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Worm Recovery and Pathology in the Olive baboon, Papio anubis, Immunized Against Schistosoma mansoni with Snail Soluble Proteins

Eunice Nkirote Kobia[1*] Dorcas Syokui Yole[2] Horace Ochanda[3] Rebecca Waihenya[4]
1. School of Biological Sciences, University of Nairobi, P.O Box 30197, 00100, Nairobi Kenya
2. The Technical University of Kenya, P.O Box 52428-00200, Nairobi, Kenya /Institute of Primate Research, P.O Box 24481-005102 Karen, Nairobi, Kenya
3. School of Biological Sciences, University of Nairobi, P.O Box 30197, 00100, Kenya
4. Jomo Kenyatta University of Agriculture and Technology, P.O Box 62000- 00200 Nairobi, Kenya

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Abstract
Schistosomiasis is estimated to infect over 200 million people worldwide. Chemotherapy remains the major means of intervention but has the challenge of rapid re-infection, high cost and risk of drug resistance. A vaccine would have a long term effect and complement chemotherapy but none is in the market. Pilot studies in mice model showed that two proteins derived from *Biomphalaria pfeifferri* RT (soluble proteins from the rest of snail tissue) and DG (soluble proteins from the digestive gland) were protective against *S. mansoni* in terms of worm reduction and reduced pathology. Both met the World Health Organization criteria of over 40% protection. This study was done to investigate the efficacy of DG and RT in olive baboons challenged with *Schistosoma mansoni*. Baboons were in three groups: DG, RT and IC (infected control). DG and RT were immunized and boosted twice with their specific soluble snail antigens in Montanide at weeks 0, 3 and 6. They were challenged 2 weeks post final booster with 600 *Schistosoma mansoni* cercariae. DG had significant worm reduction of 11.4%. It had least gross pathology and histopathology. DG offered better protection against *S. mansoni* in baboon than RT, although lower than in mouse model.
Keywords: *Schistosoma mansoni, Biomphalaria pfeifferi*, Snail soluble protein, immunization

1 Introduction
Schistosomiasis is an intravascular debilitating disease that is as a result of infection with parasitic schistosome worms, which live in the bloodstream of humans (Steinmann *et al.,* 2006). World Health Organization considers schistosomiasis to be second only to malaria and the most devastating parasitic disease in terms of socioeconomic and public health importance (WHO, 2015). Human infection is caused by three main species namely; *Schistosoma mansoni* and *S. japonicum* that cause intestinal schistosomiasis and *S. haematobium* that causes urinogenital disease (Christinet *et al.,* 2016). The main pillar for intervention is chemotherapy but rapid re-infection demands frequent re-treatment. In addition, the drug of choice, Praziquantel™, is costly and therefore not affordable to the low income earners who happen to be the main target of the disease. The other disadvantage with Praziquantel™ is its poor activity against immature schistosomes. This results in sub-optimal outcomes during mass drug administration campaigns (Cioli *et al.,* 2014). A vaccine has a long term effect, but at the moment, there is no human schistosomiasis vaccine in the market.

2 *S. mansoni* egg maturation
Upon infection with *S. mansoni,* adult parasites finally settle in the mesenteric veins. Schistosome worms have a life period of ten years but have been known to live a few more years. In a day, the worms lay hundreds of eggs. Not all of the eggs are passed out of the gut lumen. In microvasculature of the liver, a number of eggs are trapped and once there, they release antigenic products that evoke a robust immune response that result to granuloma formation (Pearce & MacDonald, 2002). Schistosome worms do not cause much pathology per se but the eggs they produce. The greater the quantity of worms, the greater the quantity of egg produced, and hence greater the pathology.

2.1 Hepatic schistosomiasis
The host's granulomatous cell-mediated immune response to the soluble egg antigen of *S. mansoni* results to Hepatic schistosomiasis. The responses normally progress to irreversible fibrosis and finally to severe portal hypertension (Van der *et al*., 2003). Schistosome eggs remain viable in the liver for approximately 3 weeks. Primarily, the eggs induce a mild Th1 response to egg antigens. This later evolves to a dominant Th2 response that is induced by egg antigens. Eosinophils are later recruited leading to granuloma formation and fibrogenesis of the liver (Wilson *et al.,* 2007).

