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NEW APPROACH FOR DETERMINATION OF SPECIFIC TREMATODA SNAIL INTERMEDIATE HOSTS

By
MOHAMED M. EL-BAHY[1]*, AND NANCY O. KAMEL[2]
Department of Parasitology, Faculty of Veterinary Medicine, Cairo University[1], and Department of Parasitology, Faculty of Medicine, October 6 University[2], Giza, Postal code: 12585, Egypt. (*Correspondence: drmelbahy@yahoo.com)

Abstract

The compatible miracidium is able to inhibit without stimulate the immune defense mechanism of its snail. So, snail failed to recognize it as foreign body. A level of antigenic similarity must be present between the miracidia and its natural snail intermediate host (IH). The present study investigated the level of compatibility or difference in molecular structures between miracidia and their specific snails IH using protein fractionation and western blot (EITB). Specific anti-snail rat hyper immune sera (RHIS) were obtained by rat vaccination. This RHIS was reacted versus different miracidial antigens (Ag) to determine the compatibility level between any miracidium and the target snail Ag using EITB.

The results showed five polypeptides bands at molecular weight (MW) of 100kda, 60kda, 50kda, 45kda & 35-38kda, common between *S. mansoni* miracidium Ag and *B. alexandrin*a feet Ag. Three fractions at MW of 58kda, 38-42kda & 22-24kda were recognized in fractionated *F. gigantica* miracidium versus anti-*L. natalensis* Ab. Six polypeptide bands at MW of 48-50kda, 38-40kda, 30kda, 25 kda, 22.5kda & 15kda were recognized between *E. revolutum* miracidia and *P. acuta* fractionated feet Ag. There was no polypeptide bands between any miracidium and snail other than its specific one.

Key words: *S. mansoni*, *F. gigantica*, *E. revolutum*, miracidia, *B. alexandrina, L. natalensis*, *P. acuta*, EITB, Compatibility.

Introduction

Snail intermediate hosts play effective roles in the transmission of snail-borne trematode infections worldwide. A good knowledge of snail-borne diseases epidemiology particularly snail intermediate host populations would provide the necessary motive to complementing actual control strategy. Thus, to determine the snail IH for new trematode parasites, trials to infect several laboratory breed snails were performed using miracidia of this trematode. This usually followed by dissection of each snail to determine the occurrence of successful infection (Brown, 1994; Lockyer *et al*, 2008). This method considered very expensive, high coast, time consuming and need special equipment and experiences. With the progress in immunological techniques concerning antigen fractionation and specificity, several authors were demonstrate similarity between each snail and its domestic parasites (Chacon *et al*, 2002; Taha *et al*, 2013) as the presence of common antigens between *Fasciola gigantica* (*F. gigantica)* rediae (ten fractions) and *Lymnaea natalensis* , as well as between *Fasciola* and *L. natalensis*. Also, Abd El Aal *et al.* (2016) showed the presence of common polypeptide bands at MW of 22, 30 & 58 KDa between *Schistosoma mansoni* worm & *Biomphalaria alexandrina* visceral antigens. So, the compatible miracidium was able to inhibit without stimulating snail immune defense mechanism and snail failed to recognize it as foreign body. Penetration of miracidium into snail other than its specific IH stimulated a variable level of defense of snail's cellular and tissue level, but the specific miracidium into its domesticated snail host did cause any response (Iwanaga, 1994). On the contrary, penetration of miracidium to abnormal host snail, stimulate gradual developed tissue and cellular (amoebocytes) response. Amoebocytes during 24-48h were filtrated around the invading miracidium and encapsulated them by fibroblasts to destroy it (Yoshino and Bayne 1983). Dissous and Capron (1989) reported that *S. mansoni* miracidium possessed surface determinants antigenically similar to *B. glabrata* hemolymph macromolecules, and that the protein fractions of

43 & 39 kda of *S. mansoni* miracidia reacted positively with rabbit anti-*B. glabrata* Ab.

