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Evaluation of Antigen Preparations Derived from *Schistosoma japonicum* Eggs for Enzyme-Linked Immunosorbent Assay (ELISA)

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Introduction

The immunological response of the host to *Schistosoma japonicum* infection is not simple, as it seems to be provoked by the cercaria, adult worm, egg and their secretion and excretion substances in the course of the establishment of infection. Each of these seems to produce different reactions in the host; and various immunological diagnostic methods have been devised to detect some of the host responses, mostly in terms of immunoglobulins, circulating antigens or cellular responses instead of detection of the parasites or eggs *per se*, which is still a surer method of parasitological diagnosis (Sadun, 1976; Matsuda and Tanaka, 1982).

The quality of each diagnostic modality is assessed in terms of its sensitivity and specificity which are determined by the properties of the diagnostic methods and the nature of antigens used. The present paper aims at evaluating the specificity and reactivity of the carbonate buffer-extracted crude antigen derived from *S. japonicum* eggs using enzyme-linked immunosorbent assay (ELISA) against the sera of rabbits infected or immunized with several parasites, including schistosomes. The attempt was also made to seek ways to reduce cross reactions of the crude antigen by means of filtration, immunoaffinity chromatography and heating.

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Materials and Methods

Sera with antibodies against parasites: Sera were obtained from rabbits infected with *S. japonicum* (Yamanashi strain) or *S. mansoni* (Puerto Rican strain) or from those immunized with the homogenates of lyophilized worms or eggs. The animals were infected transcutaneously with various numbers of cercariae. Antigens for immunization were parepared by homogenizing lyophilized worms or eggs with a Teflon homogenizer in physiological saline at a concentration of 5 mg/ml. Each rabbit was immunized weekly with 1 ml of the antigen emulsified with Freund's complete adjuvant in a total of four subcutaneous injections, followed by another booster without adjuvant. Rabbits were bled one week after the last injection. Antisera from the other

parasites were prepared by the same procedure.

Preparation of antigens for ELISA: Antigens were prepared according to the methods described by Matsuda *et al.*, 1981. Lyophilized adult worms or eggs in carbonate buffer (0.05 M, pH 9.6) were homogenized in a Teflon homogenizer in an ice bath. The homogenate was kept at 4 C for 2 days while stirring for extraction. The supernatant was stored at −70 C until it was used as crude antigen. The protein concentration of the antigens was determined by the method of Lowry and Folin (1951). All the antigen preparations, unfractionated or fractionated, were used at a concentration of 10 μg/ml.

Enzyme-linked immunosorbent assay (ELISA): ELISA was performed on Microelisa plates (Dynatech, M129A) using anti-rabbit IgG goat antibody conjugated with peroxidase (Miles-Yeda) and o-phenylenediamine (OPD) as a substrate as previously described in detail (Matsuda *et al.*, 1981; Nakao *et al.*, 1981). The reaction was read by the absorbance at 500 nm using a microplate reader (Corona MTP-12). The titer was determined by the end-point of the reaction at twice the value of the absorbance of the pooled normal rabbit serum diluted at 1:40. In some cases, the absorbance was compared with that of normal serum at a single serum dilution of 1:80.

Gel filtration: Antigens were filtered on a Sephadex G-200 column (2×95 cm). Every 120 drops of effluent were collected into a test tube.

Affinity chromatography: Fractionation of *S. japonicum* egg antigen was performed by affinity chromatography in two steps.

a) Preparation of cross-reactive antibody by affinity chromatography: *S. japonicum* egg antigen, 6 mg, was used to couple to 2 g of CNBr-activated Sepharose 4B or Epoxy-activated Sepharose 6B and the antigen-Sepharose was packed into a 10 ml plastic syringe to be used for chromatography. Anti-*Fasciola hepatica* serum, 15 ml, was applied to the column and antibodies bound to the column were eluted by acetate buffer (pH 2.5) after extensive washing with phosphate-buffered saline (PBS) (pH 7.2). The eluate was neutralized with 0.1 N NaOH immediately after elution.

b) Preparation of antibody-coupled column: The cross-reactive antibody, 1.5 ml, obtained as described above was coupled to 1 g of Sepharose (CNBr- or Epoxy-activated) and approximately 6 mg of crude antigen was applied to the column. The materials bound to the column were eluted by acetate buffer (pH 2.5). The antigenicities of both materials bound and unbound to the column were evaluated by ELISA using anti-*S. japonicum* egg and anti-*F. hepatica* worm sera.