2.2 Granuloma formation

The formation of granuloma is advantageous to the host for it blocks the hepatocytes from the toxic antigenic effects secreted from parasite eggs. However, the hosts' immunological reactions to the soluble egg antigens may result to fibrosis with high accumulation of collagen fibres and extracellular matrix proteins within the periportal space (Morais *et al.,* 2008). The formation of granuloma is mediated by helper T cell- delayed hypersensitivity reaction. This reaction is brought about by interleukin-4 (IL-4) and IL-13 cytokines whereas schistosomal induced pathology is limited by IL-10, IFN-γ, and a subset of regulatory T cells. In addition, a variety of cell types have been implicated, such as stimulated macrophages, regulatory T cells and hepatic stellate cells (Gryseels *et al.,* 2006). The magnitude of the pathology and the development of fibrosis are dependent on the balance amid TH1and TH2 cytokines (Stadecker *et al*., 2004). Eggs are detectable inside the granulomas with the subsequent formation of marked portal and peri lobular fibrosis, which is most pronounced with *S. mansoni* and *S. japonicum*.

2.3 Vaccine candidates against Schistosomiasis

Approximately a quarter of more than a hundred vaccine antigens against schistosomiasis have so far been known. They have shown some immunity in the mouse model of schistosomiasis (Siddiqui *et al.,* 2011). There are only three molecules that have gone into clinical trials in humans; *S. mansoni* tetraspanin (*Sm*-TSP-2), *S. mansoni* fatty acid binding protein (Sm14), and *S. haematobium* glutathione S-transferase (Sh28GST). The other molecule Smp80 (calpain) is currently being tested in non-human primates (Merrifield *et al*., 2016).

2.4 Common antigens between *Biomphalaria* snails and *Schistosoma* parasites

Studies by Dissous and others in 1986; Weston and others in 1994; Gamal-Eddin and others in 1996, 1997 and Chacón and 2000 have all revealed that schistosomes and snails have shared proteins. Using western blot method, they revealed that crude *Biomphalaria glabrata* antigen (S*Bg*A) agglutinated with several homologous snail molecules and that soluble crude S*Bg*A agglutinated with sera from schistosome-infected persons by ELISA with a 100% of sensitivity (Chacón *et al*., 2000).

2.5 Use of shared antigens in vaccine development

Diagnosis of schistosomes and vaccines against schistosomiasis may be achieved by use of cross-reactive antigens. Shared antigens between *S. mansoni* and *B. glabrata* snails have been studied (Chacón *et al.,* 2000). Molecules of soluble *B. glabrata* antigen (SBgA) agglutinated with sera from outbred mice that were immunized with a SBgA with that of non- infected mice. Determination of SBgA glycoprotein epitopes was done by treating the antigens with sodium metaperiodate and then comparing it with the non- treated antigen. The glycoproteins that were identified in the SBgA were; Molecules of 24, 60 and 140 kDa. The efficacy of SBgA was tested in outbred mice that were immunized with antigens together with Freund's adjuvant in two experiments. Results from the first experiment revealed (46%) protection level in the total worm burden. There was no significant protection noted in the second experiment Chacón *et al.,* 2000).

A study done on BALB/c mice immunized with soluble proteins from foot and digestive gland of the intermediate host snail and challenged with *S. mansoni* by Kuria *et al.,* (2012) showed protection of 50% of the group immunized with the digestive gland.

Kobia *et al*., (2011), immunized Swiss white mice with two soluble proteins, DG (antigens from digestive gland of intermediate host, *Biomphalaria pfeifferi*) and RT (antigens from the rest of the tissues of intermediate host, *Biomphalaria pfeifferi*). Worm reduction in RT was 60.5% while that of DG was 43.3%. The present study tested the two snail proteins, DG and RT in the olive baboon, *Papio anubis*. The olive baboon is phylogenetically closer to man than the mouse. It has been established as a model for schistosomiasis vaccine, based on work carried out at the Institute of Primate Research (Yole *et al.,* 1996; Kariuki *et al.,* 2004).

