infection, this period can be considered the latent period. From 4 to 11 days after infection, rabbits showed symptoms such as fever, anorexia and listlessness, indicating this as being the acute period. From 13 to 30 days is the subacute period without obvious symptoms, and 30 days and later can be considered the early chronic period. The OD value of CAg in rabbits increased progressively in the acute period, in which period tachyzoites propagated quickly, and then the OD value declined in subacute and early chronic periods, in which tachyzoites changed to bradyzoites and developed cyst formation. Therefore, this suggests that CAg is a good indicator of early-stage infection. CAg released from the parasite in the late stages may be mainly bound to antibodies, thus forming circulating immune complex (CIC). It is, therefore, not possible to achieve high sensitivity in the late stages. However, the new method described in this study is a much more sensitive method in detecting early or active infection of toxoplasmosis when compared with IHA and ELISA for antibody detection.

This method seemed to be useful in a survey of pregnant woman, because maternal infection in the first trimester of gestation results in a transmission rate of 10–15% and causes severe damage to the fetus. Transmission rates rise to 68% in the third trimester, and antiparasitic treatment during pregnancy can reduce transmission or sequelae in the fetus. It is, thus, important to identify the disease in the early stage (29).

From CAg-positive samples we isolated several strains of *T. gondii* through inoculation of infected tissues and serial passages in mice in our laboratory. Presence of parasites was confirmed in sera, CSF, amniotic fluid, milk, or embryonic tissue (data not shown). One strain of *T. gondii* was isolated from milk of a breast-feeding woman; it is the first case of the parasite being isolated from milk of breast-feeding women in China. Although tachyzoites are sensitive to acid and are usually killed by gastric juice (30), there is the risk of transmission of toxoplasmosis from mother to infant through breast-feeding. Attention should be paid not only to pregnant women but also to breast-feeding women, both with risk factors causing vertical transmission.

Our investigation showed that there was a significant difference of the CAg-positive rate between clinical samples with and without cerebral paralysis, suggesting the possibility of causative roles of *T. gondii* for cerebral paralysis. Another possibility that patients with cerebral paralysis might be more susceptible to *Toxoplasma* infection cannot be ruled out. It is difficult to make a diagnosis of toxoplasma encephalitis (TE) by demonstrating the parasite in CNS tissues or in CSF (31). The sandwich ELISA detecting CAg developed in the present study provides an effective way to diagnose TE using sera or CSF from patients with CNS symptoms. After treatment with azithromycin, all patients became CAg negative, and clinical symptoms of patients improved.

In conclusion, this sensitive and specific double-PcAb sandwich ELISA method is valuable in the diagnosis of acute or active *Toxoplasma* infection, and it can be extended to the survey of early-stage toxoplasmosis in humans and domestic animals.

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ワークショップ：輸入寄生虫症の現状と対策

国内問題としての Neglected Diseases

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Key Words：輸入蠕虫病，Neglected disease，症例登録

はじめに

近年の国際的な人や物流の増加は輸入感染症のリスクが増大していることを意味する。そのために，日本国内では感染症法や検疫法の改正を通じて，対策の強化が図られている。しかし，その主眼はウイルス性出血熱や新興のウイルス病，強毒性の細菌感染などに置かれており，寄生虫感染症は保健医療政策上は解決済みという立場が取られている。しかし蠕虫性疾患の国内発生動向は正式な症例登録制度がないために，行政的な捕捉は出来ないのが実状である。蠕虫感染症は先進国にあっても Neglected disease の範疇にあるのである。

最近の疾病構造の変化，特に人獣共通感染症の増加とあいまって，蠕虫感染症ではあっても，致死性の危険なものがわが国に持ち込まれる可能性や，すでに国内に定着した可能性などが論じられるようになった。しかし上述の如く，日本国内の動向を知る制度的な裏付けが一切整備されていない。そこで，厚生労働省の新興・再興感染症研究事業を通じて，輸入蠕虫症の実態把握と，診断，治療，予防など公衆衛生学的な情報整備を進める研究が進められてきた。これまでの成果の一部を紹介し，輸入蠕虫感染症の実態と今後の課題について論じることにしたい。

輸入蠕虫症の国内発生動向の情報整備

国内法で症例報告が義務づけられているのはエキノコックス症だけである。一方，途上国からの帰国邦人の寄生虫感染が決して少なくないことが知られている[1]。そこで，積極的な症例登録制度が望まれることになる。厚生労働省研究班（輸入蠕虫性疾患の監視と医療対応整備に関する研究）では疾病登録制度の立ち上げを検討した（図 1）。そこでは 2 つ

Imported Helminthic Diseases : Current Situation and Future Prospect

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図1　蠕虫症の国内登録システムとそのデータベース化

の情報ソースを考えた。第一は「蠕虫症ネット」として，日本寄生虫学会に報告のあった症例についてデータベース化し，キーワード，著者などで検索が出来るものを公開した。第二は蠕虫症登録システムである。国内の医療機関に協力を求め，症例として報告することが難しいものであっても発生情報を登録してもらうことを考えた。これは制限付き公開とし，個人情報の保護を重視している（図2）。これに本誌に詳述されている「臨床寄生虫学雑誌データベース」と併せることにより，症例捕捉効率は上昇することが期待されている。

病原体持込みのモニタリング体制

蠕虫が国外から持ち込まれる危険性を積極的に調べ，国内に定着したものについては可能な限り追跡調査を行うことが必要である。前述の研究班では蠕虫卵による食品汚染と，国外からの侵入蠕虫の動向を調査した。国外からの蠕虫持込みのルートとして可能性が高いのは虫卵や幼虫で汚染された生鮮食料品や寄生虫保有動物の国内定着である。そこで，輸入キムチの寄生虫卵汚染とネズミが保有する広東住血線虫の生活史の拡散を調査した。

キムチは輸入品と国産品を市中の商店でランダムに購入し，野菜表面の虫卵の有無を調べた。得られた虫卵様混入物は回虫の配列から作製したプライマーを用いてPCRで増幅し，配列を回虫と比較した。サンプル数は十分ではないが，国産品5商品では全て虫卵陰性であったが，輸入キムチは調べた25検体中40%が陽性であり，輸入キムチの虫卵汚染率は有意に高かった（$p < 0.01$）。形態的に回虫

図2　症例登録システムによる蠕虫症国内症例

卵と似ていたサンプルについてPCRで増幅後核酸配列を比較すると回虫のものと完全に一致した[2]。このように輸入キムチには高頻度で虫卵の混入が認められるが，ヒトへの感染性については現時点で結論は得られていない。

国内に定着した輸入蠕虫として，研究班では広東住血線虫を調査した。最近では沖縄県の疫学的背景が変化した上，致死症例も報告されている[3]。ドブネズミやクマネズミが終宿主となり，都市圏でも拡大が懸念されたことから，ペストコントロール業者の協力を得ながら調査した。その結果，首都圏の複数地域で感染ネズミが捕獲された。従来は港湾近くのネズミでこの蠕虫が維持されていることが考えられていたが，港から数十キロの地点でも感染が確認され，徐々にではあるが棲息エリアが拡大していることが考えられた（表1）。その一方で中間宿主の感染が確認できた地域は限られており，広東住血線虫の生活史がどのように維持されているかが当面の調査項目となる。

輸入蠕虫症とは国内に存在しない病原体またはその患者が国外から持ち込まれることを言うが，最近の新たな問題点は国内居住外国人が持ち込んだ食習

表1　首都圏の広東住血線虫の分布と中間宿主

都県名	捕獲場所	環境	調査年	捕獲ネズミ数	陽性個体数	調査した中間宿主	採取数	感染幼虫
千葉県	市川	遊戯施設	2001	12	7	チャコウラナメクジ	30	(−)
	銚子	漁連施設	2006	10	8			
東京都	西東京	住宅街	2002	8	8	チャコウラナメクジ	100	(−)
	墨田	住宅地	2004	1	1	チャコウラナメクジ	147	(−)
神奈川県	川崎	公園	2004	24	22	チャコウラナメクジ	27	(+)

表　2

Species	分布地	ミラシジウムの侵入
O. h. nosophora	山梨	++++
O. h. hupensis	安徽省	−
	江西省	−
O. h. robertsoni	四川省	+++

慣を日本国内の生鮮食品を用いることで蠕虫感染が発生するようになったことである。肺吸虫症は日本人の食生活の変化によって減少傾向にある蠕虫感染症であるが，一方でタイや中国からの外国人が自国の調理法で中間宿主動物を摂食する，或いは日本人に提供することで発生する事例が起こってきている。ウエステルマン肺吸虫感染についての前述の登録システムでは半数以上が外国人症例であることは最近の傾向であると言ってよい。

