. mansoni* have failed. 1b- Identifying chemically trapped cercariae by PCR was not successful for both *S. mansoni* (cercariae applied on a trap) and *S. haematobium.* 1c- The new DraI repeat family present in *S. haematobium* and in other terminal spined schistosomes (but not in *S. mansoni*)[2] , was found to be highly conservation in *S. mattheei* too. This obviated further search for target sequences suitable for differential identification of these parasites. 1d- New genomic libraries of *S. mattheei* were prepared and numerous clones exhibiting species specificity were selected as candidates for molecular species differentiation. Assay development is in progress. 1e- A Blair scientist was trained in PCR for molecular identification of schistosome species. (2) At Blair Research Laboratories (BL)- 2a- Adult *S. mattheei.* Worms were collected and shipped to Jerusalem for the molecular work. 2b- The life cycle of *S. haematobium* and *S. mattheei.* Were established for ongoing supply of biological materials. 2c- A BL scientist was trained in Jerusalem of on PCR and participated in development of the new tools. 2d- A laboratory for molecular work was established at BL. 2d.- Field sites suitable for testing newly developed molecular tools were identified. 2e-Cercarial chemical trapping was not successful (as also found in Jerusalem). Trap development seems far from completion and requires special efforts. Cercarial trapping was done by filtration and centrifugation. (3) At the Johns Hopkins Univ. (JHU)- 3a- Chemical trapping *S. haematobium* cercariae was demonstrated (but not in Jerusalem nor in Harare). 3b- *S. haematobium* cercriae were sent to Jerusalem. 3c- Regular trips made to assist work at BL. **In summary:** Molecular tools are realistically close to completion. Chemical cercarial trapping has not been found to be robust enough and its development is still needed and will probably require much further efforts.

A) Research Objectives: The general aim of the project was to develop a robust 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:

1) To use PCR techniques for detecting schistosome cercariae or remains of cercariae collected on a cercarial trap and to tests the limits of sensitivity.
2) To develop primers which will differentiate between cercariae of different species, especially *S. haematobium, S. mansoni and S. matthaei.*
3) To investigate dispersion of schistosome cercariae in pond and natural water systems, both flowing and stagnant diurnally and seasonally.

B) Research Accomplishments:

a. AT The Hebrew University

The application of a PCR assay for detecting cercariae maintained under various conditions was tested in order to determine suitability of cercariae trapped in the field and delivered to the laboratory for detection by PCR.

1) Conditions of storage of cercariae: Their effect of detectability by PCR. This has been initially investigated by employing *S. mansoni* cercariae and corresponding primers since availability of *S. haematobium* cercariae from John Hopkins U and from Blair laboratories was initially limited. For amplification of DNA from *S. mansoni* cercariae we have used tools previously described[4]. DNA preparation from cercariae was 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, then 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[3,4]:

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.[4]. 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
Dried cercariae from cercarial traps	-ve
Dried cercariae (directly)	+ve
Cercariae stored in EtOH And washed in TE	+ve
Cercariae kept frozen in deionized water	+ve
Cercariae kept frozen in TE	+ve
Cercariae kept frozen in PBS	highly inconsistent
DNA from cercariae kept frozen in PBS	highly inconsistent
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 tub

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 cannot be consistently and reliable be detected, perhaps because of the inhibitory effects of nail varnish and/or linoleic acid. This is considered one of the major obstacles for using the chemical cercarial trap.

2) Amplification of DNA from *S. haematobium* cercariae . For this purpose we employed cercariae sent by Dr. Shiff in ETOH and glass slides with trapped cercariae also sent by Dr. Shiff. 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.

Primers derived from a highly repeated tandemly arranged sequence of *S. haematobium* termed the DraI repeat[2] (found prior to this project) were employed:

Sh1: 5' GATCTCACCTATCAGACGAAAC 3' (22mer)

Sh2: 5' TCACAACGATACGACCAAC 3' (19 mer)

The PCR conditions employed for PCR with *S. haematobium* were as follows: 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 *S. haematobium* cercariae. However, a reliable detection by PCR of chemically trapped *S. haematobium* cecould not be consistently accomplished even when*S. haematobium* cercariae were dried onto glass slides covered with nail varnish and linoleic acid (cercarial trap), as described above for *S. mansoni* cercaria . Furthermore, further attempts to employ the chemical traps for *S. mansoni* (the only schistosome maintained in the Jerusalem laboratory) did not yield successful trapping, and supply of *S. haematobium* cercariae on chemical traps from John Hopkins or from Blair Laboratories was not accomplished to enable further attempts for combining PCR with Chemical trapping of cercariae. Thus, one of the main objectives (objective A) could not be achieved. One of the possible reasons for problems in trapping is the instability of linoleic acid under oxidizing conditions (when exposed to the atmosphere) and light (when exposed to UV). It should be mentioned in this regard that tetra-decenoic acid was previously found to be very active in inducing penetration behaviour of schistosomes (same behavior that leads to trapping) but also proved to be unstable for enabling a robust and long term activity[5]. Perhaps preparation of stable fatty acid derivatives and testing them for trapping purposes may lead to improvement of the trap and make it more robust for use under field conditions. A colleague from the Hebrew University

School of Pharmacy synthesized many derivatives of miristic acid (for other purposes) and they can be made available for testing cercarial attachment, but this is beyond the objectives of the present study.

3) Attempts at trapping cercariae in the laboratory in Jerusalem. Several experiments were carried out in which cercarial traps were prepared by mixing linoleic acid with nail varnish and applying the mixture onto glass slides as previously described[1]. The slides were placed in Petri dishes for 30 minutes and *S. mansoni* cercariae were then added. Various numbers of cercariae were added (50, 100, 500) and the slide was left for 1-2 hours with the cercariae and the slides were then stained with iodine and taken for microscopic examination . In 10 separate experiments we did not observe trapping. Only a few cercariae attached to the slide, and control slides with nail varnish alone or with no addition of any material also showed few cercariae attaching. There was no significant difference in numbers of attaching cercariae in the various slides used. Also it was observed that the nail-varnish-linoleic smear was easily peeled off the slides. Experiments carried out Zimbabwe by the Blair team with similar traps and using *S.haematobium* cercariae were also unsuccessful (see below). It appears therefore that a robust chemical trapping that works in laboratory and in field conditions is still needed before attempting application of molecular tools for differential identification of the cercariae trapped.

4) Employing PCR primers based on *S. haematobium* DraI repeat for differential identification of S. haematobium and S. mattheei. DraI primers mentioned above were employed for this study. The PCR optimization system (Optiprime Kit, Stratagene) was employed in an attempt to identify PCR conditions that will enable differential identification of these two schistosomes. Mr. Lincoln Chiimba, a research associate from Blair Research Laboratories, carried out this study during his training in Our laboratory (September -December 1999, a total of 3 months). The results of this study are summarized in the report provided by Blair Laboratory (see below). Consistent species differentiation could not be achieved by PCR using DraI primers and could not serve as a basis for a robust differential diagnosis. These results suggested that the DraI repeat in the genome of *S. mattheei* is very similar in its nucleotide sequence to the DraI repeat of *S. haematobium.* Further experiments were needed for

comparative analysis of the DraI repeat in both species in search of sequence differences that may enable designing of specific primers for differential identification.