3 Materials and Methods

3.1 Definitive host – Olive baboons

Olive baboons (*Papio anubis*) are permissive definitive hosts of *S. mansoni*. Wild caught juvenile baboons from the Institute of Primate Research (IPR) Animal Science Department were used in this study. After capture, they were quarantined for three months, dewormed and tested for tuberculosis (TB). They were fed with nutrient pellets (Laboratory Cho from Unga Feeds ® CO.) and supplemented with vegetables and fruit. Water was supplied *ad libitum.* During the experiment they were caged in pairs as an animal welfare requirement for companionship. Three animals (3) were allocated per group. Baboons are non-human primates and therefore ethically the minimum number for this study was three as per the 3 RRRs (replacement, reduction and refinement) (Russel *et al.,* 1959). Ethical approval to carry out the study with 3 animals per group was obtained from the IPR Institutional Review Committee (IRC). The committee is guided by International standards of animal welfare. The measured effect/ parameters included the number of worms recovered. From the previous

studies, about 450 worms are recovered per infected baboon. This means that for 3 baboons per group, an average of 1350 worms were expected to be recovered. Statistically this is acceptable and would provide the required statistical power for significant comparisons.

3.2 Intermediate host – Snails

Biomphalaria pfeifferi snails were collected from canals in Mwea Irrigation scheme in Kirinyaga County, Kenya, by scooping them out of water using a scoop with a long wooden handle. The snails were carried in plastic containers lined with damp cotton wool and transported to the snail laboratory at the IPR where they were screened for schistosomes under strong light (100 watts) for two hours for five consecutive weeks. Those that were negative were housed in the temperature controlled (25-27°C) snail room. Plastic tanks were washed thoroughly with tap water. Sand and gravel sterilized by heating at 150°C for 12 h was cooled and layered in the plastic tanks. Plastic tanks were three quarter filled with IPR tap water (chlorine free water from the IPR well). The screened snails were transferred into the tanks for maintenance as described by Yole (Yole *et al*., 1996). Water was changed twice a week. Soft lettuce (steamed and dried in oven) was added to feed the snails.

3.3 Preparation of RT and DG from the S. mansoni intermediate host Biomphalaria pfeifferi

Two hundred colony-bred (F2 and F3) *B. pfeifferi* snails were dissected under the dissecting microscope to extract the digestive gland leaving behind the rest of the snail tissues. The digestive gland and the rest of the snail tissue were placed in separate Nunc tubes containing a small volume of phosphate buffered saline (PBS). They were placed on ice. The tissues were crashed in a glass mortar with a pestle to obtain homogenates which were centrifuged in a microfuge for 1 h at 14,000/rpm at 4°C to obtain the soluble protein. The concentration of the protein was assayed using Bradford method (Bradford, 1976). Protein concentration was adjusted to 1mg/ml. The soluble protein was aliquoted and sterilized by exposure of UV light (10 minutes, 5cm from a 30 watt ultra violet OSRAM bulb). The aliquots were stored at -20°C.

3.4 Immunization of baboons

Baboons received the first immunization at the beginning of the Study (-9 wk). 1 ml of DG and RT antigens were placed in separate nunc vials. 1 ml of Montanide-ISA 51 adjuvant (Cytrx Corporation, Los Angeles CA), was drawn by the syringe and added to 1000 µg of the specific antigen in the nunc vial. The mixture was vortexed for 30 minutes until an emulsion was obtained. DG and RT groups were anaesthetized with mixture of Rompun and Ketamine 20:1 at 0.1 ml/kg body weight. The two experimental groups, RT and DG, were each injected on each of the two legs in the quadriceps muscles just above the knee with the prepared antigens intra-muscularly. Three and six weeks after the initial vaccination, DG and RT groups of baboons were boosted with 500 µg of their specific antigens in 1 ml of Montanide-ISA 51 adjuvant.