蠕虫感染症の再興の可能性

わが国では流行が終息した蠕虫感染症が再び出現する可能性を監視する必要もある。日本から消滅した蠕虫感染の代表は日本住血吸虫やバンクロフト糸状虫であるが，前者については国内に中間宿主貝が依然として棲息していることから，流行再興の懸念が払拭されていない。そのための研究として研究班ではいくつかのシミュレーションを試みている。中間宿主貝の動向については当面消滅することは考えにくい[4]。そこで，アジアの流行地から日本住血吸虫が持ち込まれた場合に起こりうる事態を検討することが必要である。日本の日本住血吸虫は日本以外に分布する *Onchomelania* 属貝には感染できず，中国本土の *O. h. hupensis* には侵入さえしないことがわかった（表2）。逆に中国の日本住血吸虫が日本のミヤイリガイに感染できるか否かが問題であるが，十分に高い適合性があることは考え難しく，その意味ではアジアからの輸入日本住血吸虫症が国内の流行再興に大きな意味を持つことはないであろうと推定される。

おわりに

わが国で行政サービス上は無視されている輸入蠕虫症の監視の望ましい方向と今度の課題について論じた。蠕虫感染症の実態が明かでない以上，適切な対応を論じることが困難である点で，Neglected disease としての特徴を持つものである。今後は情報収集の努力を継続し，診断法や治療法などの医療対応の強化を図ることが重要である。

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Proteome approach for identification of schistosomiasis japonica vaccine candidate antigen

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ABSTRACT

Experimental vaccination with radiation-attenuated cercariae (RAC) confers possible practical levels of resistance to challenge infection by humoral and by cellular mechanism. Here, we aimed to identify possible vaccine antigens by using specific IgG antibody from RAC vaccinated miniature pig. Two milligrams of soluble egg antigen (SEA) or schistosomal worm antigen preparation (SWAP) was fractionated using two dimensional liquid chromatography (proteome PF 2D) consisted of high performance chromatofocusing (HPCF) and high resolution reversed phase chromatography (HPRP). Of the 42 HPCF fractions of SEA or SWAP, 26 (61.9%) or 15 (35.7%) showed positive dot blot reaction with RAC vaccinated serum respectively. The dot blot positive fractions were applied to the second HPRP column. One hundred and seven out of 26×96 of SEA fractions and 18 out of 15×96 SWAP fractions reacted with RAC vaccinated serum. From the positive fractions we chose 17 of SEA and 10 of SWAP that had no reactivity with normal cercariae infected (NCI) sera and had single peak of 214 nm; and automated N-terminal amino acid sequence based on in situ Edman Reaction was conducted. Four sequences were obtained and applied to the homology search in NCBI database. A total of eight candidate genes were listed up and their cDNA clones from schistosomula stage were obtained. Two of the recombinant proteins (AAW27472.1 and AXX25883.1) showed strong reactivity with the RAC vaccinated serum but marginal with NCI serum. This protocol using proteome PF 2D could be applicable in identifying immunoreactive proteins from crude extract for the development of vaccines or for diagnostics.

1. Introduction

Schistosomiasis is a parasitic disease which affects more than 200 million individuals in Africa, Asia and South America. It is endemic in 74 countries causing more than 250,000 deaths per year [1]. Despite two decades of comprehensive campaign for the control, the number of individuals with active schistosomiasis worldwide remains at about 200 million annually [2,3]. Vaccine has long been expected to be developed as a novel strategic tool for the control [4–6]. A comprehensive review on current status of vaccines for schistosomiasis had provided by McManus and Loukas [7].

There are three different approaches for isolation and identification of schistosome vaccine candidates [8,9]. Selection based on protective monoclonal antibodies such as glutathione-*S*-transferase (GST) [10] and triosephosphate isomerase (TPI) [11], by unique antigen recognition by strong natural resistance in humans [12] or animals [13], or by antigen selection using the radiation-attenuated cercariae vaccine (RAC) model [14–16]. For example, a fragment of myosin of *S. mansoni* (SmIrV-5); one of the vaccine candidate antigens selected by TDR/WHO committee [17], was identified using serum from mice exposed to RAC [18]. None of the antigens identified conferred equivalent efficacy of the vaccination using RAC [5,19,20]. There is a quantitative and/or qualitative difference between the immune responses generated by RAC vaccine and those by defined subunit vaccine [21,22]. Increased immunogenicity of

Abbreviation: RAC, radiated-attenuated cercariae; NCI, normal cercariae infected; NC, healthy control; SEA, soluble egg antigen; SWAP, schistosomal worm antigen preparation; HPCF, high performance chromatofocusing; HPRP, high resolution reversed phase chromatography.

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RAC is related to delayed and truncated pattern of migration, in contrast to the normal parasite [23]. In fact, radiation induces defects in the neuromuscular coordination of the developing larvae [24]. Accordingly, delayed parasite migration through skin or skin draining lymph nodes and lungs would mean that there is a greater opportunity for interaction of parasite antigen with the immune cells at these sites which may in turn favor the priming of the protective response.

The highest levels of resistance obtained in mice after vaccination with RAC require antibodies and T cells [25,26]. It is clear that protective immunity generated by RAC vaccines is mediated by acquired immune mechanisms that require the reactivation of antigen specific CD4⁺ helper cells [27]. However, passive transfer experiments indicate that IgG antibodies are also the key mediators of this immunity [28–30].Thus, elucidation of antigens relevant to both humoral and cellular response may be critical for the development of an optimal vaccine.

Most of our knowledge on schistosomiasis is drawn from experiments in primates and rodents. Although primate model is relatively better but the high cost and ethical concerns make them difficult to be used [31]. Also the use of rodents has several problems as a model for schistosomiasis [32,33]. When we focus on RAC vaccine of *S. japonicum*, higher protection has been achieved in rhesus monkeys [34], in cattle [35,36] and in pigs [19]. Within the reservoir host animals, pig would be the easiest one compared with cattle or sheep, but still the major drawback of the domestic pig is the large body mass. For this reason we established a unique miniature pig model for human Schistosomiasis japonica [37]. In this study we aimed to identify the major antigenic molecules which are specifically recognized by RAC vaccinated serum of miniature pigs for identification of possible candidate schistosomiasis vaccine.

2. Materials and methods

2.1. Parasite

Chinese strain of *S. japonicum* was obtained from Jiangsu Provincial Institute of Parasitic Diseases Wuxi, Jiangsu Province, People's Republic of China. Cercariae were released from the infected snails by light induction as described [38]. Attenuation of the cercariae was carried out by 200 Gy of γ-irradiation at a rate of 33 Gy/min using ^{60}Co irradiator (Pony Industry CO. LTD. PS-3100sB, Osaka, Japan).

2.2. Preparation of cultured schistosomula

Cercariae were released from 100 infected snails in a beaker containing 200 ml of tap water under light for 2 to 3 h at room temperature and were passed through Sartorus Nylon spacer, mesh size 0.1 mm. The obtained suspension was centrifuged for 60 s at 1500 rpm (383 g) at 10 °C to concentrate cercariae. The cercariae were washed twice in Basal Medium Eagle (BME) (Gibco-Invitrogen Co., CA, USA) containing 15 mM of HEPES (Gibco-Invitrogen Co.), 300 units/l Penicillin (Gibco-Invitrogen Co.), 300 μg/l Streptomycin (Gibco-Invitrogen Co.) and 160 μg/l Gentamicin (Gibco-Invitrogen Co.) in aseptic condition. Transformation of cercariae to schistosomulae was carried out mechanically by shearing the tail using vortex for 3 min; and about 200–300 parasites/ml of the mechanically transformed shistosomulae were cultured in DMEM (Sigma-Aldrich Co., MO, USA) containing 1% Fetal calf serum (FCS) (Hyclone, UT, USA), 300 units/l penicillin and 300 μg/l streptomycin in 24 well plate (Corning, NY, USA) at 37 °C in 5% CO_2 incubator for 24 h. The cultured schistosomulae were collected by centrifugation, washed three times with phosphate buffered saline (PBS) and kept frozen at −80 °C until use [39].

2.3. Soluble worm antigen preparation (SWAP)

SWAP was obtained by the method described elsewhere [40]. Briefly, *S. japonicum* adult worms were obtained by perfusion of infected rabbits. After lyophilization, worms were homogenized in cold diethyl ether and centrifuged to remove lipids. The pellet was then freeze–thawed several times in PBS, pH 7.4 containing 1 mM phenyl methyl-sulphonyl fluoride (PMSF) and 2 μg/ml Leupeptin (Sigma). The homogenate was dialyzed against several changes of PBS at 4 °C, and centrifuged at 30,000 g for 50 min at 4 °C. The supernatant was filtrated through 0.22 μm filter (Millipore Co., MA, USA) and this was used as SWAP. Protein concentration was determined by BCA protein Assay Kit (Pierce Biotechnology, Inc., IL, USA).