5) Comparative analysis of the DraI repeat of *S. haematobium* and *S. mattheei.* Three Sau3a libraries of *S. mattheei* were prepared as previously described for *S. haematobium* [2]. Screening the library for clones containing repeated sequences was carried out by colony hybridization with *S. mattheei* genomic DNA labeled with ^{32}P as previously described for *S. haematobium*.. About 200 clones exhibiting positive hybridization signals with *S mattheei* total DNA, were selected as containing repeated sequences. They were, gridded and probed with DraI PCR product in search of species specific clones (see below). The results of this extensive screening is included in **Table 2 (see Appendix)**, which also summarizes additional screening of the clones as described below. Of the 80 clones which were probed with DraI PCR products 49 were DraI negative and 31 hybridized positively with DraI. Eight *S. mattheei* clones containing the DraI repeat underwent computerized sequence analysis in comparison with *S. haematobium* DraI sequence consensus (reported by us in a previous study[2]) in search of sequence differences within DraI-related sequences of the two species. The results of the DraI comparative sequence analysis are presented in **Table 3 (see Appendix).** It can be seen that the DraI sequence is very conserved not only intra-specifically (within *S. haematobium*) as previously reported[2], but is also highly conserved between *S. haematobium* and *S. mattheei* with only few nucleotide changes. Cross hybridization of the DraI sequence with several other species of terminal spined schistosomes was also previously found by us[2], however, the degree of sequence homology was not known. The present study reveals that the degree of conservation of DraI can explain why the DraI repeat cannot serve as a basis for designing primers for species specific identification. The DraI repeat however was not found in *S. mansoni*, in bird schistosomes and in echinostomes[2] and can likely be used for genus identification. Thus PCR by employing the DraI primers can be used as a first step in identifying cercariae in water also of schistosomes within their bulinid snail hosts. The second step of identification does require identification of the schistosome species to find whether they are infected with *S. haematobium* or with related terminal -spined schistosomes. In some areas exact species identification of the animal schistosome is needed but in areas where *S. mattheei* is the prevailing animal schistosome perhaps partial species specificity

is sufficient, as long as *S.mattheei* can be identified differentially from *S. haematobium* . In such cases initial screening by DraI probe/primers should be followed by an assay employing *S. mattheei* probe/ primers. If both *S. mattheei* and *S. bovis* are the major animal schistsomes (as in many areas in east Africa) then initial screening for DraI should be followed by assays capable of differentially identifying these two parasites.

6) Identification of candidate *S. mattheei* clones for a highly species specific detection by using dot hybridization. The gridded 194 *S. mattheei* clones containing repeated sequences underwent colony hybridization with ^{32}P-labeled total DNA from *S. haematobium S. magrebowiei, S. intercalatum, S, Currasoni* and *S. bovis* (wormes received from Dr. David Johnston from the Natural History Museum, London). This was done in order to select candidate clones carrying species specific sequences of *S. mattheei* that hybridize only with the homologous *S. mattheei* DNA. This type of cross hybridization was also expected to provide information on partial species specificity (see below section 5). Of the 194 candidate clones analyzed **(Table 2-Appendix)** 63 clones were found to have a high degree of species specificity by colony hybridization since they did not hybridize with the animal schistosomes DNA. Of these 63 clones 27 were not analyzed for hybridization with *S. currasoni* DNA, and 30 were not analyzed with DraI PCR products. However, since they did not cross hybridize with neither of the other 4 species of animal schistosomes' DNA the presence of DraI repeat in these clones is highly unlikely, as DraI is present in all these species[2]. The 63 clones or some of them are thus expected to identify *S. mattheei* specifically by dot hybridization. Strangely we recorded 3 clones (Smatt 70, 71 and 81) that hybridized positively with DraI but not with the schistosome species tested. They may involve an unknown version of Dra I. Sequence analysis of these clones should provide the answer to the reason of this discrepancy.

The feasibility of developing a dot hybridization assay for species differentiation has thus demonstrated. This part is close to completion (estimated a few months). We are expecting to receive from our Zimbawean partner suitable material (S. *hematobium* and *S. mattheei* cercariae) in order to further determine sensitivity (number of cercariae detectable) and specificity *(S. matthheei* Vs. *S. haematobium* and *S. mansoni)* of the hybridization method for species specific identification of *S. mattheei.*

7) Attempts at establishing a PCR assay for a highly species- specific identification of *S. mattheei*. Of the 63 *S. mattheei* clones exhibiting a high degree of species specificity by colony hybridization **(Table 2-Appendix)**, several underwent nucleotide sequencing and computerized analysis (Blast) for detecting sequence homology among them in order to identify the insert section that corresponds to a repeated sequences. This is important for determining the bounderies of the cloned repeated sequences concerned in order to enable designing PCR primers for amplifying the said repeated sequence. So far 4 clones (Smatt73, 79, 89 and194) underwent the full analysis, namely nucleotide sequencing, identification of the repeat, designing primers (**primer list is provided in table 2-Appendix**) and carrying out comparative PCR with *S. haematobium* and *S. mattheei* DNA and using 12 PCR buffers (Optiprime Kit, Stratagene, USA). None exhibited a set of PCR assay condition that may enable differentiation between the two species. Two more clones were recognized as candidates for species-specific PCR (clones Smatt7 and 53) but did not undergo yet a complete analysis. Several other clones were found unsuitable because the repeat borders could not be located and therefor PCR primers could not be designed. Many other clones in this group are still in the process of examination. A full scale analysis may take more than one year and is beyond the given timeframe even if a no-cost extension is granted.

8) Attempts at establishing a partially species specific assay for differential identification of human and animal schistosomes. While differentiation of *S. mattheei* from several terminal spined schistosomes by hybridization (as described above) or by PCR (as described above) is in progress, an assay with a lesser degree of species specificity may also be a useful practical tool. Thus, in endemic areas where *S. mattheei* is the major animal schistosome (like in Zimbabwe) differentiation from *S. haematobium* (and *S. mansoni*) alone is sufficient for practical purposes. Many *S. mattheei* clones listed in **table 2 (Appendix)** do not cross-hybridize with *S. haematobiym* DNA, and can therefore be examined for species specific identification of *S. mattheei* by dot hybridization and/or by PCR by using the screening approach and sequence analyses described above. Some of these clones also give positive hybridization signals with *S. bovis* that is distributed widely in Africa and particularly in East Africa it is present together with

S. mattheei and *S. haematobiym*.. Therefore, clones exhibiting positive hybridization signals with both *S. mattheei* and *S. bovis* (e.g. clones Smatt26, 43, 63, 66, 67, and 68) should be tested further in order to examine their usefulness for differentiating these animal schistosomes from *S. haematobium*. . This approach is realistically close to completion at a time scale from a few months to more than a year as describe in sections 6 and 7 above (depending on the scale of analysis required for finding suitable clones).

b. Field and laboratory work in Zimbabwe

1. Collection of biological materials. Several trips were made to different abattoirs in the country to collect samples from slaughtered cattle. The samples collected included cattle parts such as bladders, liver, bile and fresh cattle manure. All the samples were processed in the laboratory using either the filtration or the sedimentation techniques in order to recover schistosome eggs . The animal-schistosome eggs were difficult to recover in some of the samples. In addition, worms embedded in the walls of slaughtered cattle intestines were collected and were preserved in 80% alcohol for transportation and subsequent laboratory processing. For enabling egg collection a technique of crushing the paired worms collected from the abattoir was adopted and combined with the sedimentation technique for recovering ova . The crushed worms produced numerous eggs which were hatched for snail infectivity studies .

2. Maintenace of schistosomes in the laboratory, and testing the susceptibility of host snails (up to August 1999)-**Phase I**. A school previously identified for a parasitological survey (located in a remote area in the Eastern part of the country) was revisited for the purpose of this study. School-children aged between 8 and 15 years were screened for the presence of *S. haematobium* ova in urine. The positively identified samples of the two species (*S. haematobium* from school children and *S. mattheei* from cattle) were used to infect *Bulinus globosus* as a way of maintaining both animal and human life cycle of the parasites under study. All the experiments for infectivity studies of snails were conducted using laboratory bred F1s cultured in the snail room at Blair Research Laboratories. Snails were exposed to 3-5 miracidia to infect each *Bulinus globosus* with the animal or human

schistosome. The snails were kept at optimal conditions to induce cercarial development and shedding. Infection became patent afterseven weeks or more. Some harmsters were exposed to 300 cercariae each and were kept under optimal conditions. The harmsters were perfused for collecting adult worms after eight weeks of infection with the human and animal schistosomes. The worms were preserved in 80% alcohol for them to be used for DNA isolation for PCR. It appeared that *S.mattheei* is very difficult to cycle through harmsters and snails as compared to *S. haematobium* . This work was largely discontinued while Mr. Lincoln Chiimba was training in Jerusalem.