3.5 Challenge of baboons

The experimental groups, DG and RT, and a control group, IC, were anaesthetized with mixture of Rompun and Ketamine 20:1 at 0.1 ml/kg body weight and infected as described by Yole (Yole *et al.,* 1996). The groin area was shaved and pegs were used to create a pouch at the groin area. Wet cotton wool was used to moisten the pouch at the groin area, to enable easy penetration of cercariae. A suspension containing 600 live cercariae was dispensed in the groin pouch using 1 ml micropipette. A period of 30 minutes was allowed for cercariae to penetrate.

3.6 Perfusion and adult worm recovery

At week 6 post-challenge, baboons from each group (DG, RT and IC) were perfused to recover the adult worms. Each baboon was anaesthetized with mixture of Ketamine and Xylazine 20:1, 0.1 ml/kg body weight. Each baboon was also euthanized using a mixture of 1ml heparin (anticoagulant) in 4 ml of sodium pentobarbitone (Euthatol, May and Baker Ltd, UK). The abdomen of each baboon was opened up. The hepatic portal vein was incised. Perfusion catheter containing perfusion fluid (0.85% Sodium chloride and 1.5% Sodium nitrate) was inserted in the aorta and perfusion carried out until the liver and mesenteries were clear. The perfusate was collected in a 10 litre bucket. The perfusate was sieved using 180µm sieve into a tray. The worms on the sieve were then transferred into petri dish containing phosphate buffered saline (PBS) and then counted. Worm maturation, percentage worm recovery and reduction for each group were calculated as shown in the formulae below.

$$\text{Worm maturation} = \frac{\text{(Number of worms recovered in infected control)}}{\text{(Initial number of infecting cercariae)}} \times 100\%$$

$$\text{Percentage worm recovery} = \frac{\text{(Mean of total worms in experimental group)}}{\text{(Mean of total worms in infected control)}} \times 100\%$$

% worm reduction
=
(Mean of total worms from the infected control – (the mean of total worms from the experimental group)* 100%
(The mean of total worms from the infected control)

3.7 Gross Pathology
Gross pathology examination was done before perfusion. It focused on the general and overt appearance of the liver. The observations on the liver were inflammation, adhesions and granulomas. Granulomas appear as raised pinheads sized foci distributed over the surface of the liver lobes. 1-3 granulomas per lobe were considered few, 4-10 granulomas per lobe were considered moderate and ≥ 10 granulomas per lobe was considered severe.

3.8 Histopathology
After perfusion, liver tissue samples were obtained and were fixed immediately in 10% buffered formalin for two weeks. The fixed tissue samples of liver sections were transferred into tissue cassettes and immersed into 80%, 95% and 100% ethanol consecutively to achieve optimum dehydration. The tissues were cleared in toluene and infiltrated in hot paraffin. They were embedded on tissue- embedding paraffin wax (Sherwood Medical Co. USA). The tissues were then sectioned serially at 6 microns using a Rotary microtome (Leitz, Germany). The thin tissue sections were mounted on glass slides and stained with Haemotoxylin and Eosin. They were observed under the light microscope and only granulomas containing an ovum at the centre were enumerated and measured at x100 magnifications using calibrated ocular micrometre. The averages of the horizontal and vertical diameter were taken to be the granuloma diameter (Farah *et al.,* 2000). A total of 10 granulomas per sample were measured. Histological liver abnormalities such as fibrosis and periportal infiltration were also noted. Photomicrographs of the thin tissue sections with granuloma and other liver anomalies were taken at x400 using a microscope (Leica ICC 50).