El-Bahy *et al.* (2017) reported that there must be special behaviors between snail immune system versus its domestic miracidia, as *S. mansoni*, *F. gigantica* and *Echinostoma revolutum* miracidia penetrated and developed successfully in *B. alexandrina, L. natalinsis* and *Physa acuta*, respectively. The miracidia migrated freely to develop in snail hepato-pancreas without being recognized by the immune system. They concluded that the miracidia failed to develop in snail other than their specific IH, indicating antigenic similarity between them and their specific snail IH.

The present study aimed to investigate the level of compatibility or difference in molecular structures between miracidia of trematode parasites and their specific snails intermediate host using protein fractionation and EITB, as an easily method to determine snail suspected to act as IH of some trematodes.

Materials and Methods

Ethical approval: All study steps and procedures were approved by the Institutional Animal Care and Use ethical Committee (CU-IACUC), Cairo University. This study was conducted from August to November 2018 in the laboratory of Parasitology, Cairo Faculty of Veterinary Medicine.

Fasciola gigantica miracidia were recovered after incubation of clean eggs collected from naturally infected gal-bladders of freshly slaughtered buffaloes at Cairo Governmental Slaughterhouse. *E. revolutum* eggs were recovered after 6hr incubation of mature identified worms extracted from caeca of naturally infected ducks. The eggs were washed, sieved, collected using Fluke finder (Welch *et al,* 1987), and incubated at 28ºC as thin layer in clean Petri-dish under surface of water with Penicillin G sodium$^{(R)}$ (100U) and Streptomycin$^{(R)}$ (100 lg) per ml. in dark (Hussein *et al*, 2010). *S. mansoni* eggs with active miracidia were purchased from Theodor Bilharz Research Institute, Giza. Eggs were hatched in a darkened cylinder (500ml) containing warm (30ºc) tap water. Hatched miracidia migrated to the top lighted surface of the water were collected using suitable pipette, transferred into clean test tubes, and left for an hour in refrigerator. Miracidia were collected after centrifugation at 1500rpm.15min. by removal of supernatant fluid (Theron and Coustau, 2005).

Miracidial antigens: The sedimented miracidial masses were sonicated in 0.01 M phosphate buffered saline (PBS), PH 7.4 (PBS) for 5min. under 150 watt interrupted pulse output at 50% power cycle using a sonifier cell disrupter. The sonicated materials were centrifuged at 10.000rpm for an hour under cooling. The supernatant was concentrated in 6-8 Kda dialysis tubes against poly vinyl pylorridone and the concentrated soluble miracidial Ag were collected to measure protein content (Lowry *et al*, 1951) and stored at -70^{o}C until used. Snail antigens:

Field-collected *L. natalensis* (IH for *F. gigantica), B. alexandrina* (IH for *S. mansoni)* as well as *P. acuta* (IH of ducks flukes *E. revolutum*) were collected from the water bodies at Abou-Rawash, Giza and identified (Brown, 1994). Clean laboratory breed Albino rats were produced after cultivation of the newly deposited snail egg-masses in the laboratory (El-Bahy *et al*, 2014). Soluble crude antigens (Ag) were prepared from the feet of one-month-old laboratory breed parasite-free snails (Khalil *et al*, 1985). After crushing the selected snails, in sterile PBS, their feet were separated from its visceral hump tissue, washed several times, homogennized in an equal amount of 0.01M PBS, pH 7.4 sonicated and then centrifuged as mentioned. The supernatant was dialyzed in 6-8 KDa dialysis tubes overnight at 4^{o}C against 4 M urea buffer (Basyoni and Abd El-Wahab, 2013), concentrated and its protein content was estimated and stored as before.