3. Isolation of cercarial DNA for PCR both in Zimbabwe and in Jerusalem by a Zimbabwean scientist. The cercariae were mixed with a volume of 300ul of 1M NaOH plus 1% triton X - 100 which was freshly prepared, vortexed at room temperature . The mixture were heated for 1hr at 65 degrees .Concentrated HCl was used to neutralise the mixture by not adding more than 25ul .The mixture was subjected to a temperature of 100 degrees for 10 min and quickly cooled on ice for 1 min afterwards. The Eppendorf tube was also centrifuged at 1500 rpm for 15min to bring down the debris .Fresh tubes with supernatant containing the DNA were added with 12ul of 5M NacI. This procedure was followed by ethanol precipitation . The pellet was washed three times with cold 70% ethanol .The extraction products were dissolved 50ul of 1x TE buffer and the isolated products were stored at -20 degrees .

4. PCR Amplification (in Jerusalem by a Zimbabwean scientist Mr. Lincoln Chiimba). –Part of *Objective B.* Specific primers for *S. haematobium* and *S.mansoni* were used for the PCR (see above in the section on work at the Kuvin center). The PCR reaction was carried out in 50ul reaction mixture. The amplification components comprising 10x PCR buffers with varied volumes of Tris, MgCl ,KCl and dionised water for the prepared 12 buffers. The basic concentration of the 10x PCR buffers (10mM Tris HCl ,pH 9.2 ,3.5mMgCl and 75mM KCl),0.2mM (each) dGTP,dCTP,dATP,and dTTP,0.5 units of taq polymerase ,and 20 pmol of each of the primers. The reaction mixtures were heated to 94 degrees for 5min and were subjected to 30 cycles of 95degrees for 1min,55degrees for ,and 72degrees for 40 sec and a final extension at 72 degrees for 10min in a DNA thermal cycler . The amplified products were

analysed by 1% agarose gel electrophoresis dissolved in TAE buffer pH 8 stained with ethidium bromide . A 100 bp ladder was used as a size marker for the PCR amplicons , and the PCR products were then photographed for analysis

The research assistant on the project (Mr. Lincoln Chiimba) was attached for 3 months in Dr Hamburger`s laboratory in lsrael . He took with him some *S. haematobium* cercariae and *S. matthaei* worms which were used for The molecular studies in Jerusalem. Efforts to come up with specific primers are continuing in Jerusalem (see above). While being attached in Prof. Hamburger`s laboratory, skills in DNA extraction , PCR, and optimisation of PCR conditions for *S.matthaei* and *S. haematobium* were imparted as a technology transfer to the Blair Research team .

Several experiments were carried out. Buffers with different ionic concentrations (Optiprime Kit, Stratagene) were used in an attempt to find PCR conditions suitable for differentiation between animal and human schistosomes in the lab. PCR was repeated with *S. mattheei* as was for *S.haematobium* at different PCR buffer compositions in an attempt to select a buffer suitable for differential identification of the schistosome species concerned. The principal investigator from Blair (Dr. Steven Chandiwana) also visited the research assistant while he was training in Jerusalem, to supervise progress of the technology transfer training for 2 weeks at the Kuvin centre for tropical disease in Jerusalem. The Zimbabwean scientists have carried out similar experiments at Blair Laboratories and the technique is now established at at Blair research institute. Reagents like primers and the reagent cocktail for PCR were supplied to Blair laboratories by the laboratoy in Jerusalem. It is important that supplies be sent for continuity and uniformity of reaction conditions . However thisawaits completion of the development of the molecular identification tools.

Table 4: Comparative PCR amplification of *S.haematobium* (S. S.hem) ,*S.mansoni* (S.man) and S .matthaei (S. matt) DNA at 10pg and 1ng using *S. haematobium* DraI primers.

Buffer	10pg S.haem	10pg S.matt.	10pg S.mansoni	1ng S.haem	1ng S.matt	1ng S.mansoni
1	-	++	+/-	+	+	+
2	+++	++	+/-	+	+	+
3	++	++	+/-	+	++	-
4	++	++	+/-	+	++	+
5	++	++	+/-	-	++	-
6	++	-	+/-	+	++	+
7	+	++	+/-	+	-	-
8	+++	+	+/-	+	++	+
9	++	+	+/-	+	++	-
10	++	+	+/-	+	+	+
11	+++	+	+/-	+	+	+
12	+++	+	+/-	+	++	+
+ control	+++	++	+/-	+	++	+
-control	-	-	-	-	-	-

The results presented are representative. Consistent differentiation between S. *haematobium* and *S. mattheei* was not possible using the DraI primers. This can be explained by the high conservation of the DraI sequence in these two species (see above in results obtained in Jerusalem). Reactions with *S. Mansoni* (which does not contain the DraI sequence) are borderline or negative and can be considered non-specific. Although in general PCR signals with *S. hematobium* were stronger than with *S. mattheei*. the diagnosis on the basis of dilution experiments cannot be considered robust enough for enabling a clear species identification. Efforts to complete development of the molecular tools are ongoing in Jerusalem (see above). Once prepared and standardized they are expected to be transferred to Blair Laboratories.

Table 5: *Schistosoma mattheei* adult worms taken to Jerusalem for preparing genomic libraries towards development of molecular tools for schistosome species identification (see above – Research at the Hebrew University)

No of S. matthaei male worms	No. of matthaei female worms	Total No. of Paired matthaei worms	Total No. of collected matthaei worms
1287	540	735	2562

5. Setting up a molecular laboratory at Blair Research Institute. Briefly, A biochemistry laboratory was modified and converted into molecular laboratory . The lab consist of the main lab were DNA isolation is normally carried out. The other adjacent room is for PCR experiments. The PCR room has a cabinet fitted with U.V light. . A thermocycler is also available in the same room . The laboratories have the following equipment :
A dark room is also available within the same building . Inside the darkroom is a U.V trans-illuminator and polaroid camera. Major equipmet are thus available for molecular work at Blair . These plus electrophoresis equipment can be used for PCR. Identification by dot blot hybridization will require a dot blot block. While PCR is expected to be well incorporated in Blair the hybridization technique especially if involving radio-labeling is more complex and therefor dot blots should be prepared at BL and examined in Jerusalem.

6. Maintenace of schistosomes in snails and hamsters (since 2000)-**Phase II**. Most snails in the snail room died when the assistant on the project (Mr. Anthony Chiimba) visited Prof. Hamburger`s laboratory. So continued collection of more samples from the abattoir was undertake . The team shifted emphasis from collecting other parts where animal schistosome could be recovered and a technique for collecting worms only was adopted based on previous experience attained in Phase I (see above). The paired worms were processed using a micro sieve 0.25 um . The worms were crushed using ice cream sticks. Normal saline was continuously applied to prevent the eggs from hatching during the isolation process. The filtrate was collected in a flask and left undisturbed for 30mins . This was repeated until the supernatant was clear. The process was followed by distributing the mixture in test tubes for

spinning at 1000rpm for 30 sec and discarding the supernatant . Eggs in samples drawn using a Pastuer pipette were enumerated by microscopical examination using 10x objective lens. Finally, the tubes containing eggs were identified and freshwater was added to induce hatching of eggs . numerous snails were infected using the miracidia harvested . The female and the male worms were preserved in ethanol for DNA extractions . The team routinely shed snails for cercarieae and preserve the cercariae. We are hoping to receive some primers from Israel as we have already made our request to resume some experiments.