3.9 Statistical analysis
Data analysis was done using t- test and ANOVA test. T-Test was used to find out whether there were variances in worm recovery between DG and RT. ANOVA was performed with the help of SPSS to determine whether there were differences in worm recovery among DG, RT and IC. T-Test is used to compare two means while ANOVA is used to compare more than two means. Significance level/probability level used in calculation was $p<0.05$

4 Results
Baboons in DG group were immunized with antigen derived from the digestive glands of the intermediate host then infected with *S. mansoni*. Baboons in RT were immunized with antigens derived from the rest of the body tissues of the intermediate host and then infected with *S. mansoni*, while baboons in IC group were infected with *S. mansoni* only, i.e. the infected control. The mean number of *S. mansoni* worms that were recovered from baboons in the three groups, percentage worm recovery and percentage worm reduction are shown in Table 1. Worm maturation was 78.8%. The mean number of worms for RT group was 443±10.69, DG group 418 ± 11.37 and IC was 473 ± 7.51. This indicates that DG had a lower mean number of worms than the other vaccinated group, RT and also the infected control. The percentage worm reduction was 11.4% in DG while in RT it was 6.1%.

DG had significantly lower mean worm reduction than RT ($p<0.05$). There was no significant difference in the mean worm reduction between RT and IC ($p>0.05$). However, there was a significant difference in the mean worm reduction between DG and IC ($p<0.05$).

Treatment Groups	Mean number of worms (mean, s.e)	% worm recovery	% worm reduction
DG	418±11.37	88.5	11.4
RT	443±10.69	93.8	6.1
IC	473±7.51		

Table 1: Worm recovery and reduction in baboons immunized with soluble proteins from *Biomphalaria pfeifferi* and challenged with *Schistosoma mansoni*

Key: DG-Baboons immunized with soluble proteins from the digestive gland of the intermediate host and then challenged**; RT-** Baboons immunized with soluble proteins from the rest of the body tissues of the intermediate host and then challenged; **IC**-Infected control.

4.1 Gross pathology
Gross pathology was done by physical observation of the liver surface to detect adhesions; inflammation and presence of granulomas. In DG, adhesions were absent while in RT they were present in only one member.

However, adhesions were observed in all the members of IC. Inflammation in the liver was categorized as no inflammation, moderately inflamed and severely inflamed. In DG, two animals had moderate inflammation and the other one had no inflammation. In RT, all the baboons had moderately inflamed livers. In the IC, two baboons had severe inflammation while one had moderate inflammation. 1-3 granulomas per lobe were considered few, 4-10 granulomas per lobe were considered moderate and ≥ 10 granulomas per lobe was considered severe. In DG, two animals had moderate granulomas while one had few granulomas. In RT, all the baboons had moderate granulomas. In IC, two baboons had severe granulomas while one had moderate granulomas.

4.2 Histopathology

Figure 1A shows the granuloma sizes in the three groups. Among the three groups, DG had the average granuloma size of 25.7 ± 0.82 µm followed by the RT group whose average granuloma size was 28.57 ± 1.87 µm. The IC had the largest average granuloma size of 34.7 ± 3.54 µm. The average granuloma size for DG and RT were similar $p>0.05$. There was a statistical significant difference between the two immunized groups and the IC $p<0.05$.