2.4. Soluble egg antigen preparation (SEA)

Preparation of SEA was previously described [41]. Briefly, *S. japonicum* eggs were isolated from infected liver and intestine of rabbit. The purified eggs were finally adjusted to a concentration of 50,000 eggs/ml of PBS with 1 mM of PMSF, and 2 μg/ml Leupeptin (Sigma) and sonicated three times on ice for 10 min. The suspension was freeze–thawed several times and centrifuged at 30,000 g for 50 min at 4 °C. The supernatant was filtrated through 0.22 μm filter; and this was used as SEA. Protein concentration was determined by BCA protein assay.

2.5. Experimental animals, parasitological examinations and serum sample collection

Six-week-old male CLAWN strain miniature pigs (Japan farm, Kagoshima, Japan) weighing between 2.5 kg and 3 kg were used in this study. The pigs were fed with standard feed based on their body weights, with water ad libitum. Seven pigs were used, three for immunization with radiation-attenuated cercariae (RAC) of *S. japonicum*, three for infection with 200 cercariae (NCI) and one as a healthy control (NC). Sera were collected four weeks after vaccination and four weeks after challenge by drawing blood from auricular vein at indicated times. Before taking blood, pigs were anesthetized by intramuscular injection with 0.2 mg/kg midazolam (Yamanouchi Pharmaceutical Co., Ltd. Tokyo, Japan) and 40 μg/kg medetomidine (Orion Corp., Espoo, Finland). The first group of pigs was subjected to a single percutaneous exposure of 400 RAC using a cover slip as described [37]. Four weeks later, the first and second groups of pigs were challenged with 200 normal cercariae (NCI). The third group was used as a healthy control. Feces were collected every week and the number of eggs excreted into feces was counted as previously described [37]. Adult worms were recovered from the liver and mesenteric veins as described [20]. The experimental protocol was approved by the Animal Ethical Committee of Nagasaki University (No.0204250127-3).

2.6. SDS-PAGE and western blot analysis

SWAP and SEA were boiled for 5 min in reducing sample buffer containing 4% sodium dodecyl sulfate (SDS) and 50 mM Dithiothreitol (DTT), and electrophoresed by using 5–20% gradient SDS polyacrylamide gel (E-pagel®, Atto Co., Tokyo, Japan) [42]. For the western blot analysis, SEA and SWAP proteins separated by the E-pagel were transferred onto PVDF membrane (Millipore Co.). After blocking in 5% skimmed milk in Tris-buffer (TBS-Tween 20) (20 mM Tris–HCl, pH 7.5; 500 mM NaCl; 0.1% Tween 20) for 1 h at room temperature, the protein transferred-membrane was incubated with the 3000 times diluted test serum for 2 h at room temperature. After washing three times with TBS-Tween 20, the membrane was incubated in 10 ml of a secondary antibody solution containing 40,000 times diluted affinity Purified anti-porcine IgG antibody that is horseradish peroxidase

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Table 1
Fecal egg excretion of RAC immunized and none immunized miniature pigs after challenge infection with 200 cercariae of *S. japonicum* Chinese strain

Examination time after challenge infection	EPG (egg per gram of feces)		P[c]
	RA[a] (n=3)	NC[b] (n=3)	
5th week	74.34±82.26	102.81±81.47	NS
(EPG of individual)	(190.65, 18.65, 13.71)	(45.03, 196.00, 67.4)	
6th week	265.03±151.25	413.42±171.92	0.028
(EPG of individual)	(438.78, 193.48, 162.82))	(610.00, 291.13, 339.14)	
7th week	180.68±104.29	389.85±254.99	NS
(EPG of individual)	(298.61, 142.86, 100.57)	(681.03, 206.42, 282.10)	
8th week	156.24±99.28	558.26±181.23	0.026
(EPG of individual)	(265.91, 130.36,72.46)	(686.22, 637.68, 350.88)	
9th week	207.42±79.60	480.61±76.57	0.003
(EPG of individual)	(280.49, 219.18, 122.95)	(528.69, 520.83, 392.31)	

The values in parentheses represent the EPG for each pig.
[a] RA: vaccinated with 400 RAC.
[b] NC: no vaccination control.
[c] P values were calculated by two tailed Student's *t*-test.

(HRP) conjugated (Anti pig IgG (H+L)-HRP, AP166P, Chemicon International, CA, USA) and TBS-Tween 20 for 1 h at room temperature. After washing three times with TBS-Tween 20, reactive protein bands were visualized by exposing an x-ray film using ECL-Plus Western blotting Detection system (G. E. Healthcare, Amersham Biosciences, Buckinghamshire, UK).

2.7. Protein purification and fractionation

The protein purification and fractionation of SWAP and SEA were performed using Proteome Lab PF 2D system (Beckman Coulter, CA, USA) that is designed for two dimensional liquid chromatography consisted of a high-performance chromatofocusing (HPCF) in the first dimension followed by high-resolution reversed-phase chromatography (HPRP) in the second dimension. One milliliter of 2 mg/ml of either SWAP or SEA was introduced with a manual injector into the column for the first dimensional HPCF. The protein was bound to a strong anion exchanger followed by elution with a continuously decreasing pH (8.5–4.0) gradient (Beckman Coulter). The proteins were eluted based on their isoelectric point (pI), collected in a 96 deep-well plate (Beckman Coulter) [43]. The first dimensional fractions were directly applied to the second HPRP in a C18 column. The mobile phase consisted of two buffers, the first one 0.1% Trifluoroacetic acid (TFA) in water and the second one 0.08% TFA in acetonitrile. Separation was performed according to the Manufacturer's instruction protocol.

2.8. Dot blot ELISA

The fractions were blotted onto a PVDF membrane (Millipore Co.) using the Bio-Dot ® SF Micro filtration apparatus (Bio Rad Laboratories, Inc., CA, USA). Briefly, 20 μl of each fraction were blotted onto the PVDF membrane and the membrane was soaked in 5% skimmed milk in Tris-buffer (TBS-Tween 20) for 1 h at room temperature. The membrane was incubated with 1000 times diluted test serum for 2 h at room temperature. The reactivity of each dot with test serum was estimated by the dot blot ELISA using the same method described above in the western blotting. We selected positive dots when they showed increased intensity compared with those incubated with normal control serum. The criteria that used for selection of the positive/negative results of dot-ELISA are using two positive controls, the crude antigens of SWAP and SEA, and PBS as a negative control. All the positive fractions of the first dimension were sequentially applied to the second dimensional HPRP.

Table 2
Worm numbers recovered from each group of miniature pigs

Pig group	Worm burden			
	Total	Male	Female	Reduction rate[b]
RA 1	5	4	1	96.4%
RA 2	42	36	6	69.7%
RA 3	51	44	7	63.2%
Average	32.67	28.0	4.67	76.4%
NC 1	160	135	25	–
NC 2	117	87	30	–
NC 3[a]	–	–	–	–
Average	138.5	111.0	27.5	–

NC3[a]: this pig died at the 9th week after infection due to sudden death.
Reduction rate[b]: The worm reduction rate=(the average worm burden in the control group−the worm burden in the vaccinated group)/(the average worm burden in the control group)×100.

2.9. Amino acid sequencing

Fractions from the second dimensional HPRP were analyzed by dot blot ELISA and positive fraction was subjected to amino acid sequencing. Two hundred microliters of positive fraction was spotted onto Polybrene-coated glass fiber discs. Subsequently proteins were sequenced with an automated protein sequencer (ABI Model cLc; Applied Biosystem, CA, USA).

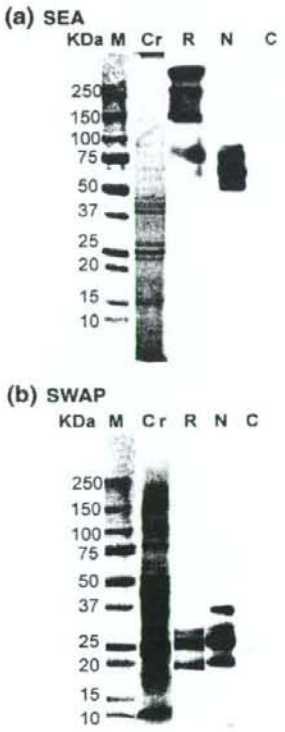

Fig. 1. SDS-PAGE and western blot analysis of RAC (R), NCI (N) and NC (C) sera of miniature pig for the specific IgG against (a) SEA and (b) SWAP. Lane M: molecular weight marker; lane Cr: CBB staining of the crude extract; lanes R, N and C: Western blot pattern probed with a panel of sera; lane R: RAC serum; lane: N. NCI serum and lane: C NC serum. Sera were diluted 3000 times.