Table 6. Showing the breakdown of snails exposed to both animal and human schistosomes

Snails exposed to S. mattheei	Shedding with S.mattheei infection	Snails exposed to S.haemat	Shedding with S.haem infection	S.mattheei male worms	S.matheei female worms
1000+	9	103	11	113	58

Collcting eggs for reestablishing the *S. haematobium* life cycle required collection of ova from urine of infected school children. This was accomplished and the results of the screening in the school selected are presented in table 7.

Table 7. Prevalence of S.haematobium in school children

Pupils Screened	Positive	negative
275	38 (14.8%)	237

7. Attemps at cercarial trapping. This was carried under the guidance Dr. Schiff when he visited Blair in July 2000 . Chemical traps made of glass slides smeared with linoleic acid on one end were employed. In addition an apparatus was constructed with three filters of varying size for filtering cercariae from water. Following filtration the last filter (made of Whattman paper) was stained with either iodine or light green stain on Dr Schiff's recommendation. These filtration

experiments were repeated several times . At first the recovery rate was very low, and then adjustment of the filtration apparatus was undertaken.

A 1000ul micro pippette to draw cercariae from a shedding tube. This was repeated three times and an average was obtained. . A range of 1000ul -5000ul containing about 20-100 cercariae was introduced in beakers containing 1000mls of fish water . The cercariae were left to stand briefly to distribute themselves freely. The water mixed with cercariae in a 1000ml beaker was then intermittently poured through the sieves . The final filters were removed and dried with a pore size of 0.7um. Either light green stain or iodine was used for staining the filters . A dissection microscope was used to visualise the retained cercariea on a stained filter.

The glass slide smeared with linoleic acid at one end did not yield very positive results under laboratory conditions . In cases where we trapped cercariae it was difficult to differentiate them with artifacts. It is also a problem after trapping the cercariae to try and recover them for DNA extraction when they adhere to the cutex.

8. Pond trials. These experiments were carried out in outdoor ponds available on Blair laboratories ground. Snails *(Bulinus globosus)* were each infected separately with 5 miracidia by overnight exposure. The snails were incubated for more than 6 weeks. The experiments were conducted in such a way that 10 snails were placed in each floating cage. Two floating cages were strategically placed in the pond using a string and a nail driven in the ground to keep hold of the floating cages. The internal diammeter of the pond was determined as well as the water column and recorded. The water temperature pH and turbidity were also measured at the same time. Floating time for the cages was also important and recorded. Before the cercariae were put in water there were kept in the dark for 2 hours. The snails after 2 hours were returned to the aquaria. Three experiments were carried out to determine if there is any presence of shed cercariae in the pond after exposure time. A filtration apparatus was used to filter 2, 10 and 20 litres of water separately using a filter paper of 40um pore size in each case. The filter paper after passing the sample were each removed and dried. Light green stain was later used to stain the filters for microscopic examination using the dissection tmicroscope. The trapped cerrcariae were then enumerated and recorded.

Results of trapping experiments are presented in **Tables 8 through 15 in the Appendix**

It was thus demonstrated by the Zimbabwean team that trapping by filtration is possible. Cercariae trapped by filtration can be processes for DNA extraction and PCR as has been previously demonstrated (Hamburger et al.[4]). It is therefore likely that cercariae trapped by filtration in Zimbabwe will be suitable for molecular species identification once the tools are ready (see above). However it was cercarial trapping by a chemical trap (due to attraction to linoleic acid) that are the subject of this project and this has not been accomplished. Thus, modifications of the chemical trap are needed by changing the attractant and the attachment matrix to more suitable substances.

c. 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 the initial 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.

3) The major activity of Dr. Schiff in subsequent years was to facilitatethe various operations in Blair research laboratories. His position as the previous director and as a frequent visitor at Blair enabled facilitation of the variousoperations reported by Blir (see above). In particular:

i. Helping setting up life cycles of *S. haematobium* and *S. mattheei* and sample collection.
ii. Helping selecting a suitable trainee from Blair Laboratories for training in Jerusalem.
iii. Helping in trapping experiments (mentioned above).
iv. Helping in pond experiments (mentioned above).

4) Dr. Shiff has recently described a modified chemical trap developed for another study. Modifications of the chemical trap are indeed needed by changing the attractant to a more stable one and the attachment matrix (nail varnish) to a more suitable one. Such modifications have been described by Dr. Schiff [6], but have not been incorporated for in the present study.

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C) Scientific Impact of Collaboration:

The main scientific rationale for building the collaboration of our three groups was the respective scientific advantages of the participants. Thus Dr. Schiff from JHU previously developed a chemical trap for cercariae potentially of great importance for determining risk of infection in schistosomiasis transmission sites. It required the addition of a molecular tool for identifying cercariae by species, since cercariae of human schistosomes and those of animal schiasrtosomes cannot be differentiated by their morphology. In the Hebrew University we previously developed PCR assays for monitoring cercariae of *S. mansoni* and of snails infected with this parasite, and also identified repeated sequences of *S. haematobium* , the DraI repeat, potentially suitable for identifying this parasite. These technological advantages were taken as a basis for further development of molecular tools for differentiation between the human schistosomes and *S. mattheei* the main animal schistosome found in Zimbabwe. Blair research Laboratories in Harare have been involved in schistosomiais research for decades and Dr. Chandiwana, the director, participated in the development of the cercarial chemical trap with Dr. Schiff. Well organized laboratories close to the field where schistosomiasis transmission occurs, plus a vast experience in studying factors of transmission in the laboratory, in outdoor ponds and in natural transmission sites in the field, were all provided by Blair Laboratories. Dr. Schiff's long lasting connection with the Blair Laboratory and his regular visits to Israel positioned him as a liaison between the Blair laboratory and Jerusalem. Collaboration **during the first year** was accomplished by combining procurement of biological materials at Blair and JHU on the one hand, with optimizing PCR conditions for detecting cercariae of *S. haematobium.* While this was accomplished cercarial trapping on chemical traps in Jerusalem did not work out with *S. mansoni* Cercariae. Collaboration enabled receiving traps with *S. haematobium* cercariae from JHU. But still recovery of DNA and detection by PCR were not successful. At Blair in the meantime parasites were collected and preparations were made for maintaining in the laboratory the life cycles of *S. haematobium* and of *S. mattheei* in snails and in hamsters. This was an essential requirement for continuation of the molecular work in Jerusalem. **During the second year** adult *S. mattheei* worms were painstakingly collected from materials collected in abattoirs, and the life cycles of the parasites were established at Blair. Dr. Schiff from JHU visited Blair laboratories for helping major decisions on communication and on selecting a candidate for

training in Jerusalem. He also helped in the establishment of the parasites in the laboratory as mentioned above. Later on during the second year a research associate from Blair (Mr. Lincoln Chiimba) visited for 3 months in at the Hebrew University. He brought with him *S. mattheei* adult worms, and carried out a study on the use of S. *haematobium* DraI primers for differential diagnosis between *S. haematobium S. mansoni and S. mattheei.* His work helped determine that primers derived from the DraI repeat are unsuitable for differential identification of *S. haematobium* and *S. mattheei.* This initiated at the Hebrew University a comparative analysis of the DraI repeat family in these parasites as well a search for sequences more suitable for differential identification. *S. mattheei* adult worm collected in Zimbabwe and brought by Mr. Chiimba were used for DNA extraction and construction of genomic libraries of *S. matteei.* Screening of the libraries for clones representing repeated sequences was undertaken and194 such clones were selected. **During the 3rd Year** Blair Research Laboratories undertook reestablishing of the life cycles of *S. haematobium* and *S. matheei* to enable trapping experiments at Blair and supply of biological materials for the molecular work in Jerusalem. Trapping experiments at Blair established that the chemical trap still needs improvement. Although trapping by filtration was accomplished and its coupling with molecular identification highly probable (as previously demonstrated), it is the chemical trapping approach that targeted for this study. Dr.Schiff from John Hopkins participated in the trapping experiment in Zimbabwe both by chemical traps and by filtration and demonstrated together with the Zimbabwean partner that the chemical trap is apparently not sufficiently robust. Cercariae trapped by filtration from these experiments still need to reach the laboratory in Jerusalem. At the Hebrew University, comparison of the DraI-repeat family in *S. haematobium* and *S. mattheei* was undertaken in an attempt to identify sequence differences that may allow designing of specific primers for differential identification. This was done with the intention of rapid completion of the development of suitable molecular tools for their establishment at Blair Laboratories in order to proceed with trapping experiments coupled with molecular identification. Howevet the DraI-sequence was found to be highly conserved between the species, so that specific PCR primers for differential identification could not be designed. This led to further search for clones carrying species specific sequences. This was started during the third yesr and is still continueing. **During the Fourth Year** at the Hebrew University an extensive search for clones suitable for differential