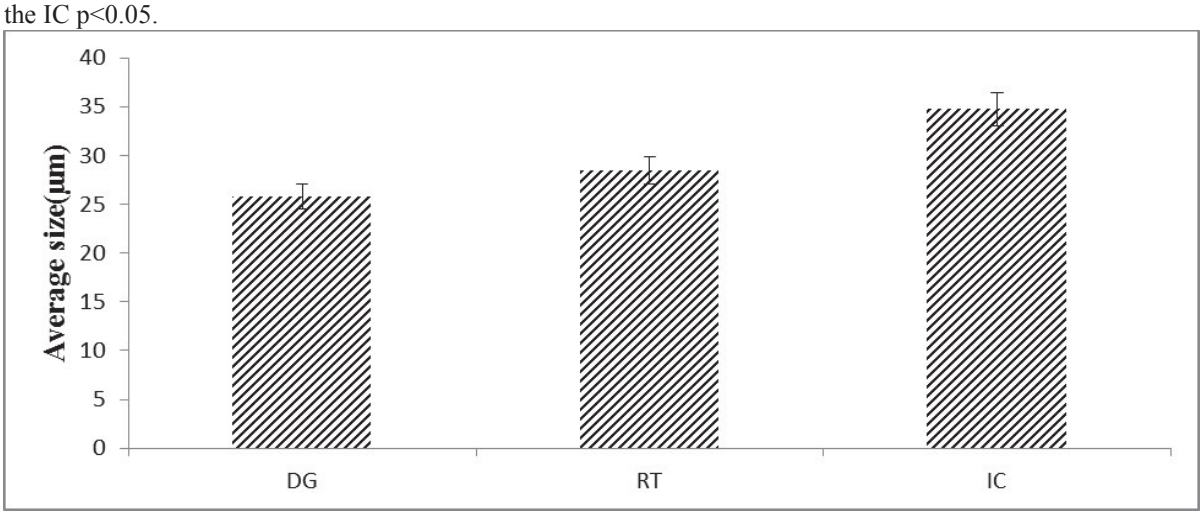

Figure 1A: Granuloma Size in Baboons Immunized with snail soluble Proteins and Challenged with *Schistosoma Mansoni*

Key: RT- Baboons immunized with snail soluble proteins derived from the rest of the body tissues and then infected; **DG-**Baboons immunized with snail soluble proteins from the digestive gland and then infected**; IC-** Infected control

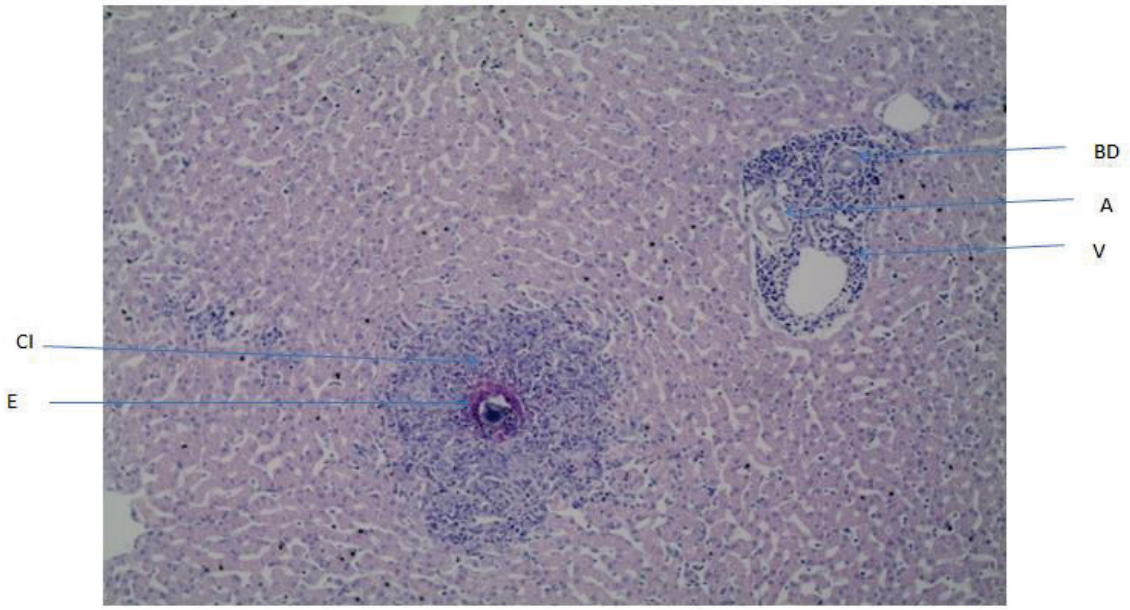

Figure 1B: A section (x 400) from RT showing a centrally placed *S. mansoni* egg and a portal triad with infiltrated bile duct

KEY: A-Artery BD-Bile duct E-Egg CI-Cellular infiltration V-Vein

5 Discussion

World tropical disease load caused by schistosome infections and geohelminthes with the exclusion of malaria is above 40% (WHO, 2014). Baboons in DG group were immunized with soluble proteins derived from the digestive glands of the intermediate host then challenged with *Schistosoma mansoni* cercariae while Baboons in RT were immunized with soluble proteins derived from the rest of the body tissues of the intermediate host and then challenged with *Schistosoma mansoni* cercariae. The baboons in IC group were infected only, i.e. the infected control. The dose used to challenging baboons was 600 *Schistosoma mansoni* cercariae. In the IC, 473 of the infecting cercariae were recovered as adults, a worm maturation of 78.8%. This compares well with a worm maturation of 80% in olive baboons (*Papio anubis)* as previously reported (Yole *et al*., 1996).