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2.10. Homology search

The obtained amino-terminal sequences were blast-searched for their homology with the genes deposited in the *S. japonicum* database in NCBI using BLAST/blastp suite programs, in non redundant Gene Bank Coding Sequence (CDS). After homologous genes were listed up, further selection was performed according to the following criteria; the homologous area should be located at the N-terminus sequences and its deduced pH must be within the pI range of first dimensional fraction.

2.11. mRNA analysis

To confirm mRNA expression of the candidate genes in the different stages of the parasites, total RNA was extracted from cercariae, 24 h cultured schistosomulae, eggs, and adult worms of *S. japonicum* according to the instruction manual of Micro-to-midi total RNA purification system kit (Invitrogen Co.). The first strand cDNA was synthesized from the total RNA by using oligo (dT) primer according to the instruction manual of high capacity cDNA reverse transcription Kit (Applied Bio systems) and was used as template for reverse transcript PCR using a set of primers that were designed from the candidate gene as indicated in the Table 4. *S. japonicum* actin gene primers were used as an internal reference. RT-PCR was performed by the following condition of 42 cycles of 30 s at 94 °C, 50 s at 55 °C and 2 min at 72 °C for all samples. PCR products were subsequently separated on 1% agarose gel, stained with ethidium bromide and visualized under UV light. The resulting PCR products were cloned into pCR 2.1 using TOPO-TA cloning Kit (Invitrogen Co.), sequenced using Big-Dye V.1.1 terminator cycle sequencing Kit (Applied Biosystem) and analyzed on an ABI 3710 DNA Sequencer (Applied Biosystem) for confirmation with database sequences.

2.12. Production of recombinant protein

Reverse-PCR products from cercarial mRNA purified from agarose gel were cloned into the pET/100 D-TOPO expression vector (Invitrogen Co.) and transformed into chemically competent TOP10 *Escherichia coli* (Invitrogen Co.) according to the manufacturer's protocol. Cells were plated onto LB-Ampicillin (50 μg/ml) plates and incubated for 18 h at 37 °C. Ten positive clones were identified and were grown for 18 h in 6 ml LB medium containing Ampicillin (50 μg/ml) and plasmids prepared using a Gene Elut™ plasmid Miniprep kit (Sigma). Plasmids were then digested with Nhe I and Sac I (New England Biolabs, MA,USA) for 2 h at 37 °C and the inserts detected by separating the DNA on a 1% agarose gel stained with ethidium bromide and visualized under U.V. light.

To confirm the correct orientation and in frame, the inserts were sequenced using Big-Dye V.1.1 terminator cycle sequencing Kit (Applied Biosystem) and analyzed on an ABI 3710 DNA Sequencer (Applied Biosystem). Plasmid DNA containing expression constructs was transformed into BL21 Star™ (DE3) One Shot® *E. coli* (Invitrogen Co.) for recombinant protein expression. Briefly, 10 ng of plasmid DNA was transformed into the bacteria by heat shocking at 42 °C for 30 s. Transformed bacteria were grown overnight at 37 °C in LB medium supplemented with either 100 μg/ml ampicillin or 50 μg/ml

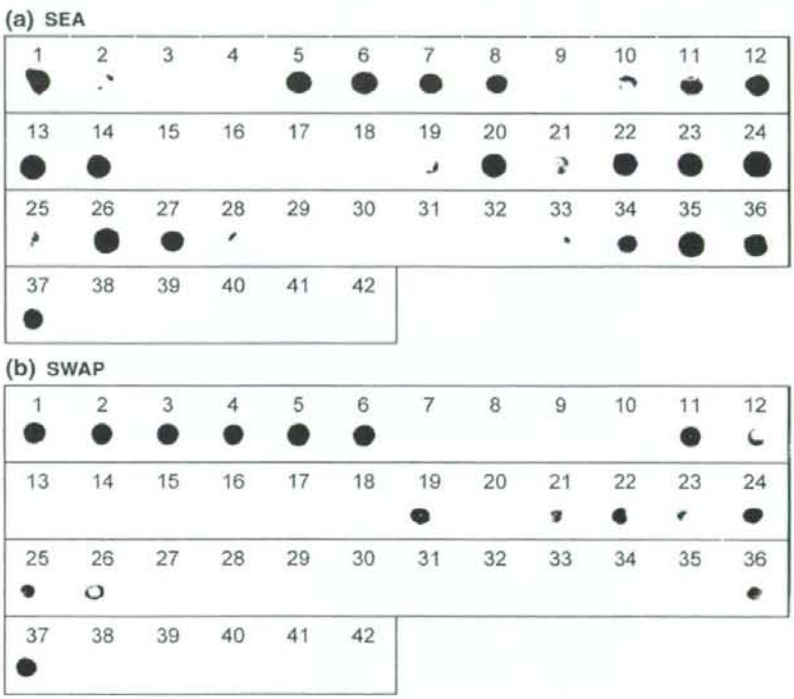

Fig. 2. Dot blot analysis of (a) SEA and (b) SWAP fractions of HPCF using pooled RAC vaccinated sera were diluted 1000 times. Dot blot has been repeated twice and the representable positive fractions to RAC serum were selected.

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Table 3
Dot blot analysis of the first and second fractions of SEA and SWAP that reacted with RAC vaccinated and/or NCI serum from miniature pigs

Sequential screening steps		No. of the RAC positive fractions (%)	
		SEA (%)	SWAP (%)
1st dimension	(42 Frs. of SEA, 42 Frs. of SWA)	26 (61.9)	15 (35.7)
2nd dimension	(2496 Frs. of SEA, 1440 Frs. of SWA)	107 (4.3)	18 (1.3)
NCI (−)	(107 Frs. of SEA, 18 Frs. of SWA)	61 (57)	10 (55)
Single peak (+)	(61 Frs. of SEA, 10 Frs. of SWA)	17 (27.87)	10 (100)

Frs.: Fractions.
NCI (−): Dot blot fractions that did not react with Normal Cercaria Infection serum.
Single peak (+): Dot blot fractions that have single retention peak were selected to amino acid sequencing.

carbenicillin prior to pilot expression by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer according to the manufacturer's protocol (Invitrogen Co.). Briefly, 10 ml of LB medium containing 100 μg/ml ampicillin or 50 μg/ml carbenicillin was inoculated with 500 μl of an overnight culture and allowed to grow for 2 h at 37 °C with shaking until they reached mid-log phase of growth. IPTG was then added to a final concentration of 0.1 mM to 0.5 mM and a 500 μl aliquot was removed from the culture, centrifuged at 10,000 g in a micro centrifuge for 30 s. The supernatants were removed and the cell pellets frozen at −20 °C. Remaining cultures were incubated at 37 °C with shaking and 500 μl aliquots were removed after 1, 2, 4 and 6 h post-induction. Individual sample was analyzed by SDS-PAGE and western blot for detection of recombinant protein using Anti-His G-HRP Antibody (Invitrogen Co.).

3. Results

3.1. Protective response in miniature pig vaccinated with radiation-attenuated cercariae (RAC)

The mean of fecal egg excretion of RAC vaccinated group was significantly reduced at 6th, 8th and 9th weeks post infection, when compared to the control group as shown in Table 1. The recovered worm number after the perfusion also indicated the vaccination effect of RAC as shown in Table 2.

3.2. Antigen recognition by sera from the vaccinated miniature pigs

We aimed to identify the antigenic molecules reactive with RAC vaccinated serum of miniature pigs but not with serum from those just infected with 200 cercariae for 4 weeks. As a target antigen preparation, we used SEA and SWAP antigens [16].

Pooled sera obtained 4 weeks after the RAC vaccination recognized antigenic molecules of 263, 255, 155, 130, 78, 55 and 20 kDa from SEA as shown in lane (R) of Fig. 1a. Whereas sera obtained 4 weeks post challenge with 200 normal cercariae, recognized antigens of 253, 155, 130, 74, 60, and 37.5 kDa (lane N of Fig. 1a). When we used SWAP, pooled sera obtained 4 weeks after the RAC vaccination recognized antigens of 70, 27, 21 and 10 kDa as shown in lane R of Fig. 1b. Sera obtained 4 weeks post challenge with normal cercariae recognized antigens of 150, 71, 36, 27, 21 and 10 kDa (lane N, Fig. 1b). The pooled sera used in this experiment were diluted 3000 times.

3.3. First dimension: high-performance chromatofocusing (HPCF)

Following the HPCF fractionation of SEA and SWAP 42 fractions were obtained. All the 42 fractions from SEA or SWAP were tested for reactivity with RAC vaccinated pooled serum as shown in Fig. 2a and b. Totally 26 SEA and 15 SWAP fractions were reacted to RAC vaccinated pooled serum as shown in Table 3.