Reference rat hyper-immune serum (RHIS): After Langley and Hillyer (1989), RHIS were raised against the 3 tested snails Ag (*L. nataliensis, B. alexandrina* & *P. acuta*). Rats were initially injected S/C and followed by

three consecutives intramuscularly injections in an equal amount of mineral oil at one week interval. One week after last injection the antibodies level in sera of the immunized rats was evaluated. The rats were sacrificed and sera were collected. Blood samples were collected from these rates before vaccination to raise sera used as negative control.

Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot (EITB) techniques: **T**hree prepared snail feet and miracidia Ag were fractionated using 12% SDS-PAGE (Laemmli, 1970) associated with low molecular weight standards (MWS) (Pharmacia Biotech). After Comassi blue staining of the first prepared gel piece another one was prepared for each antigen type and its containing protein fractions were transferred onto nitrocellulose (NC) sheet for western blot technique (Towbin ***et*** *al*, 1979). Four mm width NC strip represent fractions of each antigen was arranged in groups of 4 strips each, associated with one of MWS. The 1st strip with fractionated *B. alexandrina* Ag, 2nd strip with fractionated *S. mansoni* miracidia, 3rd strip with frac-tionated *F. gigantica* miracidia, and 4th strip with fractionated *E. revolutum* miracidia. Four strips treated in the same time by *B. alexandrina* RHIS RHIS using EITB (Figs. 1, 2, 3 & 4).

NC strips with t three fractionated miracidia and 1st strip with fractionated *L. natalensis* feet Ag, and group treated by *L. natalensis* RHIS using EITB. Also, the NC strips the three fractionated miracidia and the 1st strip with fractionated *P. acuta* feet Ag, and the group treated using *P. acuta* RHIS sera using EITB. The polypeptides reacted under the same condition in snail and any of the other 3 miracidial fraction was considered snail and miracidia related. Others without combination between snail and its target miracidia were non-relat-ed fractions (Pharmacia Medical Company, Cairo, Egypt)

Results

Staining of the produced gel piece using comassi blue stain (Fig. 1) showed the presence of a structural homology between the snail and its compatible miracidium. From ten protein bands recorded corresponding to MWS at the level of 18-150 kda in fractionated *B. alexandrina* feet Ag three fractions at MW of 50-55 kda, 38-40 kda & 22 kda were in fractionation of domestic *S. mansoni* miracidia (Fig. 1, lanes 1 & 2,). Also, *L. natalensis* showed 6 fractions at MWS of 15-150 kda, three fractions of them at MWS of 58kda, 25kda & 20kda were recorded in the domestic miracidia of *F. gigantica* (lanes 3 & 4). From six fractions recorded in *P. acuta* in range of 18-120 kda, three fractions were recorded in the domestic miracidia of *E. revolutum* corresponded to MWS at 55-58 kda, 23kda & 18kda (lanes 5 & 6).

The similarity between different miracidial fractions and that of each snail species, fractions of each miracidium on NC strips reacted versus anti-snail Ab developed after vaccination of rats by snail feet Ag via EITB.

Different fractions reacted positive with anti-*B. alexandrina* Ab in RHIS vaccinated by *B. alexandrina* feet Ag. Five polypeptides corresponded to MWS at 100kda, 60 kda, 50kda, 45kda & 35-38kda were common reacted bands between *S. mansoni* miracidium Ag and *B. alexanderina*. No common polypeptide bands were between same snail and miracidial Ag of *F. gigantica* or *E. revolutum*. Miracidia were unable to develop in *B. alexandrina* (Fig. 2, Tab. 1).

Three broad polypeptide fractions at MW of 58 kda, 38-42 kda & 22-24 kda were recognized in *F. gigantica* miracidium Ag similar to those bands in *L. natalensis* fractionated feet Ag after injecting both NC strips by RHIS with anti-*L. natalensis* Ab. One fraction at 23 kda and another one at 25 kda were recognized in fractionated *S. mansoni* and *E. revolutum* miracidium respectively after injecting NC strips by anti-*L. natalensis* Ab with rat sera vaccinated by *L. natalensis* feet Ag (Tab. 2, Fig 3).