The mean number of worms for DG was significantly lower ($p<0.05$) than the other immunized group, RT and also the infected control. The percentage worm reduction for DG was 11.4% while in RT it was 6.1%. These results indicate that DG was efficacious against *S. mansoni* while RT was not. The results from this study agree with a study done on BALB/c mice that were immunized with soluble Proteins from foot and digestive gland of the intermediate host, *Biomphalaria pfeifferi*, and challenged with *S. mansoni* by Kuria *et al.,* (2012). The results from the mice study showed protection of 50% of the group immunized with the digestive gland.

In work done by Kobia *et al*., (2011), Swiss white mice were immunized with soluble proteins from the digestive gland and from the rest of the tissues of intermediate host, *Biomphalaria pfeifferi*. Worm reduction in RT was 60.5% while that of DG was 43.3%. In these two mice studies, DG was efficacious against *S. mansoni* and this is in agreement with the present baboon study. However, the protection was higher in the mouse model compared to the baboon, most probably because of genetic differences.

DG was efficacious in this study with a protection level of 11.4%. Since the baboon is phylogenetically closer to man than the mouse, there is a high probability of DG being protective in man. However the protection level is below the World Health Organization goal of 40% protection (WHO, 1996). Further work should be done to find out how the efficacy of DG can be increased, for example using more refined protein like molecules from the digestive gland.

Gross pathology

Baboons immunized with DG soluble proteins had least gross pathology, in terms of reduced number of granulomas, inflammation and adhesions, followed by those immunized with RT soluble proteins and then those of the IC. Therefore the DG baboons were better protected than RT.

Histopathology

Granulomas are areas of cellular infiltration consisting mainly of eosinophils, macrophages, fibroblasts, and lymphocytes surrounding a tissue-trapped schistosome egg (Hagan *et al*., 1998). Florid granulomas with a centrally placed conspicuous egg at the centre are characteristic of the acute phase of infection of *Schistosoma mansoni.*

In DG and RT, the average granuloma sizes were similar ($p>0.05$). However, there was a significant reduction in granuloma sizes between the two immunized groups and the IC ($p<0.05$). Murine studies showed that larger lesions are detrimental while smaller ones are an ideal compromise between egg-sequestration and tissue pathology (Hagan *et al*., 1998). Observations from this study showed that liver tissues from the DG and RT group had mild cellular infiltration whilst those from the IC had intense cellular infiltration as results of diffusing egg antigens (Hagan *et al.,* 1998).

Conclusion

DG was efficacious against *Schistosoma mansoni.* Baboons immunized with DG had a protection level of 11.4 % while that of RT was 6.1%. DG immunized baboons had least gross pathology, in terms of having a reduced number of granulomas, inflammation and adhesions compared to RT immunized baboons and IC. However, RT demonstrated less pathology than IC. Since the baboon is phylogenetically closer to man than the mouse, there is a high probability of DG being protective in man. Further work should be done to find out how the efficacy of DG can be increased, for example using more refined protein like molecules from the digestive gland.