3.4. Second-dimension: high performance reversed-phase chromatography (HPRC)

Those 26 and 15 dot blot positive fractions of SEA and SWAP respectively, from the first dimension (HPCF) were subjected to further fractionation by the HPRC. The Proteome maps of the second dimensional separation are shown in Fig. 3a and b.

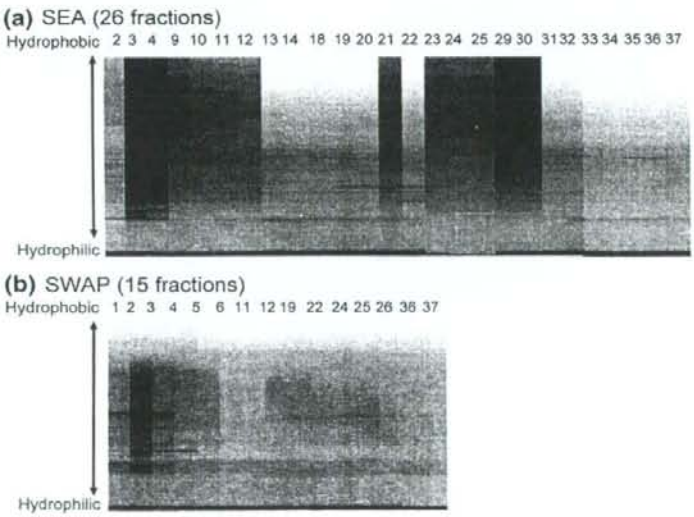

Fig. 3. The Proteo Vue® MAP of the second dimensional separation for the positive dot blot fractions from SEA (a) and SWAP (b). Hydrophobicity of each fraction is expressed by its retention time. Each band represents Optical Density of a peptide at 214 nm. The fraction numbers of the first column are shown on top of the figures.

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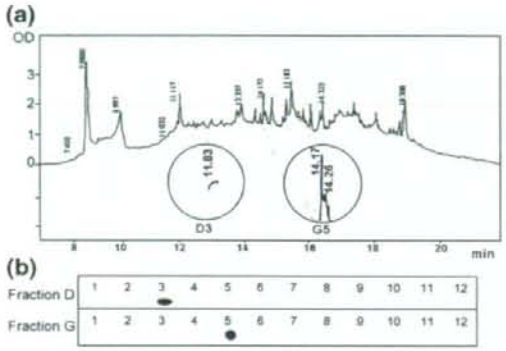

Fig. 4. Representative of the second column separation of the first fractions (fraction No.2 of SEA) (a) and the dot blots analysis for the fraction No. 2 of SEA (b). (a) The optical densitometry pattern (OD at 214 nm) is represented. The x axis represents retention time in min according to the concentration of acetonitrile in the mobile phase. (b) D3 and G5 are the two fractions obtained from the 96-well plate that was used for collection of the second column separation.

Elution pattern of the fraction number 2 of SEA after the second column is shown in Fig. 4a, as an example. All the fractions obtained after the second column were tested for reactivity against the RAC vaccinated serum. Out of the 2496 SEA and 1440 SWAP fractions, 107 and 18 fractions reacted with the RAC vaccinated serum respectively, as shown in Table 3. These 107 and 18 positive fractions were further tested for reactivity with normal cercariae infected (NCI) serum. Out of the 107 SEA and18 SWAP fractions, 46 and 8 fractions reacted with NCI serum respectively (Table 3). Dot blot pattern for fraction number 2 of SEA after second column separation was shown in Fig. 4b as an example. D3 and G5 fractions showed strong reactivity and D3 fraction had single retention peak while G5 fraction had two peaks as shown in Fig. 4a. The pooled sera used in dot blotting experiments were diluted 1000 times.

3.5. Amino acid sequencing

As shown in Table 3, after identification of 107 SEA and 18 SWAP fractions, we selected 61 SEA and 10 SWAP fractions by excluding the fractions reactive to NCI serum. For amino acid sequencing, we analyzed their retention peak patterns as shown in Fig. 4a, and picked 17 from SEA and 10 from SWAP that had single peak. Finally we obtained four N-terminal sequences, 2 from SEA and 2 from SWAP as shown in Table 4.

3.6. Homology search

The four identified N-terminal amino acid sequences were applied to the NCBI/BLAST/blastp suite programs. After the homology search

Table 4
The amino acid sequences obtained from the fractions and the candidate genes with their homology and pI range

Fraction no.	pH range	N-terminal seq.	Identified homology	Accession no.	pI[a]
F29.2E. SEA	pH<4.80	MCVLPVD	60 CILPVD 65	AAX24607.2	4.70
			39 CVLP–43	AAW27155	4.60
F2.3D.SEA	8.49–8.4	MAVLPPIYKYL	3——PILYKYL 9	AAW27472.1	8.03
			281 MAVLP———— 285	AAW26143.1	8.02
F12.H1. SWAP	8.40–8.10	VPTNQN	415-PTNQ-418	AAW27566	8.39
			211-PTSQN 215	AAW26607.1	8.40
F12.F1. SWAP	8.40–8.10	KRRGPPGEER	131-RRSNPPTEE-139	AAW27690.1	8.49
			19——PPSEE-23	AAX25883.1	8.49

[a] pI: has been calculated from DNAstar software (DNASTAR Inc., Madison, WI, USA).

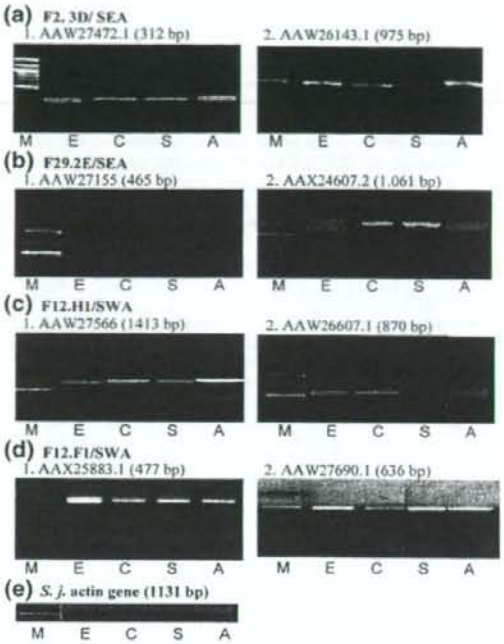

Fig. 5. mRNA expression of the selected candidate genes from different developmental stages of *S. japonicum*. PCR fragments with expected size amplified from the different stages of the *S. japonicum* were obtained using primer sets designed for the eight candidate genes and the actin gene as a control. Lane M: molecular weight maker; lane E: egg; lane C: cercariae; lane S: 24 h cultured schistosomula; lane A: adult worm. (e) *Schistosoma japonicum* actin gene.

we set the selection criteria of the candidate genes as follows, (1) high homology on the N-terminal sequences (2) deduced pH compatible with the pI range of first dimension. Two candidate genes for each amino acid sequence were selected as shown in Table 4.

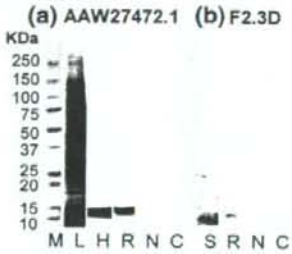

Fig. 6. Reactivity of the recombinant protein of AAW27472.1 (a) and its original fraction, F2.3D/SEA (b) with RAC vaccinated serum. (a) Lane M: molecular weight marker; lane L: CBB stained pattern of total lysate after induction with IPTG. Lanes H, R, N and C Western blotting patterns of the total lysate probed with a panel of sera; lane H: Anti histidine tag antibody; lane R: RAC serum; lane N: NCI serum; lane C: NC serum. (b) Lane S: Silver staining patterns of the original fraction, F2. 3D fraction. Lanes R, N and C: Western blotting patterns of the F2. 3D fraction probed with a panel of sera; lane R: RAC serum; lane N: NCI serum and lane C: NC serum.

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3.7. Expression of mRNA encoding the candidate genes in different developmental stages

mRNA from eggs, cercariae, 24 h culture schistosomulae and adult worms of the Chinese strain of *S. japonicum* was used for RT-PCR amplification of the selected candidate genes. All the candidate genes were expressed in all the stages of the parasite, as shown in Fig. 5, except for the genes AAW27155 that was expressed only in the egg stage and AAW26143.1 that is expressed in the egg, cercariae and adult but not in schistosomulae (Fig. 5). All the amplified PCR fragments had exactly the expected molecular weight as in the NCBI database.