Treatment of NC strips containing *E. revolutum* miracidium fractions versus anti-*P. acuta* Ab in RHIS vaccinated by *P. acuta*

feet Ag showed six similar polypiptied bands between these miracidia and their snail IH. These fractions corresponded to MW of 48-50kda, 38-40kda, 30kda, 25kda, 22.5kda &15kda. Only two fractions at MW of 22.5kda & 15-18kda were positive in fractionated *S. mansoni* miracidia and another two fractions at 30-38kda & 22-25kda were in fractionated *F. gigantica* miracidia after injection with corresponded NC strips by anti-*P. acuta* Ab prepared after rat vaccination with snail Ag (Fig. 4, Tab. 4).

Table 1: Specific reacted protein fractions in different miracidia versus *B. alexandrina* HIS using EITB

Kda fractions reacted on NC strip contain Ag of			
B. *alexandrina* foot	S. *mansoni* miracidia	F. *gigantica* miracidia	E. *revolutum* miracidia
150 kda	100 kda	70 kda	70 kda
100 kda	60 kda	58 kda	58 kda
60 kda	50 kda	55 kda	
50 kda	45 kda	40 kda	35 kda
35-48 kda	35 -38 kda	20 kda	10 kda
26 kda	30 kda	15 kda	
25 kda	18-25 kda		
18-23 kda			

Table 2: Specific reacted protein fractions in different miracidia versus *L. natalensis* HIS using EITB

Kda fractions reacted on NC strip contain Ag of			
L. natalensis foot	*F.gigantica* miracidia	*S.mansoni* miracidia	*E. revolutum* miracidia
50-58 kda	100 kda	100-150 kda	70 kda
42 kda	80 kda	50 kda	50 kda
38 kda	70 kda	40 kda	38 kda
30 kda	58 kda	33 kda	32 kda
23 kda	38-42 kda	23 kda	25 kda
20 kda	22-24 kda	10 kda	
12 kda	15 kda		

Table 3: Specific reacted protein fractions in different miracidia versus *P. acuta* HIS using EITB

Kda fractions reacted on NC strip contain Ag of			
P. acuta foot	*E.revolutum* miracidia	*S.mansoni* miracidia	*F.gigantica* miracidia
48-50 kda	100 kda	150 kda	80 kda
38-40 kda	80 kda	90 kda	50-55 kda
30 kda	70 kda	55 kda	30-38 kda
25 kda	60 kda	52 kda	22-25 kda
22.5 kda	48-50 kda	40 kda	20 kda
15 kda	38-40 kda	22.5 kda	18 kda
	30 kda	15-18 kda	
	25 kda		
	22.5 kda		
	20 kda		
	15 kda		

Discussion

Common antigens relation between snails and trematode parasites were reported (Iwanaga, 1994: Taha *et al,* 2013; Abdel Aal *et al,*. 2016). Compatible miracidium did not stimulate the immune defense mechanism of its specific snail intermediate host that failed to recognize this miracidium as foreign body (El-Bahy *et al,* 2017). Consequently, antigenic similarity level must be present between the miracidia and its natural snail IH.

In the present study, electrophoretic profile of *S. mansoni* Ag compared with *B. alexandrina*, and *F. gigantica* compared with *L. natalensis* and miracidia Ag of *E. revolutum* compared with *P. acuta* using SDS-PAGE was illustrated. Comassi blue stained gel piece showed that three fractions at MW of 50-55kda, 38-40kda & 22kda, were common between *S. mansoni* miracidia and *B. alexandrina* Ag. Also, three fractions at MWS of 58kda, 25kda & 20kda were common be-

tween *F. gigantica* and *L. natalensis* feet Ag. Another three fractions of MWS at 55-58kda, 23kda & 18kda were recorded between *E. revolutum* and *P. acuta*.