identification of the schistosome species concerned was carried out. Many clones exhibiting a high degree of species specificity or partial species specificity were identified. Sequence analysis of several of them was carried out and primer designed for attempting species specific detection By PCR. Development of a suitable PCR assay has not been achieved yet and there are many more clones to be tested. However, many clones potentially suitable for species specific identification by dot hybridization have been identified. This part requires procurement of cercariae of *S.haematobium* and *S.mattheei* from Blair Laboratory. This collaborative step is being awaited for completing development of the said molecular tools and for publishing the results. Dr. Schiff from John Hopkins University has been involved in another study which led to improvement of the cercarial chemical trap. For *S. mansoni*. These studies open a possibility that improvement of the trap for *S. haematobium* and *S.mattheei* cercariae may still be possible but this aspect of the work seems to still require much studies and redefinition of goals and plans.

D) Description of Project Impact:

The impact of the project can be examined in relation to the expectations and the goals presented. It is hereby presented by the following main points:

1. It was demonstrated that cercariae can be identified by PCR without prior extraction of DNA. This should present a major advantage for field application.
2. The DraI repeat[2] of *S. haematobium* was also found in *S. mattheei*. It was cloned and characterized to demonstrate a high degree of similarity with that of *S. haematobium*. These results obviate that species differentiation cannot be based on this sequence for probing or for primer design for PCR.
3. About 200 clones of *S. mattheei* containing repeated sequences were identified and are available for a thorough search for clones that can serve as a basis for species specific identification of *S. mattheei* .
4. A large-scale cross-hybridization study was carried out and enabled identification of about 60 clones carrying repeated sequences that can potentially be used for species identification of *S. mattheei* . Species specific identification by dot hybridization is thus realistically possible following some additional work.

5. *S. mattheei* is the major animal schistosome in Zimbabwe and in other countries in Africa where human schistosomes are cohabiting the same water bodies with animal schistosomes and obscure the results of surveys attempting to determine the risk of infection with the human schistosomes. Completion of the development of the molecular tools should solve an important problem for determining factors of transmission of schistosomes in Africa.
6. The study enabled to determine that the chemical trapping method is not robust enough and that the development of this important cercarial trapping approach still needs further development before attempting combining it with molecular tools for species identification.
7. The present study has demonstrated that even when cercariae are trapped on the chemical trap proposed (at JHU but not at HU nor at BL), it is not possible to harvest DNA and to perform PCR on product of "DNA extraction" from the trap. It is thus suggested that the problem is not just trapping but also suitability of the materials employed in the trap for enabling DNA extraction and PCR.
8. Training was again proven to be mutually beneficial to the trainee and his host laboratory. Thus, results obtained by the trainee from Blair Laboratories (BL) enabled shifting of the emphasis from DraI repeat family to other repeated sequences for the work on species differentiation.
9. The Training enabled establishing new concepts and technologies related to the development and application of molecular tools at Blair Laboratories.
10. The project enabled establishment of the life cycle of *S. mattheei* and *S. haematobium*, including improving practices for sample collection from humans and cattle. This is essential for further development of molecular tools, chemical traps and for the sustainability of using these technologies.

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. Training was accomplished with results beneficial to the developing country and the host laboratory. The instrumentation and facility needed for technology transfer was established at Blair laboratories. When the technology development is completed it will be possible to transfer it to Blair Laboratories without too much preparations. This package will

enable further refinement of the new molecular tools on location as well as their adaptation for field surveys. The present project also enabled the reestablishment of facilities tools and practices for collecting biological materials needed for further development of the molecular tools, as well as for future sustainable implementation of these tools in epidemiological studies towards control.

F) Future work:

1) Future work at HU will involve screening of candidate clones for their usefulness in identifying *S. mattheei* cercariae differentially from *S. haematobium* and *S. mansoni* cercariae. This will require shipment of cercariae of *S. mattheei* and *S. haematobium* (in ethanol) from Blair Laboratories (and some perhaps also from John Hopkins). Development of dot hybridization for this purpose and the determining the sensitivity and specificity of detection is expected within several months (pending supply of the biological materials required). Development of PCR assays for differential identification will take more time and funds and is beyond the scope of this project. Considering that as the DraI repeat was found to be unsuitable for differential identification, a whole new search was initiated for identifying other sequences for this purpose.
2) Future work at Blair Laboratories is required for the supply of biological materials required further development of the molecular tools concerned. When the molecular tools (species differentiation by hybridization) are completed they should be introduced at Blair Laboratories (based on the technology transferred) for evaluation in the laboratory and in the field towards sustainable implementation. Radiolabeling is not expected to be feasible at Blair at this stage but if dot blots are prepared there they can be shipped to Jerusalem for testing.
3) At John Hopkins and at Blair laboratories. Further development of the chemical trap is required for trapping human and animal schistosome cercariae at similar rates. At HU we have collaborated with a colleague who synthesized derivatives of fatty acids (for another project). These may be more stable than linoleic acid and should be tested for trapping activity. They should be tested for attempting improvement of the cercarial trapping capabilities.

SECTION III

A) Managerial Issues:

For the first Year and (from the first report): 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 the BL 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 Blair 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.

Major issues during the remainig 3 years of the project were communication and reporting: The first annual report (1999) was issued in time. Reporting to AID has not been accomplished thereafter until the present report. It should be mentioned that coverage of the reporting period from December 2000 up todate was not yet received from the partners (except for the dates of visits of Dr. Schiff to Blair laboratories). **A supplement to this report will be sent as soon as more information is received from the partners.**

Retrospective examination of the reporting failure was made for enabling the design of possible preventive / alert measures in the future. In this context it should be taken into account that the co-PI from Zimbabwe had a severe car accident close to the time of the second reporting (this was only indirectly reported to the PI), and his report for the second year arrived on February 2001. It included data until Decamber 2000 (part of the 3rd year too). The PI from Jerusalem was partially incapacitated from August 2000- through April 2001 because of a health problem (which was since solved). Work on the project in Jerusalem continued all this time with the PI's supervision, and was even intensified (in search of new repeated sequences, see above). Reporting however was not accomplished due to unsuccessful attempts to manage an overwhelming workload under the difficult circumstances. The situation was reported by the PI to Dr. Schiff. Also, communication with Zimbabwe was not successful for a long while and then discontinued until recently. Dr. Schiff (the official liaison between the Hebrew U and Blair laboratories) visited Blair Laboratories several times during this period but detailed reporting has not followed. It should also be taken into account that Zimbabwe has been for the last two years

under political turmoil which may have affected communication and other aspects of the project, but an evaluation of whether this was a real possibility has not been undertaken.

Expenditure reports. These are handled entirely by the audits of the Authority of Research and Development (Ms. Elinor Slater). Lags in expenditure reporting became evident to the PI only recently. It is believed that this aspect was also affected by communication problems.

B) Budget:

The reporting on this aspect requires the attention of Ms. Elinor Slater of the Authority for Research and Development of the Hebrew University.

c) Special concerns:

Considering the above-mentioned special circumstances, which have contributed to the formation of the reporting gap, it appears that administrative tools for crisis management are most important. However, crisis-management measures have not been implemented at the various levels concerned, and for a long period of time. Perhaps the availability and functionality of crisis management tools (actual or potential) require evaluation.