3.8. Recombinant and native protein reactivity with RAC vaccinated serum

Protein expression in *E coli* using recombinant pET100/D-TOPO expression vector bearing each PCR fragment was confirmed by the detection of a band in Coomassie Brilliant blue stained SDS-PAGE after IPTG induction and by western blotting probed with Anti His-tag Antibody or with vaccinated sera (data not shown). Two candidate genes named AAW27472.1 (Fig. 6) and AAX25883.1 (Fig. 7) with recombinant proteins having molecular weights 15.5 kDa and 24 kDa respectively were strongly recognized by RAC vaccinated sera but not reactive with NCI or NC sera. While the recombinant protein AAW27690.1 whose molecular weight was 35 kDa showed weak reactivity with RAC vaccinated serum but strong reactivity with NCI as shown in Fig. 7. Original native protein fractions of AAW27472.1, AAX25883.1 and AAW27690.1 that were F2.3D for AAW27472.1 and F12.1F for the latter two genes were analyzed by the western blotting analysis to compare with the recombinant protein patterns. As is shown in Fig. 6, native protein that corresponds to the same molecular weight around 11 kDa of the recombinant was detected by RAC serum.

Fig. 7 also shows strong reactive band of around 18 kDa in the F12.1F native fraction with RAC serum but not with NCI serum that may correspond to the AAX25883.1 recombinant protein of 21 kDa. Interestingly F12.1F fraction contained reactive band of 25 kDa with NCI serum that almost correspond to AAW27690.1 recombinant pattern.

4. Discussion

We have already reported that high levels of protective immunity were obtained by UV-attenuated cercaria vaccination in Chinese domestic pigs [20]. In this study we used CLAWN miniature pigs for vaccination with RAC and observed a comparable level of protective immunity as seen in the domestic pigs (Table1). Previous studies have shown that serum of RAC vaccinated mice can transfer protective immunity to naive recipients [25,44]. We concentrated on the analysis of antibodies in the development of resistance in this RAC vaccine miniature pig model.

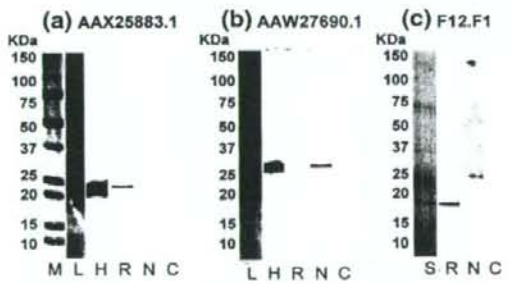

Fig. 7. Reactivity with RAC vaccinated serum of the recombinant proteins of AAX25883.1 (a) and AAW27690.1 (b) and that original fraction, F12.1F SWAP (c). (a) and (b) Lane M: molecular weight marker; lanes L: CBB stained pattern of total lysate after induction with IPTG. Lanes H, R, N and C: Western blot patterns of the total lysate probed with a panel of sera; lanes H: Anti histidine tag antibody lanes R: RAC serum; lanes N: NCI serum and Lanes C: NC serum. (c) Lane S: Silver staining patterns of the original fraction F12. F1 fraction). Lanes R, N and C: Western blotting patterns of the F12. F1 fraction probed with a panel of sera; lane R: RAC serum; lane N: NCI serum and lane C: NC serum.

In the present study, sera from RAC vaccinated pigs reacted with a number of proteins of SEA whereas sera collected from NCI showed different pattern of reaction (Fig. 1a).

The strong reactivity to SEA by the RAC vaccinated and NCI pigs could be explained by cross-reactivity of the provoked antibodies against components of cercaria to schistosomula stage worms or by the existence of common molecules shared between young worm and eggs. The significant difference of the reactive SEA proteins against RAC vaccinated and NCI sera suggests that the mode of immunization is different between those two methods [45,46].

It is well known that much of the antibodies of infected subjects are directed against carbohydrate determinants from the adult worms and the eggs [47]. We confirmed our major RAC vaccinated antibodies were directed against peptide by treating crude SEA and SWAP with glycopeptidase A, to remove the glycoconjugates. This treatment did not abolish the basic reactivity detected by the western blotting patterns of SEA or SWAP (data not shown).

Then we tried to identify a series of unique proteins that are recognized by RAC vaccinated sera but not by normal cercaria infected sera (NCI). As shown in Fig. 2 and Table 3, two-dimensional (2D) liquid chromatography successfully resolved the peptides that were shown to be reactive to RAC vaccinated serum. Although it is not clear that the 2D-LC has significant advantage over the 2D-SDS PAGE, using 2nd dimensional column, we could physically separate totally 2496 SEA fractions from 42 positive fractions of the first dimensional column and identified 107 fractions that were specifically reactive with RAC serum. Separation of intact proteins coupled with fraction collection was another advantage for this method [43]. Although our Edman degradation method could not identify many peptides, the discrimination ability from such a crude extract can be tolerated for further study.

We successfully obtained enough length of N-terminal sequences; 4 fractions out of 27 (Table 4). As shown in Figs. 6 and 7, even after the 2D column separation, each fraction contained several proteins as visualized by silver staining or by western blotting. The overall efficiency of the N-terminal sequencing of the fractions by the Edman Reaction is dependant on the purity and quantity so that we might have picked up major peptide's sequences in terms of quantity. Of course there is a fact that many schistosomal proteins were glycosylated and were possibly N-terminally blocked for the Edman Reaction. There is no doubt that MS-based protein identification is much faster and technically easier and currently the first choice over Edman degradation. Despite the fact that most of biological samples are a mixture even after physical separation as used in this study, the power of MS-based protein identification currently available should enable estimation of likeliness and relative abundance of identified proteins based on the number of peptides and coverage. The MS-based method must be more promising for this kind of identification.

After we got four sequences, the current database of ESTs from *S. japonicum* was used for the homology search. Unexpectedly, we have got only a limited level of homology to all the input sequences as was also noticed in the previous study [48]. Because of a large size of *S. japonicum* genome, it has not been subjected to full scale genome sequencing; [49,50]. Of course ESTs do not cover full length of the coding regions and a limited portion of transcripts are likely deposited in the EST database [51]. In order to increase the probability of selection of the candidate genes that were picked by their homology, pI was set as a second criterion for the selection (Table 4). His-tag fused recombinant proteins from the entire listed candidate genes were confirmed by western blotting using anti-his tag antibody and by

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their expected molecular weights, and only three recombinant proteins of AAW27472.1, AAX25883.1 and AAW27690.1 showed reactivity to RAC vaccinated serum.

The recombinant proteins obtained from the cloned AAW 27472.1 and AX25883.1 were highly reactive to RAC vaccinated serum but marginal with NCI serum that exactly reproduced the original fraction's behavior (Figs. 6 and 7). While the recombinant protein obtained from the cloned AAW27690.1 showed weak reactivity with RAC vaccinated serum, it showed strong reactivity with NCI as shown in Fig. 7. Therefore, AX25883.1 is more probable to match with the obtained N-terminal sequence.

The expected molecular weight of AAW27472.1 was 12.475 kDa but the protein in original purified fraction showed about 11 kDa as shown in Fig. 6. This may be a result of post-translation modification that often occurs in schistosomes [52]. AAW27472.1 was revealed to be a hypothetical protein, but had 23% homology with Cathepsin B endopeptidase (*S. japonicum*), and had 26% homology with cathepsin B endopeptidase (*S. mansoni*). In contrast to AAW27472.1, the expected molecular weight of AAX25883.1 was 18 kDa, and the protein in original purified fraction showed same molecular weight as shown in Fig. 7. AAX25883.1 was revealed to be a Syntaxin N-terminus domain that is a neuron system-specific protein implicated in the docking of synaptic vesicles with the presynaptic plasma membrane and had 23% homology with crystal structure of the 26 kDa glutathione S-transeferase of *S. japonicum*. The expected molecular weight of AAW27690.1 was 25 kDa, and the protein in original purified fraction, showed the same molecular weight as expected as shown in Fig. 7. AAW27690.1 was revealed to be a NADH ubiquinone oxidoreductase subunit of NDUFA12 and had 26% homology with "NADH dehydrogenase subunit 5". As the original purified fraction showed a reactive band around 25 kDa, this may be explained by the same reason as AAW27472.1.

In the present study, we have focused on the identification of soluble proteins and did not analyse the membrane protein that is expected to be another source of vaccine candidates. This 2D column system is applicable to the detergent solubilized membrane proteins using 2% Trion X-100 [53]. Membrane protein must be the next target for our study.

Although we have not yet examined the efficacies of these subunit candidate vaccines, we concluded that our 2D column protein fractionation system was simple and effective to identify the immuno-reactive proteins from crude extract.