Heating of antigen: Antigen solution was heated in a water bath in test tubes with screw-tops to avoid evaporation.

Results

1. Cross Reactivity of Crude Antigens of Different Parasites

Reactions with sera from rabbits infected with S. japonicum or S. mansoni: The antibody titers of the sera from rabbits infected with *S. japonicum* or *S. mansoni* against eight different antigens are shown in Table 1. The crude *S. japonicum* egg antigen reacted strongly to the serum from rabbits infected with *S. japonicum* and even to that with *S. mansoni*. *S. mansoni* worm antigen reacted more poorly than the *S. japonicum* egg antigen to all the sera tested. Virtually no cross reaction was observed in schistosomal antibodies with antigens derived from *F. hepatica* or other non-schistosomal parasites, except occasional low reactions with antigen derived from *Clonorchis sinensis* or *Toxocara canis*.

Reactions with immune sera: Reactions of the sera from rabbits immunized with different parasite antigens to all the anti-

Table 1 ELISA titers of sera from schistosomiasis infections

Rabbit number	Number of cercariae	Sera collected in	Antigens							
			S. japonicum egg	*S. japonicum* worm	*S. mansoni* egg	*S. mansoni* worm	*F. hepatica* worm	*P. philippinensis* worm	*C. sinensis* worm	*T. canis* worm
1	1,000 (*S. monsoni*)	7th week	640	40	320	—	—	—	—	—
		12	640	80	320	40	—	—	—	—
		18	640	40	160	—	—	—	—	—
2	1,000 (*S. mansoni*)	7	2560	160	1280	—	—	—	—	—
		12	1280	80	640	80	—	—	40	—
		18	640	40	160	40	—	—	—	—
3	300 (*S. japonicum*)	7	5120	1280	1280	—	—	—	—	—
		12	5120	1280	5120	—	—	—	40	80
4	100 (*S. japonicum*)	7	1280	320	160	—	—	—	—	—
		12	5120	640	1280	—	—	—	—	—
		18	5120	160	320	—	—	—	—	—

ELISA title: reciprocal of the dilution of the serum with the smallest absorbance which is larger than twice the value of the absorbance of the normal control serum diluted at 1 : 40; (—) indicates titers less than 40.

Table 2 ELISA titers of immune sera

Immune sera \ Antigens	*S. japonicum* egg	*S. japonicum* worm	*S. mansoni* egg	*S. mansoni* worm	*F. hepatica* worm	*P. philippinensis* worm	*C. sinensis* worm	*T. canis* worm
S. japonicum egg	**5120**	320	5120	1280	1280	—	—	—
Anti-*S. japonicum* worm	2560	2560	320	**5120**	1280	160	80	160
Anti-*S. mansoni* egg	2560	40	**5120**	2560	—	—	—	—
Anti-*S. mansoni* worm	320	**5120**	1280	**5120**	1280	80	—	40
Anti-*F. hepatica* worm	1280	640	320	1280	**5120**	2560	640	160
Anti-*P. philpinensis* worm	—	160	40	320	320	**5120**	640	320
Anti-*C. sinensis* worm	—	160	—	320	160	160	**640**	—
Anti-*T. canis* worm	—	—	—	80	—	—	—	**5120**

gens were studied using ELISA (Table 2). The antigens which gave the highest titer to the sera are printed in bold type. These results show that various parasite antigens cross-reacted widely with the immune sera, but that antiserum generally produced the highest titer against the antigen used for immunization except for the combinations of *S. mansoni* worm antigen and anti-*S. japonicum* worm serum and of *S. japonicum* worm antigen and anti-*S. mansoni* worm serum. However, there was no clear distinction between the immune rabbit sera with *S. japonicum* egg or worm

Table 3 Evaluation of *S. japonicum* egg antigen filtered on Sephadex G-200 by ELISA in terms of absorbance at 500 nm

Serum \ Antigen	fraction 1	2	3	4	5	6	unfractionated *S. japonicum* egg	*F. hepatica* worm
Normal	.151	.166	.071	.060	.115	.300	.071	.078
Anti-*S. japonicum* egg	.816	.665	.580	.235	.337	.622	.731	.363
Anti-*F. hepatica*	.585	.394	.120	.080	.182	.481	.543	.828

N.B.: "Normal" was pooled negative sera from 12 rabbits. All the sera were diluted at 1:80.