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REFERENCES

1) Bradford M. M (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of

Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry* 72(1-2):248-254.
2) Chacon, N. (2000). Common Antigens between Different Species of *Schistosoma* and their Intermediate Hosts. *Southeast Asian Journal of Tropical Medical and Public Health*, 25(3):509-515.
3) Christinet V, Lazdins-Helds JK, Stothard JR, Reinhard-Rupp J. (2016). Female genital schistosomiasis (FGS): from case reports to a call for concerted action against this neglected gynaecological disease. *Int J Parasitol.;* 46(7):395–404.
4) Cioli D, Pica-Mattoccia L, Basso A, Guidi A. (2014). Schistosomiasis control: Praziquantel™ forever? *Mol Biochem Parasitol.* 195(1):23–9.
5) Farah, I.O., Nyindo, M., King, C.I., and Hau, J., (2000). Hepatic granulomatous response to Schistosoma mansoni eggs in BALB/c mice and Olive baboons (*Papio cynocephalus anubis*). *Journal of Comparative Pathology*. 123, 7-14.
6) Gryseels B, Polman K, Clerinx J, Kestens L (2006). Human Schistosomiasis.*Lancet*: 1106–1118.
7) Hagan P; Ndhlovu, P.K. and Dunne, D.W (1998). Schistosome immunology: More question than answers. *Parasitology Today* 14: 407-411.
8) Kariuki TM, Farah IO, Yole D. S (2004). Parameters of the attenuated Schistosome vaccine evaluated in the olive baboon. *Infection and Immunity* 2004; 72: 5526–5529.
9) Kobia, E.N., Ochanda, H. and Yole, D.S. (2011). The effect of immunizing Swiss mice with soluble proteins from the intermediate host Biomphalaria *pfeifferi* and challenging them with *schistosoma mansoni*. *Internet journal of Tropical and Infectious Diseases.*
10) Kuria Kennedy M. 1; Rebecca Waihenya; Hellen Kutima; Peninnah Njoki and Dorcas S. Yole (2012). Cellular responses against *Schistosoma mansoni* in immunized Balb/c mice with soluble proteins from intermediate host, *Biomphalaria pfeifferi*. *Tanzania Journal of Health Research Doi: http://dx.doi.org/10.4314/thrb.v14i4.7 Volume 14, Number 4,*
11) Merrifield M, Hotez PJ, Beaumier CM, Gillespie P, Strych U, Hayward T (2016). Advancing a vaccine to prevent human schistosomiasis. *Vaccine*. 34(26):2988–2991.
12) Morais C.N., J.R. Souza, W.G. Melo, M.L. Aroucha, P. Miranda, A.L. Domingues (2008). Cytokine profile associated with chronic and acute human *Schistosomiasis mansoni. Mem Inst Oswaldo Cruz,* 103: 561–568
13) Russel W. M. S & Burch R. L. (1959). The principles of human experimental technique. Methnen, London, ISBN.
14) Siddiqui AA, Siddiqui BA, Ganley-Leal L. (2011). Schistosomiasis vaccines. *Hum Vaccin*. 7(11):1192–1197.
15) Stadecker M.J, H. Asahi, E. Finger, H.J. Hernandez, L.I. Rutitzky, J. Sun (2004). The immunobiology of Th1 polarization in high pathology schistosomiasis. *Immunol Rev*, 201 pp. 168–179.
16) Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J (2006). Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. *Lancet Infect Dis*.; 6:411–425.
17) Weston D, Allen B, Thakur A, LoVerde PL, and Kemp WM (1994). Invertebrate host-parasite relationship: convergent evolution of a tropomyosin epitope between *Schistosoma* sp., *Fasciola hepatica* and certain pulmonate snails. *Exp Parasitol 78*: 269-278.
18) World Health Organization (1996) World Health Organization Fact Sheet 115.
19) World Health Organization (2015). Schistosomiasis. *In: Schistosomiasis Fact Sheet No 115.* http://www.who.int/mediacentre/factsheets/fs115/en/.
20) World Health Organization. (2014). WHO schistosomiasis fact sheet. Available from: http://www.who.int/mediacentre/factsheets/fs115/en
21) Yole, D. S., Pemberton. R. Reid, G. D. and Wilson, R. A. (1996). Protective immunity to *Schistosoma mansoni* induced in the Olive baboon, *Papio Anubis*, by the irradiated cercariae vaccine. *Parasitology*. 112, 37-46