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VERSITA

RESEARCH NOTE

Isolation of the cDNAs encoding secreted and membrane binding proteins from egg of *Schistosoma japonicum* (Chinese strain)

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Abstract

The granulomatous reaction which occurs around egg trapped in the intrahepatic venules ultimately may lead to fibrosis, which is the main pathogenesis of schistosomiasis. The excreted proteins from eggs play an important role during this process, and they may be a target for developing new strategies to control the hepatic pathogenesis caused by schistosome infection. In this study, fifteen genes encoding secreted or membrane binding protein were identified with the signal sequence trapping method by retrovirus mediated expression screening (SST-REX) of cDNAs from the egg of *Schistosoma japonicum* (Chinese strain).

Keywords

Schistosoma japonicum, secretory or membrane binding protein, signal sequence trapping (SST)

Schistosomiasis is spreading throughout South America, Africa and Asia with the implementation of irrigation programs, and thus hundreds of millions are exposed to infection (Chitsulo *et al.* 2000). Severe disease is associated with high rates of infection and represents an important health problem for a number of subtropical countries. In persons infected by schistosomes, severe clinical forms are mainly characterized by hepatosplenomegaly, high portal hypertension and ascites, which are the consequences of a chronic inflammation caused by the egg products (Phillips and Lammie 1986). Proteins excreted from the egg surface of schistosomes are exposed to the host liver tissue, which may play a critical role in the process of egg granuloma and fibrosis formation through mediating inflammation; they thus present as potential targets for developing new methods to prevent liver granulomatous hypersensitivity, and are of additional interest as candidate vaccine antigens of anti-pathogenesis.

Although there are about 100 000 *Schistosoma japonicum* (Chinese strain) expressed sequence tags (ESTs) and 17 000 *Schistosoma mansoni* expressed sequence tags (ESTs) in the dbEST database (LoVerde *et al.* 2004, Liu *et al.* 2006), recent research shown that some important genes are not included (Smyth *et al.* 2003, Wuhrer *et al.* 2006). Moreover, many schistosome mRNAs do not share sequence identity with proteins of known function only about 20–25% of the total schistosome genes have been discovered, and there is no effective technique available to identity target genes for developing an anti-pathogenesis vaccine for schistosomiasis.

Schistosome egg excreted proteins posses N-terminal hydrophobic signal peptides that direct the traffic of these proteins through the secretory pathway to the cell surface. The secretory signal sequences of proteins from different organisms are functionally interchangeable, highlighting the conserved nature of the secretory apparatus. These observations led to the development of many signal sequence trapping (SST) methods of identifying secretory molecules by expression screens in heterologous expressing systems (Tashiro *et al.* 1993, Klein *et al.* 1996, Kojima and Kitamura 1999, Moffatt *et al.* 2002, Smyth *et al.* 2003).

This is the first report of using signal sequence trapping method to identify the secreted proteins from *S. japonicum* (Chinese strain) egg cDNAs possessing a signal sequence

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Table I. Genes harbouring signal sequence isolated from egg of *Schistososoma japonicum* (Chinese strain)

Name	Accession no.	Kozak seq[a]	Putative signal sequence[b]	SignalP-NN C	Y	S	s	Signal P-HNN	No. of internal transmembrane domain
SjP4001	AY570756	GAA_	MFKMRIINLVNISTVLLLINLLQTKSQ GH···	N	Y	Y	Y	SP	1
SjP1531	AY570742	TAA_	MFKMRIINLVNISTVLLLINLLQTKSQ VN···	N	Y	Y	Y	SP	2
SjP3842	AY570748	GAA_	MFKMRIINLVNISTVLLLINLLQTKSR LV···	N	Y	Y	Y	SP	3
SjP3611	AY570744	AAA_	MRIIILGIISTVLLLINLLQTKSQ TQ···	Y	Y	Y	Y	SP	2
SjP1084	AY570737	AAA_	MRIINLVIISTALLLINLLQTKSQ AN···	Y	Y	Y	Y	SP	2
SjP3742	AY570746	AAA_	MQMLNFVKIPTLLLLLHVISINA QL···	N	Y	Y	Y	SP	1
SjP1183	AY570783	AAA_	MQMLNFVKISTMLLLPQLISTNT QH···	N	Y	Y	Y	SP	1
SjP171	AY570743	TCA_	MIPTIKLLSLVCLLSYVKA GV···	Y	Y	Y	Y	SP	1
SjP1412	AY570741	GAA_	MYPLLICLIVLSSMITLKSQS VQ···	Y	Y	Y	Y	SP	1
SjP391B	AY570749	GAT_	MKMRGSANQLVTCILTLVIFTGTTLTQS SD···	Y	Y	Y	Y	SP	1
SjP4071	AY596288	CAA_	MLYLFSVLFVVNITLA VT···	Y	Y	Y	Y	SP	1
SjP3782	AY570747	AGA_	MIMFDPTILMKQFLVVFVSFSIF IC···	N	Y	Y	Y	SP	1
SjP422	AY570752	GTG_	MHECMIVFFIFAVVSIYYADA ES···	Y	Y	Y	Y	SP	1
SjP122	AY570740	ATA_	MMNKLWLIVAIFTMIVVTNQTNA QA···	Y	Y	Y	Y	SP	1
SjP3811	AY596287	GCG_	MSFNLNQMNSLLYWLIPSIYFNILYLDNSVA LF···	Y	Y	Y	Y	SP	4

[a]The Kozak consensus sequence for eukaryotes is RCC_, where R is A or G_, represent initial codon ATG; [b]the putative cleavage point of each signal peptide is denoted by a space followed by the first two N-terminal residues of the processed protein.

were isolated with a signal sequence trap by retrovirus-mediated expression screening (SST-REX). In this SST-REX system, the vector pMX-SST was constructed by Kojima and Kitamura (1999), a truncated MPL^M (ΔMPL^M, which belongs to the cytokine receptor family and is known to transmit a proliferation signal in interleukin-3 dependent cell lines, including the mouse pro-B cell line Ba/F3) which is used as a reporter. When a foreign cDNA possessing a signal sequence is inserted upstream of ΔMPL^M to replace the ΔG^M sequence in the vector pMX-SST, the fusion expressing ΔMPL^M will be transported onto the cell membrane and transmits a proliferation signal, which permits Ba/F3 cell growth in the IL-3 free medium otherwise, the Ba/F3 cells die as they lack a proliferation signal. Then the foreign cDNA fragments containing a signal sequence can be recovered from the genomic DNA of the factor independent growth Ba/F3 cells by PCR.

The life cycle of *S. japonicum* (Chinese strain) was maintained in *Oncomelania hupensis* snails and New Zealand white rabbits in the Department of Schistosomiasis Control Jiangsu Institute of Parasitic Diseases. Eggs were collected from the liver tissue of New Zealand rabbits infected with cercariae of *S. japonicum* (1500 per rabbit) after 45 days (Dalton *et al.* 1997) method. Egg mRNA extracted and purified by oligo (dT)-cellulose chromatography using a QuickPrep@ mRNA Purification Kit (Phamarcia) following the manufacturer's instructions was transcribed into double cDNA with random primers by SuperScript™ Choice System (Invitrogen). cDNA fragments (>400 bp) were excised from electrophoresis gels purified by digesting the low melting agarose gel with β-agarase I (NEB) and precipitated with cold ethanol then the purified cDNAs were added to the BstX1 adapter at its 3', 5' end and cloned into the BstX1 digested pMX-SST vector (Kojima and Kitamura 1999). The resultant *S. japonicum* (Sj) egg cDNA-ΔMPL^M SST library was electroporated into Ecoli.DH10B competent cells and plated onto LB plates containing ampicillin (100 μg/ml). A 0.66×10^6 cfu of the Sj egg cDNA-ΔMPL^M SST library was obtained inferred from restriction analysis of the plasmid DNA of 20 colonies selected randomly by EcoR1 digestion. High titer retroviruses representing the Sj cDNA-ΔMPL^M SST library were produced using the packaging cell line 293T by co-transfecting the Sj cDNA-ΔMPL library plasmids into 293T cells together with plasmid pE-eco expressing Ecotropic env protein and plasmid pGP expressing gag-pol protein (TaKaRa Retrovirus Packaging Kit Eco) using the Fugene 6 reagent (Roche) (Pear *et al.* 1993).

About 1×10^7 Ba/F3 cells were infected by 10 ml of the retroviral supernatant containing 3×10^6 virus particles, following incubation at 24 hours with retroviruses in the presence of polybrene and IL-3. The infected Ba/F3 cells were washed with PBS (-) three times, then the cell was seeded in twelve 96 multiwell plates (1152 wells) in the absence of IL-3. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 to 2 weeks factor-independent growth was observed in 210 wells. These clones were expanded, and their genomic DNA was isolated. Integrated cDNAs were recovered by PCR with a pair of specific vector primer (GGGGG TGGACCATCCTCTA and CGCGCAGCTGTAAACGGT AG) one hundred and fifty nine cDNA fragments were produced and sequenced after being TA cloned with PCR2.1 vector (Invitrogen).