During the whole period Dr. Schiff made 10 visits to Zimbabwe and two visits to the laboratory in Jerusalem (see below). Perhaps A more balanced liaison activity could have been helpful for transfer of biological materials and for coordination of various matters (including reporting).

It should be mentioned that coverage of the reporting period from Dec. 2000 up to date was not yet received from the partners (except for the list of visits of Dr. Schiff to Blair laboratories). **A supplement to this report will be sent as soon as more information is received from the partners.**

D) Collaboration, Travel, Training and Publications:

Collaboration involved visits, supply of biological materials (cercariae in alcohol or on traps) by Dr. Schiff to Jerusalem, and supply of adult *S. mattheei* worms from Blair Laboratories to the laboratory in Jerusalem. Also, Dr. Schiff attempted with the Jerusalem team and with the

Zimbabwean team trapping of schistosome cercariae. Hebrew University provided training to a Blair scientist and hosted Dr. Chandiwana the head of Blair Laboratories for acquiring detailed information on the technologies transferred during the training. The Hebrew University benefited from the supply of biological materials which enabled development of molecular tools for schistosome species identification (still requires completion). Implementation of these tools at Blair Laboratory is pending completion of development and a future visit by JH to Blair.

Travel

a) Visit to Zimbabwe March-April 1998 (Schiff and Hamburger). 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. Shiff 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.

d) Visit of Dr. Schiff to Zimbabwe April 1999. A full week spent on the project in discussion organizing field trips-snail facilities for mainrtaining *S. haematobium* and S. *Mattheei.*

e) September 1999- Repeat visit of Dr Schiff to Zimbabwe (alternative funds used). a. To discuss visit of the research assistant from Blair Laboratories)Mr. Lincoln Ciimba) to Jerusalem for training in molecular methodology. b. To help establish snail culture. c. To help arrange collection of *S. mattheei* adult worms for Mr. Chiimba to take to Jerusalem.

f) Training visit of Mr. Lincoln Chiimba to Jerusalem, Sept 1999 through December 1999. Mr. Chiimba brought with him adult worms of *S. mattheei* collected in Zimbabwe for the molecular work in Jerusalem.

g) Visit of the Director of Blair Laboratories, Dr.Stevcen Chandiwana, to Dr. Hamburger's laboratory to oversee training of Mr. Chiimba-October 1999.

h) April 2000- After return of Mr Chiimba from Jerusalem, Dr. Schiff visited Blair Laboratories helping set up cercarial trapping experiments. Material collected was was prepared for shipment to Jerusalem. Delivery not accomplished.

i)July 2000- A visit to Zimbabwe by Dr. Schiff. Follow-up on above visit for helping establish at Blair the facility for carrying out PCR.

j) April 2001 A visit of Dr. Schiff to Zimbabwe discussions with Dr. Chandiwana and Lincoln .

k) July 2001- A trip of Dr. Schiff to Zimbabwe. Activity unspecified.

(Another visit of Dr. Schif to Israel was carried out in 1999 during Dr. Chimba's training –an exact date was not recorded. During this visit Dr Schiff attempted to demonstrate chemical trapping of cercariae. These attempts were not successful).

Training. Training of Mr. Chiimba from Blair laboratories took 3 month in Jerusalem. He trained in DNA preparation, PCR optimization, comparative analysis of PCR primers for schistosome species identification. He received some background knowledge in cloning in bacteria. One Israeli technitian and two student were trained in Jerusalem in PCR and molecular technologies related to the project. A Ph.D. student also took part in these studies.

Publications. The results on differential identification of *S. haematobium*, *S. mattheei* and *S.mansoni* by dot blot hybridization will be published following completion of experiments for examining candidate clones for suitable detection sensitivity and repeating specificity tests.

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

Transfer of funds is requested for reimbursement of expenses.

Permission is requested for a no cost extension of the project in order to enable completion of the development of the tools for differential species identification by dot hybridization and for travelling to Zimbabwe for establishing the technology. The purchase of a dot blot block and hybridization membranes and the supply of chemicals will be required. Radiolabeling is not expected to be feasible at Blair at this stage but if dot blots are prepared there they can be shipped to Jerusalem for testing.

APPENDIX

Table 2- A summary of the analysis of S. mattheei clones.

194 *S. mattheei* clones initially selected for demonstrating presence of repeated sequences were gridded and hybridized as follows:

1. With DraI PCR products- for selecting clones for comparative sequence analysis of the DraI repeat (presented in table 3).
2. With DNA from several other schistosome species for searching candidate clones for species specific identification of *S. mattheei.*

33 clones exhibiting a high or partial degree of species specificity underwent nucleotide sequencing (data available). Sequence analysis with several of thos exhibited repeated sequences boundaries (numbers of base pares indicated), and served for designing PCR primers (presented at the end of the table). None of those examined by PCR (at 12 different conditions each) provided a robust species specific identification. This type of search continues.

Table 3- Comparative sequence analysis of DraI sequence from 8 separate clones of *S. mattheei* with the DraI consensus of *S. haematobium* (previously published[2]).

Intra and inter specific base differences are highlighted in white or grey.

Tables 8 through 12- Filtration experiments for trapping cercariae in the laboratory (Zimbabwe).

Tables 13 through 15-Filtartion experiments-Pond water (Zimbabwe).

Table 2: A summary of the analysis of *Schistosoma mattheei* clones.

	Hybridized by						Sequence analysis				Potential candidates
Clone No	DraI	*S. h.*	*S. inter*	*S. marg*	*S. cur.*	*S. bov.*	Dir.	Rev.	Comp.	DraI	for species specific ID
Smatt1	ND	-	+	-	ND	-					
Smatt2	ND	-	-	-	ND	-					
Smatt3	ND	-	+	-	ND	-					
Smatt4	ND	-	+	-	ND	-					
Smatt5	ND	-	+	-	ND	-					
Smatt6	ND	+	+	-	ND	-					
Smatt7	ND	-	-	-	ND	-	D	R	C	No	300-500
Smatt8	ND	+	+	-	ND	+					
Smatt9	ND	+	+	-	ND	+					
Smatt10	ND	-	-	-	ND	-	D		C	No	
Smatt11	ND	-	-	-	ND	-					
Smatt12	ND	-	+	-	ND	-					
Smatt13	ND	-	-	-	ND	-	D		C	Yes	
Smatt14	ND	-	-	-	ND	-					
Smatt15	ND	-	+	-	ND	-					
Smatt16	ND	+	+	-	ND	-					
Smatt17	ND	-	-	-	ND	-					
Smatt18	ND	-	-	-	ND	-					
Smatt19	ND	-	+	-	ND	-					
Smatt20	ND	-	+	-	ND	-					
Smatt21	ND	-	-	-	ND	-	D		C	No	
Smatt22	ND	-	+	-	ND	-					
Smatt23	-	-	+	-	ND	-	D		U	No	
Smatt24	-	-	+	-	-	-	D		U	No	
Smatt25	-	-	+	+	+	+					
Smatt26	+	-	+	-	-	+					
Smatt27	-	-	+	-	-	-					
Smatt28	-	-	-	-	-	-	D	R	U	No	
Smatt29	-	-	+	-	-	-					
Smatt30	-	-	-	-	-	-	D	R	C	No	
Smatt31	-	-	-	-	-	-	D		U	No	
Smatt32	-	-	-	-	-	-	D		U	No	
Smatt33	+	-	-	-	-	-					
Smatt34	-	-	-	-	-	-	D	R	U	No	
Smatt35	-	-	-	-	-	-					
Smatt36	-	-	-	-	-	-					
Smatt37	-	-	-	-	-	-					
Smatt38	+	-	+	-	+	-					
Smatt39	+	+	+	-	-	-					
Smatt40	-	-	+	-	-	-					
Smatt41	+	-	+	-	-	-					
Smatt42	+	+	+	-	-	+					
Smatt43	-	-	+	+	+	+					
Smatt44	-	-	-	-	-	-	D	R	U	No	
Smatt45	-	-	+	-	-	-					
Smatt46	-	-	+	-	-	-					