Table 4 Anti *F. hepatica* immune serum fractionated on the CNBr-activated Sepharose 4B coupled with *S. japonicum* egg antigen

Serum \ Antigen	*S. japonicum* egg	*F. hepatica*
Normal	.165	.253
Anti-*F. hepatica*	1.100	1.457
Anti-*F. hepatica* effluent	.416	1.467
Anti-*F. hepatica* eluate	1.074	.950
Anti-*S. japonicum* egg	1.419	.687

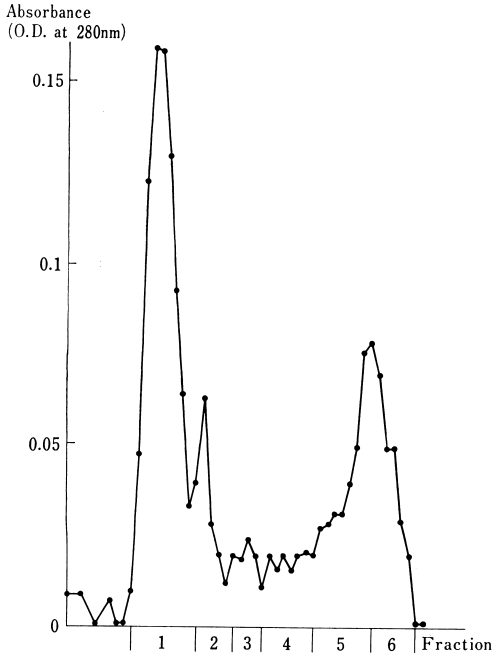

Fig. 1 Filtration of *S. japonicum* egg antigen on Sephadex G-200.

antigen and those with *S. mansoni* egg or adult antigen. Nevertheless, anti-egg immune sera produced high titers with the egg antigens of *S. japonicum* and *S. mansoni*, and anti-adult worm immune sera, with the worm antigens of both species. These findings indicate that immune sera can be differentiated using a number of different parasite antigens, even if crude.

Antigens derived from *S. japonicum* eggs and *S. mansoni* adult worms produced strong reactions with anti-*F. hepatica* immune serum. And the antigen derived from *F. hepatica* produced strong reactions with anti-*S. japonicum* adult and anti-*S. mansoni* adult sera. Based on these findings, anti-*S. japonicum* egg and anti-*F. hepatica* sera were selected as reference sera in the study of the cross reactions produced by the antigens from *S. japonicum* eggs and *F. hepatica* worms.

2. Partial Purification of Specific *S. japonicum* Egg Antigen

Separation by gel filtration: The *S. japonicum* egg antigen was filtered on a Sephadex G-200 column. The elution profile is shown in Figure 1. The protein concentration of each fraction was determined by the Lowry-Folin method and each fraction was dialysed and examined by ELISA for the antigen activities against anti-*S. japonicum* egg and anti-*F. hepatica* adult worm sera (Table 3). The absorbance of ELISA at 500 nm of the sera diluted at 1:80 was measured instead of the serum titer,

Fractions 1, 2, 5 and 6 produced higher absorbance reacting with the normal control serum in comparison with the unfractionated *S. japonicum* egg antigen. Fraction 3 seems to be the best in differentiating anti-*S. japonicum* egg serum from anti-*F. hepatica* worm serum. It produced strong coloration with the former serum and reduced coloration with the normal or the latter serum in comparison with the unfractionated *S. japonicum* egg antigen.

Separation by affinity chromatography: Anti-*F. hepatica* immune serum was filtered on the Sepharose column coupled with *S. japonicum* egg antigen. The column was prepared using CNBr-activated Sepharose 4B. The unbound and bound materials, effluent and eluate, respectively, produced the absorbance as shown in Table 4. In this procedure, unbound material or effluent is expected to be more specific to *F. hepatica*. The effluent had a lower absorbance in reacting with *S. japonicum* egg antigen in comparison with the unfractionated anti-*F. hepatica* serum, while still resulting in a high absorbance with the *F. hepatica* antigen.