All the sequences were analyzed with the Editseq program of DNAstar (DNASTAR Inc., Madison, WI, USA) software to find the fusion open read frame (ORF) between egg cDNA and ΔMPL^M, and matched to the Genbank and dbEST databases with the blast program of the National Center for Biotechnology Information (NCBI). The signal sequence prediction was performed using the SignalP3.0 server (Nielsen *et al.* 1997). The internal transmembrane domains were predicted with the TMpred server (Hoffmann and Stoffel 1993). The results of DNA sequence analysis showed that the 159 egg cDNA fragments belonged to 35 kinds of different sequences, each with its own fusion open read frame of the egg cDNA

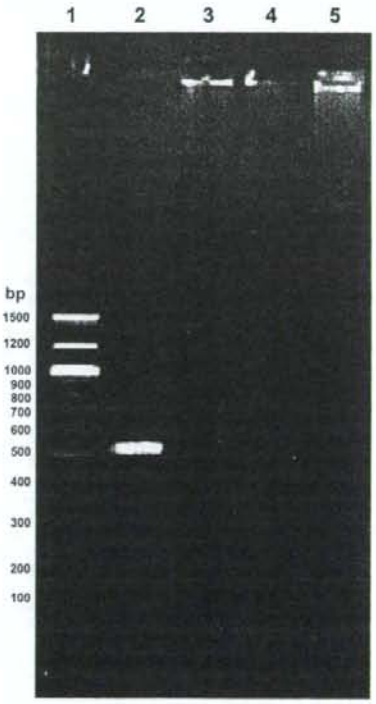

Fig. 1. The PCR product of *Schistosoma japonicum* genomic DNA amplified by gene specific primer of gene possessing signal sequence: Lane 1 – 100 bp DNA molecular weight marker. Lane 2 – the PCR product amplified from genomic DNA of *S. japonicum* by gene specific primer of SjP1183. Lane 3 – the PCR product amplified from genomic DNA of *S. japonicum* by gene specific primer of SjP3742. Lane 4 – the PCR product amplified from genomic DNA of *S. japonicum* by gene specific primer of SjP1531. Lane 5 – the PCR product amplified from genomic DNA of *S. japonicum* by gene specific primer of SjP4001

and ΔMPL^{M}, and encoding a hydrophobic domain at the N-terminal. These sequences could be attributed to two different groups: sequences homologous to schistosome ESTs (31 cDNA fragments), and sequences of genes with known function (4 sequences).

We also found that the internal hydrophobic domain of each gene could function as a signal sequence, an example being the sequence SjP110 (sequence data not published), which encodes the partial amino acid sequence of *S. japonicum* actin; translation was initiated at Met(134thaa) of the actin ORF. The amino acid sequence from 134aa to 154aa produced a positive C and S score as predicted by the SignalP-NN server, implying that the internal hydrophobic domain of this protein could also be able to trapped by the signal sequence trapping method. Thus, the signal sequence of an unknown gene selected by SST must be confirmed further by checking its full length cDNA sequence. To investigate the full length cDNA sequence of each cDNA fragment trapped by SST in this study, we designed and synthesized the gene specific primer and nested gene specific primer following each egg cDNA sequence trapping by SST to amplify the 5' or 3'end sequence of each SST trapped cDNA fragment by a rapid amplification cDNA end method using GeneRacer Kit (Invitrogen). The PCR products were purified and again amplified by nested PCR with the nested gene specific primer. The specific band produced by the nested PCR was sequenced after being TA cloned. The full length cDNA sequences of egg genes were determined by overlapping the 5' end cDNA sequence, SST trapping cDNA sequence and 3' end cDNA sequence of each egg gene possessing a signal sequence. In total, fifteen full length *S. japonicum* egg cDNA sequences were acquired and their signal sequence and internal transmembrane domains again determined with SignalP3.0 and Tmpred; the results were identical to the original prediction

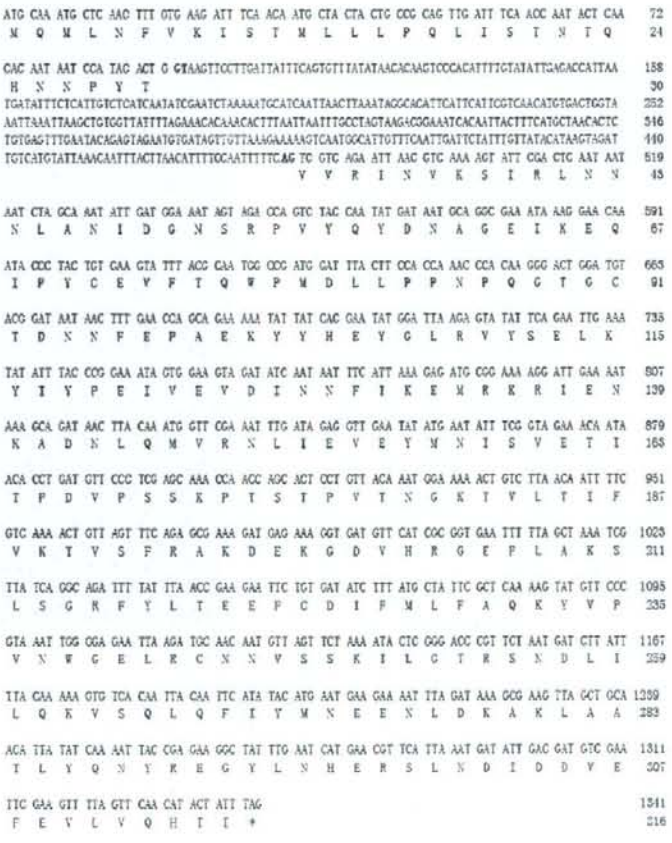

Fig. 2. The genomic DNA sequence graphic of gene SjP1183. The underlined letters present an intron sequence

(Table I). Among them, 11 genes expressed excreted proteins, while 4 were membrane binding proteins.

By aligning the deduced amino acid sequences of these fifteen genes trapped by SST, we found that the signal peptide sequence of gene SjP1531 was the same as gene SjP4001 and similar to genes SjP3842, 3611, and 1084, and the signal peptide sequence of gene SjP3811 was very similar to gene SjP3742 (Table I). To provide insight into the relationship between the signal peptide sequence and mature peptide sequence of these genes, 4 pairs of gene specific primers were designed and synthesized following to flanking sequence of the signal peptide cleavage site of these genes (primer SjP 1183 forward: 5'-TGCTACTACTGCTGCAGTTGATTTC-3', primer SjP1183 reverse: 5'-CGCCTGCATTATCATATTG GTAGA-3', primer SjP3742 forward: 5'-ATAATTGAGATT TTCCTGTGAACGTTTACTC-3', primer SjP3742 reverse: 5'-CAATCGAGCGGAGAAATCACTT-3', primer SjP4001 forward: 5'-TTCTGTGAACGTTTTCGTATCTGAG-3', primer SjP4001 reverse: 5'-ACTGACCCTTTAGGAATCTGA GATT-3') and primer SjP1531 forward: 5'-ACTTTGTGA ATATTTCAACTGTGCTACTT-3', primer SjP1531 reverse: 5'-GGATTCCACTCGACCAAAACTTC-3'). The forward primer was located upstream of the cleavage site of the gene, and the reverse primer was located downstream of the cleavage site of the gene. The genomic DNA of *S. japonicum* (Chinese strain) was amplified with these gene specific primers, a 542 bp specific DNA band was produced when the genomic DNA was amplified with the gene specific primer of SjP1183 (Fig. 1) this band is longer than the expected cDNA length (149 bp) between the forward primer and the reverse primer on the cDNA fragment, and the DNA sequencing data showed that there was an intron of 393 bp between the signal sequence and the mature portion of the gene SjP1183. No DNA band was produced when the genomic DNA were amplified by the gene specific primers of gene SjP1531, SjP4001 or SjP3742 (Fig. 1). These results suggest that the splicing model between the signal sequence and mature portion of gene SjP1183 involves alternative splicing, whereas the signal sequence of gene SjP3742, SjP1531 and SjP4001 might come from independent transcription of mRNA and their splicing model might be due to *trans* splicing (Sutton and Boothroyd 1986), but this requires confirmation. If true, this means that both an alternative splicing model and a *trans* splicing model exist during the transcribing process of egg genes of *S. japonicum* (Chinese strain) simultaneously. As the signal sequence of SjP4001 is same to the one of SjP1531 completely, this phenomenon confirmed further that different gene could share same signal sequence and a same signal sequence could also be spliced with different gene through alternative splicing model or *trans* splicing model.

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