Hybridized by						Sequence analysis					Potential candidates
Clone No	DraI	*S. h.*	*S. inter*	*S. marg*	*S. cur.*	*S. bov.*	Dir.	Rev.	Comp.	DraI	for species specific ID
Smatt47	-	-	-	-	-	-	D		U	No	
Smatt48	-	+	+	+	-	-					
Smatt49	-	-	-	-	-	-	D		U	No	
Smatt50	+	-	+	-	-	-					
Smatt51	-	-	-	-	-	-					
Smatt52	-	-	-	-	-	-					
Smatt53	-	-	-	-	-	-	D	R	U	No	470-670
Smatt56	-	-	+	-	-	-					
Smatt57	-	-	-	-	-	-					
Smatt58	-	-	-	-	-	-	D		U	No	
Smatt59	-	-	-	-	-	-					
Smatt60	-	-	-	-	-	-					
Smatt61	-	-	-	-	-	-					
Smatt62	-	-	-	-	-	-					
Smatt63	-	-	+	-	+	+					
Smatt64	-	-	-	-	-	-	D	R	C	No	
Smatt65	-	-	+	-	-	-					
Smatt66	-	-	+	+	+	+					
Smatt67	+	-	+	-	-	+					
Smatt68	-	-	+	-	+	+					
Smatt69	-	-	+	-	-	-					
Smatt70	+	-	-	-	-	-					
Smatt71	+	-	-	-	-	-					
Smatt72	-	-	+	-	-						
Smatt73	-	-	-	-	-	-	D	R	U	No	230-400: PCR(1)
Smatt74	-	-	+	-	-	-					
Smatt75	-	-	+	-	-	-					
Smatt76	-	-	-	-	-	-					
Smatt77	-	-	-	-	-	-					
Smatt78	-	-	-	-	-	-					
Smatt79	-	-	-	-	ND	-	D	R	U	No	230-400: PCR(1)
Smatt80	-	-	-	-	ND	-					
Smatt81	+	-	-	-	ND	-					
Smatt82	-	-	-	-	ND	-					
Smatt83	+	+	+	-	ND	-	D	R	C	Yes	
Smatt84	+	+	+	-	ND	-					
Smatt85	+	+	-	-	ND	-					
Smatt86	+	+	+	-	ND	-					
Smatt87	+	-	+	-	ND	-	D	R	C	Yes	
Smatt88	+	-	+	-	ND	-	D	R	C	Yes	
Smatt89	+	-	+	-	ND	-	D	R	C	Yes	PCR(2)
Smatt90	+	-	+	-	ND	-	D	R	C	Yes	
Smatt91	+	-	-	-	ND	-					
Smatt92	+	-	-	-	ND	-	D	R	C	No	
Smatt93	+	-	+	-	ND	-					
Smatt94	+	-	-	-	ND	-	D	R	C	Yes	
Smatt95	+	-	+	+	ND	-					
Smatt96	+	-	+	-	ND	-					

Hybridized by						Sequence analysis					Potential candidates
Clone No	DraI	*S. h.*	*S. inter*	*S. marg*	*S. cur.*	*S. bov.*	Dir.	Rev.	Comp.	DraI	for species specific ID
Smatt97	+	-	+	-	ND	-					
Smatt98	+	-	-	-	ND	-	D	R	C	No	
Smatt99	+	-	+	-	ND	-	D	R	C	Yes	
Smatt100	+	-	+	-	ND	-	D	R	C	Yes	
Smatt101	+	-	-	-	ND	-					
Smatt102	+	-	+	-	ND	-					
Smatt103	ND	+	-	-	ND	-					
Smatt104	ND	-	-	-	ND	-					
Smatt105	ND	-	-	-	ND	-					
Smatt106	ND	+	+	-	ND	+					
Smatt107	ND	+	+	+	ND	-					
Smatt108	ND	-	-	-	ND	-					
Smatt112	ND	+	+	-	ND	+					
Smatt113	ND	+	+	-	ND	+					
Smatt114	ND	-	-	-	ND	-					
Smatt115	ND	-	+	+	ND	-					
Smatt116	ND	-	-	-	ND	-					
Smatt117	ND	-	-	-	ND	-					
Smatt118	ND	-	+	-	ND	-					
Smatt119	ND	+	+	-	ND	-					
Smatt120	ND	-	+	-	ND	-					
Smatt121	ND	-	-	-	ND	-					
Smatt122	ND	-	-	-	ND	-					
Smatt123	ND	+	+	-	ND	-	D	R	C	No	
Smatt124	ND	-	-	-	ND	-					
Smatt125	ND	-	-	-	ND	-					
Smatt126	ND	-	-	-	ND	-					
Smatt127	ND	+	+	-	ND	-					
Smatt128	ND	+	+	-	ND	-					
Smatt129	ND	+	+	-	ND	-					
Smatt130	ND	-	-	-	ND	-					
Smatt131	ND	-	+	+	ND	-					
Smatt132	ND	-	+	+	ND	+					
Smatt133	ND	+	+	+	ND	+					
Smatt134	ND	+	+	-	ND	+					
Smatt135	ND	-	-	-	ND	-					
Smatt136	ND	-	-	-	ND	+					
Smatt137	ND	-	-	-	ND	+					
Smatt138	ND	-	-	-	ND	-					
Smatt139	ND	-	+	+	ND	-					
Smatt140	ND	--	-	-	ND	-					
Smatt141	ND	-	+	-	ND	-					
Smatt142	ND	+	+	-	ND	-					
Smatt143	ND	+	+	-	ND	-					
Smatt144	ND	-	+	-	ND	-					
Smatt145	ND	-	+	-	ND	-					
Smatt146	ND	-	+	-	ND	-					
Smatt147	ND	-	+	-	ND	-					

Hybridized by						Sequence analysis					Potential candidates
Clone No	DraI	*S. h.*	*S. inter*	*S. marg*	*S. cur.*	*S. bov.*	Dir.	Rev.	Comp.	DraI	for species specific ID
Smatt148	ND	-	+	-	ND	-					
Smatt149	ND	-	-	-	ND	-					
Smatt150	ND	-	-	-	ND	-					
Smatt151	ND	-	-	-	ND	-					
Smatt152	ND	-	+	-	ND	-					
Smatt153	ND	-	+	-	ND	-					
Smatt154	ND	-	-	-	ND	-					
Smatt155	ND	+	+	-	ND	-					
Smatt156	ND	-	+	-	+	+					
Smatt157	ND	-	-	-	+	-					
Smatt158	ND	-	+	-	+	-					
Smatt159	ND	-	+	-	+	-	D	R	C	No	
Smatt160	ND	-	+	-	-	-					
Smatt161	ND	-	-	-	-	-					
Smatt162	ND	-	+	-	+	-					
Smatt163	ND	+	+	-	+	-					
Smatt164	ND	-	-	-	-	-					
Smatt165	ND	-	+	-	+	-					
Smatt169	ND	+	+	-	+	-					
Smatt170	ND	+	+	-	+	-					
Smatt171	ND	-	+	-	+	-					
Smatt172	ND	-	+	-	+	+					
Smatt173	ND	+	+	-	+	-					
Smatt174	ND	-	+	-	+	-					
Smatt175	ND	-	+	-	-	-					
Smatt176	ND	+	+	+	+	-					
Smatt177	ND	-	+	-	-	-					
Smatt178	ND	-	+	+	+	+					
Smatt179	ND	-	+	-	+	-					
Smatt180	ND	-	+	-	-	-					
Smatt181	ND	+	+	-	-	-	D	R	C	No	
Smatt182	ND	+	+	-	+	+					
Smatt183	ND	-	+	-	+	-					
Smatt184	ND	-	+	-	+	-					
Smatt185	ND	+	+	-	+	+					
Smatt186	ND	+	+	-	+	-					
Smatt187	ND	+	+	+	+	-					
Smatt188	ND	-	+	-	+	-					
Smatt189	ND	-	+	-	+	-					
Smatt190	ND	-	+	-	+	-					
Smatt191	ND	-	+	-	+	+					
Smatt192	ND	+	+	-	-	-					
Smatt193	ND	-	-	-	-	-					
Smatt194	-	-	-	-	-	-	D	R	C	No	PCR (3,4)