The cross-reacting components of anti-*F. hepatica* serum were then coupled to CNBr-activated Sepharose 4B, to which the crude *S. japonicum* egg antigen was then applied. The reactions of the effluent and eluate with the immune sera are shown in Table 5. In spite of the above fractionation, the effluent from the *S. japonicum* egg antigen was still reactive with anti-*F. hepatica* serum, similarly to the unfractionated *S. japonicum* egg antigen.

Because of the unsatisfactory results with CNBr-activated Sepharose 4B, Epoxy-activated Sepharose 6B was employed so that carbohydrate components could be coupled to the column. The *S. japonicum* egg antigen was first coupled to Epoxy-activated Sepharose 6B, to which anti-*F. hepatica* serum was applied. The cross-reacting portion in anti-*F. hepatica* serum was eluted from the column and coupled to CNBr-activated Sepharose 4B. The crude *S. japonicum* antigen was then applied to this column. In this procedure, more specific antigenicity was expected in the effluent. The antigenicities of the effluent and eluate as examined by ELISA are

Table 5 *S. japonicum* egg antigen fractionated on CNBr-activated Sepharose 4B to which the cross-reactive component of anti-*F. hepatica* immune serum that had been prepared on CNBr-activated Sepharose 4B was coupled

Serum \ Antigen	*S. japonicum* egg	*S. japonicum* egg, effluent	*S. japonicum* egg, eluate	*F. hepatica*
Normal	.063	.044	.059	.065
Anti-*S. japonicum* egg	.745	.711	.678	.321
Anti-*F. hepatica*	.426	.400	.495	·733

Table 6 *S. japonicum* egg antigen fractionated on CNBr-activated Sepharose 4B to which the cross-reactive component of anti-*F. hepatica* immune serum that had been prepared on Epoxy-activated Sepharose 6B was coupled

Serum \ Antigen	*S. japonicum* egg	*S. japonicum* egg, effluent	*S. japonicum* egg, eluate	*F. hepatica*
Normal	.044	.040	.179	.045
Anti-*S. japonicum* egg	.638	.541	.527	.370
Anti-*F. hepatica*	.344	.144	.217	.658

shown in Table 6 together with those with the untreated antigens derived from *S. japonicum* eggs and *F. hepatica* adult worms. The effluent had a reduced absorbance with anti-*F. hepatica* serum in comparison with the original antigen, while retaining most of its activity on anti-*S. japonicum* egg serum. The eluate reacted strongly even with the normal control serum. The effluent was further fractionated on the column of Sepharose 4B coupled with the cross-reacting component in anti-*F. hepatica* serum which had been prepared on CNBr-activated Sepharose 4B. The final effluent was examined for its sensitivity and specificity by ELISA in comparison with other materials. The results were basically the same as those found with its original effluent (Table 7).

Heat treatment: Since the *S. japonicum* egg antigen seemed to be coupled to Epoxy-activated Sepharose 6B more efficiently than to CNBr-activated Sepharose 4B, it was suspected that some of the major components of the antigen cross-reactive to anti-*F. hepatica* serum consisted of carbohydrate. The antigen was heated for denaturation of the protein portion at different temperatures for different durations. It is shown in Table 8 that as the temperature is raised, the absorbance tended to decrease and this tendency is more marked in reaction to anti-*F. hepatica* serum than to anti-*S. japonicum* egg serum.

Table 7 Further fractionation of *S. japonicum* egg antigen on CNBr-activated Sepharose 4B to which the cross-reactive component of anti-*F. hepatica* immune sera that had been prepared on CNBr-activated Sepharose 4B was coupled

Serum \ Antigen	*S. japonicum* egg	*S. japonicum* egg, effluent 1	*S. japonicum* egg, effluent 2	*S. japonicum* egg, effluent 3	*F. hepatica*
Normal	.044	.040	.042	.042	.045
Anti-*S. japonicum* egg	.638	.541	.598	.592	.370
Anti-*F. hepatica*	.344	.133	.214	.126	.658

Effluent 1: fractionated using the cross-reactive component of anti-*F. hepatica* immune serum prepared on Epoxy-activated Sepharose 6B.
Effluent 2: fractionated using the cross-reactive component of anti-*F. hepatica* immune serum prepared on CNBr-activated Sepharose 4B.
Effluent 3: fractionated using the above two procedures.