Ag compatibility degree versus NC strips with fractionated miracidial Ag showed five polypeptides at MWS of 100-kda, 60kda, 50 kda, 45kda & 35-38kda as common bands between *S. mansoni* miracidium Ag and *B. alexandrina.* These agreed with Sulahian *et al.* (2005) and Abd el Aal *et al.* (2016).

Treatment of fractionated *F. gigantica* miracidium on NC strips versus anti-*L. natalinsis* Ab in RHIS showed three broad fractions at MW of 58kda, 38-42kda & 22-24kda similar to corresponding bands in *L. natalensis* fractionated foot Ag. Also, six polypiptied bands at MW of 48-50kda, 38-40kda, 30kda, 25kda, 22.5kda & 15kda were common between *E. revolutum* miracidia and *P. acuta* fractionated feet Ag. Specific fractions were between the miracidia and their natural snail IH agreed with Dissous and Capron (1989) and Chacon *et al.* (2002).

Chacon *et al.* (2002) reported band fractions at MW of 85, 54, 45, 34, 28 & 22kda common bands between *L. natalensis* feet and *F. gigantica* miracidial antigens. At MW of 78, 54 & 45kda were common specific bands between *B. alexandrina* feet and *Paramphistomum microbothrium* (cattle rumen fluke) miracidial antigens. No doubt, miracidium is a difficult stage to get enough amounts for experimental study vaccination.

In the present study, no common polypeptide bands were between any miracidia and snail other than its natural snail IH. This agreed with Adema (2002) who found peptides from snail antigen against heterologous (foreign) miracidia hyper immune sera present enzymes as myeloperoxidase or fibrinogen-related proteins from foreign snails.

Conclusion

There is an antigenic similarity degree between each miracidia and its susceptible snail IH. This antigenic similarity let snail immune system to fail in identifying them as foreign body. For this reason, miracidia migrate freely inside its snail IH and complete its parthenogenic development. This finding is essential for prediction of snail IH suitable for a trematode of unknown life cycle.

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Explanation of figures

Fig. 1: Fractions of snails and its domestic miracidia using SDS-PAGE (Comassi-blue stained gel): Lane 1 = Fractionated *B. alexandrina* feet Ag, Lane 2 = fractionated *S. mansoni* miracidia Ag, Lane 3 = Fractionated *L. natalinsis* feet Ag, Lane 4 = fractionated *F.gigantica* miracidia Ag, Lane 5 = Fractionated *P. acuta* feet Ag. Lane 6 = fractionated *E. revolutum* miracidia Ag. MWS = Molecular weight protein standard

Fig. 2: Treatment of *B. alexandrina* fractionates feet Ag and different miracidial Ag versus *B.alexandrina* RHI using EITB: Lane 1 = Fractionated *B.alexandrina* snail feet Ag, Lane 2 = fractionated *S.mansoni* miracidia Ag, Lane 3 = fractionated *F.gigantica* miracidia Ag, Lane 4 = fractionated *E.revolutum* miracidia Ag, MWS = Molecular weight protein standard

Fig. 3: Treatment of *L. natalensis* fractionate feet Ag and different miracidial Ag versus *L. natalinsis* RHI using EITB: Lane 1 = Fractionated *L.natalensis* snail feet Ag, Lane 2 = fractionated *F. gigantica* miracidia Ag, Lane 3 = fractionated *S. mansoni* miracidia Ag, Lane 4 = fractionated *E.revolutum* miracidia Ag, MWS = Molecular weight protein standard

Fig. 4: Treatment of *P. acuta* fractionated feet Ag and different miracidial Ag versus *P. acuta* RHI using EITB: Lane 1 = Fractionated *P. acuta* snail feet Ag, Lane 2 = fractionated *E. revolutum* miracidia Ag, Lane 3 = fractionated *S. mansoni* miracidia Ag, Lane 4 = fractionated *F.gigantica* miracidia Ag, MWS = Molecular weight protein standard