S. h. : Schistosoma haematobium.
S. inter.: S. Intercalatum
S. marg.: S. margrebowiei
S. cur. : S. curassoni
S. bov.: S. bovis
Smatt: S. mattheei

1 . PCR was done using the following primers:
SmattD (2601): TTA TTC ACT ATC AAG TGT TCA AT
SmattR (2602): TGT ATA TTG AAC ATT GTG AAC

2 . PCR was done using the following primers:
SmaD (3308): CTT CCA TTG GTA AAC CG
SmaR (3309): CGG AAT TTA ATG AGG CCA ACC

3 . PCR was done using the following primers:
First set of primers:
SmzD (2603): GAG CAT GAC TCA ACA TTT ATA
SmzR (2604): ACA TTA TAT ACT CAT ATA CC

Second set of primers:
Smz 4 D (2605): ACA TAA TCA TTT TTA TAG AAG
SmzR (2606): CGC AAG ACT CCA CAA CCA CTA

Table 3: *S. mattheei* : alignment of DraI sequences against consensus DraI (*S.haematobium*)

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           1                   20                  40                  60
smatt83    TCACCTATCAGACGAAACAA AGAAAATTTTAAAATTGNTG GTGGAAGTGCCTGTTTCGCA
smatt88    TCACCTATCAGACGAAACAA AGAAAATTTTAAAATTGTTG GTGGAAGTGCCTGTTTCGCA
smatt87    TCACCTATCANACNAAACAA AGAAAATTTTAAAATTGTTG GTGGAAGTGCCTGTTTCGCA
smatt87/2  TCACCTATCAGACGAAACAA AGAAAATTTTAAAATTGTTG GTGGAAGTGCCTGTTTCGCA
smatt90    TCACCTATCAGACGAAACAA AGAAAATTTTAAAATTGTTG GTGTAAGTGCCTGTTTTGCA
smatt94    TCACCTATCAGACGAAACAA AGAAAATTTTAAAATTGTTG GTGTAAGTGCCTGTTTTGCA
smatt89    TCACCTATCANACGAAACAA AGAAAATTTTAAAATTGTTG GTGTAAGTGCCTGTTTTGCA
smatt99    TCACCTATCANACTAAACAA AGAANATTNTCAAATTGTNG GNGTAAGTGCCTGTTTCCCA
consensus  TCACCTATCAgACgAAACAA.AGAAaATTtTaAAATTGttG GtG.AAGTGCCTGTTTcgCA

           61                  80                  100                 120
smatt83    ATATCTCCGGAATGGTTGGT CGTATCGTTGTGAAAATTGT TTCATATTATTGGTGACGAT
smatt88    ATATCTCCGGAATGGTTGGT CGTATCGTTGTGAAAATTGT TTCATATTATTGGTGACGAT
smatt87    ATATCTCCGGAATGGTTGGT CGTATCGTTGTGAAAATTGT TTTATATTATTGGTGACGAT
smatt87/2  ATATCTCCGGAATGGTTGGT CGTATCGTTGTGAAAATTGT TTTATATTATTGGTGACGAT
smatt90    ATATCTCCGGAATGGTTGGT CGTATCGCTGTGAAAATTGT TTTATATTATTGGTGGCGAT
smatt94    ATATCTCCGGAATGGTTGGT CGTATCGTTGTGAAAATTGT TTTATATTATTGGTGGCGAT
smatt89    ATATCTNCGGAATGGTTGGT GCNATCNCTGNGAAAATNGT NTTATATTATTGGTGGCGAT
smatt99    ATATCTCCNGAATGGTTGGC CGTNTCNTTGTGAAAATTGT TTTATATTATTGGTGGCCNT
consensus  ATATCTcCgGAATGGTTGGt cGtaTCgtTGtGAAAATtGT tTtATATTATTGGTGaCgaT

           121
smatt83    C
smatt88    C
smatt87    C
smatt87/2  C
smatt90    C
smatt94    C
smatt89    C
smatt99    C
consensus  C
```

The tables below show results of repeated experiments using a filtration apparatus with filters stained with either light green stain or very dilute iodine solutions using *S. haematobium*

Table 8. EXPERIMENT 1

FILTER	CERCARIAE INTRODUCED	CERCARIAE RECOVERED	IODINE STAINED	FILTER	CERCARIAE INTRODUCED	CERCARIAE RECOVERED	LIGHT GREEN	pH	H_20 TEMP
1	20	9	–	1	20	15	–	7.4	23
2	20	7	–	2	20	18	–	7.4	23
3	30	8	–	3	30	22	–	7.4	23
4	40	9	–	4	40	31	–	7.4	23
5	50	11	–	5	50	37	–	7.4	23
6	100	18	–	6	100	91	–	7.4	23

Table 9. EXPERIMENT 2

FILTER	CERCS INTRODUCED	CERCS RECOVERED	IODINE STAINED	FILTER	CERCS INTRODUCED	CERCS RECOVERED	LIGHT GREEN	pH	H_20 TEMP
1	20	7	–	1	20	16	–	7.4	24
2	20	6	–	2	20	18	–	7.4	24
3	30	9	–	3	30	26	–	7.4	24
4	40	11	–	4	40	39	–	7.4	24
5	50	19	–	5	50	43	–	7.4	24
6	100	27	–	6	100	72	–	7.4	24

TABLE 10. EXPERIMENT 3

FILTER	CERCS INTRODUCED	CERCS RECOVERED	IODINE STAINED	FILTER	CERCS INTRODUCED	CERCS RECOVERED	LIGHT GREEN	pH	H_20 TEMP
1	20	11	–	1	20	11	–	7.4	24
2	20	7	–	2	20	19	–	7.4	24
3	30	14	–	3	30	29	–	7.4	24
4	40	9	–	4	40	31	–	7.4	24
5	50	11	–	5	50	36	–	7.4	24
6	100	13	–	6	100	75	–	7.4	24

TABLE 11. EXPERIMENT 4

FILTER	CERCS INTRODUCED	CERCS RECOVERED	IODINE STAINED	FILTER	CERCS INTRODUCED	CERCS RECOVERED	LIGHT GREEN	pH	H_20 TEMP
1	20	7	–	1	20	18	–	7.4	23
2	20	6	–	2	20	20	–	7.4	23
3	30	5	–	3	30	20	–	7.4	23
4	40	11	–	4	40	35	–	7.4	23
5	50	17	–	5	50	42	–	7.4	23
6	100	36	–	6	100	82	–	7.4	23

Table 12. CONTROL

FILTER	CERCS INTRODUCED	CERCS RECOVERED	IODINE STAINED	FILTER	CERCS INTRODUCED	CERCS RECOVERED	LIGHT GREEN	pH	H_20 TEMP
1	0	0	–	1	0	0	–	7.4	24
2	0	0	–	2	0	0	–	7.4	24

Pond Trial

Table 13 using 2L of pond water for filtration

Filter	Cercs trapped	Water temp	pH	Light green	Turbidity
1	11	24	7.6		15
2	14	24	7.6		15
2	12	24	7.6		15

Table 14 using 10L

Filter	Cercs trapped	Water temp	pH	Light green	Turbidity
1	11	24	7.6		15
2	17	24	7.6		15
3	29	24	7.6		15

Table 15 using 20L

Filters	Cers trapped	Water temp	pH	Light green	Turbidity
1	27	24	7.6		15
2	18	24	7.6		15
3	21	24	7.6		15