Table 8 Heating of *S. japonicum* egg antigen for different time intervals

Serum \ Antigen	*S. japonicum* egg	*F. hepatica* worm	70 C				80 C				90 C			
			minutes				minutes				minutes			
			10	20	30	60	10	20	30	60	10	20	30	60
Normal	.046	.050	.048	.048	.047	.044	.035	.036	.036	.035	.037	.039	.035	.044
Anti-*S. japonicum* egg	.481	.048	.573	.503	.506	.342	.433	.452	.349	.396	.362	.328	.333	.337
Anti-*F. hepatica* worm	.105	.308	.148	.130	.128	.089	.109	.103	.074	.064	.068	.058	.047	.061

Discussion

Various antigens have so far been proposed for use in the diagnosis of schistosomiasis: the cercaria, adult worm, egg and their excretory and secretory products with different extraction methods (Schinski *et al.*, 1976; Melcher, 1943; Bocter *et al.*, 1979; Carter and Colley, 1978; Tsang *et al.*, 1981; Murrell, 1974; Rotmans *et al.*, 1981). The methods are basically composed of destruction of solid parasite materials using homogenation, sonication or freeze-thawing and extraction with different solvents. In this study, carbonate buffer-extracted antigens from alternately frozen-thawed adult worms or eggs of parasites were used for ELISA. The carbonate buffer of 0.05 M at pH 9.6 was chosen for extraction because of the pH requirement for the ELISA technique.

Superiority of the crude *S. japonicum* egg antigen to the *S. japonicum* adult, *S. mansoni* egg and *S. mansoni* adult antigens at the same protein concentration has been demonstrated with respect to the reactivity with serum from rabbits infected with *S. japonicum* or *S. mansoni*. It was also shown that *S. japonicum* and *S. mansoni* infections could not be distinguished from each other with the crude antigens, be they derived from *S. japonicum* eggs or adults or *S. mansoni* eggs or adults. These results coincide with the findings of Kitani *et al.* (1979) who used the acidic protein fraction of adult worms. For the seroepidemiological survey of schistosomiasis japonica, this crude antigen extracted with carbonate buffer from *S. japonicum* eggs will be sufficient, since schistosomiasis mansoni is not present in *S. japonicum*-infected areas. More than 90% of egg excreters were detected with this antigen as demonstrated by Matsuda *et al.* (1981). This does not mean, however, that there is no necessity for developing purified or defined antigens such as MSA_1, which is said to be stage- and species-specific (Hamburger *et al.*, 1976; Pelley *et al.*, 1977), as it is quite possible for international travelers to acquire diseases which do not exist in their native places. From a clinical point of view, however, identification of schistosomiasis, be it japonica, mansoni or haematobia, will be sufficient, for the drug therapy is not so specific. Defined antigens will be most useful in analysing the immunological pathology of schistosomiasis as well as in developing vaccines for the prevention and control of these infections.

In spite of the cross reactivity with anti-*F. hepatica* serum (Table 2), the crude *S. japonicum* egg antigen did not react with the sera of rabbits immunized with *Paragonimus philippinensis, C. sinensis,* or *T. canis*. These results differ from that which Hillyer and Gomez de Rios (1979) found with the soluble egg antigen derived from *S. mansoni*. They found extensive cross reactivity with the sera from humans with *F. hepatica, Trichinella spiralis, Taenia solium* and *Echinococcus granulosus* infections. In other words, their soluble egg antigen was even reactive in cestode infections as well as to nematode infections. Such a wide range of reactivity was not found by other researchers (Bout *et al.*, 1976; MacLaren *et al.*, 1978). The cross reactions between antigens and anti-parasite immune sera both derived from *S. japonicum, S. mansoni* and *F. hepatica* were, however, clearly demonstrated in the present study, similarly to the findings of Pelley and Hillyer (1978) and Hillyer *et al.* (1979).

Although the immunological properties of serum from infection and anti-parasite immune serum are considered to be different, it has been shown in this experiment that the problem of cross reaction in parasitological diagnosis will possibly be solved even without purified antigen, if comparative examination with different parasite antigens is performed. Since immune serum

seems more complex than infection serum, actual diagnosis of parasitic infection may be simpler than differentiating different immune sera.

The three procedures for reducing the cross reactivity of the crude *S. japonicum* egg antigen with respect to anti-*F. hepatica* serum resulted equally in improved specificity and reduced reactivity.

A preliminary infra-red spectrophotometric analysis of our crude antigen extracted from *S. japonicum* eggs showed that it was composed of proteins, fatty acids, phosphates and sugars (unpublished data). The analysis by gas chromatography of sugar components showed that the crude antigen is composed of glucose, galactose, innositol, mannose and fucose (unpublished data). The presence of fucose is of interest, as this is often identified as an antigen determinant in immunology. The major components of schistosomal crude antigen were reported to be non-protein substances (Fujinaga *et al.*, 1981; Carter and Colley, 1981). The antigen was better purified in this study using Epoxy-activated Sepharose 6B than CNBr-activated Sepharose 4B. The results obtained with the further purified antigen are consistent with the above findings. The heat-stable nature of our antigen is important and this reminds one of the heat-resistant nature of the substance responsible for the circumoval and intraoval precipitations (COP and IOP) (Kamiya, 1980; Kamiya, 1981). It will be of significance to study how much of the heat-resistant component of our crude antigen is related to the antigenic substance in the space between the miracidium and the vitelline membrane of the schistosome egg, which is considered to be essential for the COP or IOP tests. Hillyer and Pelley (1980) have shown that the circumoval precipitinogen is the MSA_1 in the case of *S. mansoni* using the hybridoma technique.

Summary

Carbonate buffer-extracted crude antigen from *Schistosoma japonicum* eggs was examined for its specificity against the sera from rabbits infected or immunized with *S. japonicum* or *S. mansoni* as well as those immunized with the crude antigens derived from *Fasciola hepatica, Paragonimus philippinensis, Clonorchis sinensis* and *Toxocara canis*. Marked cross reactivity was observed between the crude *S. japonicum* egg and *F. hepatica* antigens. Attempts were made to separate *S. japonicum* egg antigen into the specific antigen and the antigenic component cross-reactive to anti-*F. hepatica* serum.

The crude *S. japonicum* egg antigen was filtered on Sephadex G-200, fractionated by affinity chromatography, or heated. Fraction 3 from the Sephadex G-200 elution was superior to the unfractionated *S. japonicum* egg antigen in differentiating anti-*S. japonicum* egg and anti-*F. hepatica* worm sera, whereas fraction 6 reacted strongly even with the normal serum. The two sera could be better distinguished using antigen prepared on Epoxy-activated Sepharose 6B than on CNBr-activated Sepharose 4B, whereas the final eluate obtained using Epoxy-activated Sepharose 6B reacted strongly with the normal control. The crude antigen heated at 90 C for 30 minutes produced basically the same result as fraction 3 on Sephadex gel filtration and the antigen partially purified by affinity chromatography.

The antigens from these three different procedures equally demonstrated increased specificity, although reactivity was reduced to certain degrees as far as the two reference immune sera were concerned. These findings suggested that important determinants of the *S. japonicum* egg antigen were composed of non-protein substances such as polysaccharides and/or glycoproteins.

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ELISA に用いる日本住血吸虫卵抗原の研究

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炭酸緩衝液で抽出した ELISA 用寄生虫抗原の特異性を家兎血清を用いて検討した．日本住血吸虫及びマンソン住血吸虫で，感染血清及び免疫血清を作成した．肝蛭，フイリピン肺吸虫，肝吸虫，犬回虫で免疫血清を作成した．日本住血吸虫卵抗原は，肝蛭成虫免疫血清に交叉反応が強かった．

ゲル ろ過，イムノアフイニティークロマトグラフイー，加熱の三法で，日本住血吸虫卵抗原の精製を試みた．日本住血吸虫卵及び肝蛭成虫免疫血清に対して検定した．セフアデックス G-200カラムの第3分画，エポキシ活性化セフアローズによる分画，90C30分間の加熱によって，ほぼ同程度に特異性の向上が見られたが感受性は低下した．

日本住血吸虫卵抗原の重要な部分は，熱抵抗性非蛋白部分にあることが示唆された．