MOLECULAR CHARACTERIZATION AND TISSUE DISTRIBUTION OF 23 KDA MEMBRANE PROTEIN ENCODING GENE FROM *SCHISTOSOMA MEKONGI*

By
Ms. Kaisone CHANDA

A Thesis Submitted in Partial Fulfillment of the Requirements
for Master of Science (BIOSCIENCE FOR SUSTAINABLE AGRICULTURE)
Graduate School, Silpakorn University
Academic Year 2020

การศึกษาคุณลักษณะทางโมเลกุลและการกระจายตัวของยีนโปรตีนเมนเบรนขนาด 23 kDa จากพยาธิใบไม้เลือด *Schistosoma mekongi*

โดย

Ms.Kaisone Chanda

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต

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By	Kaisone CHANDA
Field of Study	(BIOSCIENCE FOR SUSTAINABLE AGRICULTURE)
Advisor	Assistant Professor Narin Preyavichyapugdee , Ph.D.

Graduate School Silpakorn University in Partial Fulfillment of the Requirements for the Master of Science

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MS. KAISONE CHANDA : MOLECULAR CHARACTERIZATION AND TISSUE DISTRIBUTION OF 23 KDA MEMBRANE PROTEIN ENCODING GENE FROM *SCHISTOSOMA MEKONGI* THESIS ADVISOR : ASSISTANT PROFESSOR NARIN PREYAVICHYAPUGDEE, Ph.D.

The objectives of this study are: (1) To clone and characterized 23 kDa membrane protein gene from *S. mekongi*. (2) To predict the potential immunogenic epitope of 23 kDa membrane protein from *S. mekongi*. (3) To study the distribution of Smek23 in parasite tissues. *Schistosoma mekongi* is one important human parasitic which causes liver damage in South-east Asia. 23 kDa protein is an integral membrane protein of the blood fluke genus *Schistosoma* and its expressed in all parasite stages. The cDNA encoding Smek23 of adult *S. mekongi* was cloned and sequenced. The nucleotide sequence of Smek23 was 686 bp in length. The nucleotide sequence of Smek23 showed an open reading frame encoding 23 kDa integral membrane protein containing 218 amino acids. The expected molecular weight of Smek23 determined from its constituent amino acids is 23.62 kDa. Smek23 has four transmembrane domains (TM). The localization of Smek23 demonstrates that the molecule is localized to tegument membrane compartments. The Smek23 amino acid sequences showed the highest degree of identity with the *S. turkestanicum* 23 kDa. The identity of Smek23 amino acid sequences with other schistosome species (*S. mansoni, S. japonicum* and *S. haematobium* showed at 87.6-89.9% respectively. Phylogenetic analysis in this study revealed that Smek23 exhibited a distant evolutionary relationship from the Tetraspanin of mammalian host species (CD63, CD81). The low degree of conservation observed from amino acid sequences of mammalian hosts could reveal its applicability for use as the vaccine candidate against the schistosome infection which may not interfere with the hosts' Tetraspanin molecule during the vaccination. Three candidate B cell immunogenic epitopes were predicted by four programs (Hopp and Woods, Welling, Parker, and B-EpiPred). These regions were 111-KIDA-114, 125-DHP-127, and 150-PNDYKGSVPDSCKEGQVPYT-169. All of these three regions were located in extracellular domain 2 (EXT 2), which is a large hydrophilic domain (LHD) of Smek23 molecule. The predicted epitopes provide promising vaccine candidates and could be tested by a wet laboratory as a targeted vaccine against *S. mekongi* infection.

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CHAPTER I

INTRODUCTION

1.1 Introduction to the research problem and its significance

Schistosomiasis is a disease caused by *Schistosoma* species, a parasitic blood-dwelling fluke which are causative agents in human and animal in tropical region. In terms of its public health, schistosomiasis ranks second after malaria. The majority of cases are occurring in South America, the Caribbean, Middle East, sub-Saharan Africa and Asia. At least 6 schistosome species are causative agent that infected in humans: *Schistosoma haematobium, Schistosoma intercalatum, Schistosoma guineensis Schistosoma japonicum, Schistosoma mansoni,* and *Schistosoma mekongi* (Gordon et al., 2019). There are more than 200 million people are infected, more than 700 million people are at risk of infection worldwide and approximately 200,000 deaths annually (Nelwan, 2019). In Asia three schistosome are endemic, all are cause of intestinal schistosomiasis: *S. japonicum* is the most prevalent (occurring in China, the Philippines and some area in Indonesia), *S. malayensis* (Malaysia) and *S. mekongi* (Mekong river basin including Lao People's Democratic Republic (Lao PDR) and Cambodia), Along Mekong river near the border between southern Lao People's Democratic Republic (Laos) and northern Cambodia, there are approximately 1.5 million people are at risk of infection by *S. mekongi* (Attwood, 2008).

S. mekongi was first identified in 19 century (Kitikoon, 1980). Even though the morphology is very similar to *S. japonicum*, but the differences between two species are geography distribution and morphology. *S. mekongi* are zoonotic that can infect both human and animal (dogs and pig are reported to be a natural and experimental host, respectively). The lifecycle of *S. mekongi* is complex involving sexual reproductive phases in vertebrate hosts (often a mammal) and asexual reproductive phases in invertebrate hosts (fresh water snails). Due to the requirement of an intermediate host snail, schistosomiasis mekongi is endemic in Mekong river basin where snail habitats and susceptible transmitting snails are present. The intermediate host of *S. mekongi* is *Neotricula aperta* (previously *Lithoglyphopsis aperta*).

The transmission is strongly associated with human sanitation, open defecation is a main factor of disease dissemination. Poor knowledge about the disease, poor sanitation, and a lack of effective health policies promote the transmission of schistosomiasis in endemic area. So successful strategy to control this schistosomiasis are required, including management of the human definitive host, animal reservoir hosts and environment.

Clinical feature of schistosomiasis expresses as two distinct stages: First, the prepatent stage begins as a skin rash caused by an immune response to the penetrating cercaria. Second the patent stage relates with schistosome egg production and can be further divided into acute and chronic schistosomiasis (Da Silva et al., 2005). Acute disease is commonly seen in naïve persons, whereas chronic disease, is more likely seen in resident in schistosome-endemic areas. Chronic disease occurs due to retention of eggs in the liver, spleen, and intestinal walls. Entrapment of eggs in the tissues is the beginning of inflammation and causes of granuloma in Schistosomiasis. Eggs in the tissues secret product that elicit immune reaction that end with granuloma formation and severe fibrosis in various tissue. This can result in hepatosplenomegaly, portal hypertension, abdominal pain, and bloody diarrhea (Da Silva et al., 2005).

At present, the control measure of schistosomiasis still depend on a single drug praziquantel (PZQ), which World Health Organization recommended Praziquantel (PZQ) recommended as the drug of choice for treatment and control of human schistosomiasis and using as drug for mass administration programs since1970s (Gönnert & Andrews, 1977). Praziquantel, a pyrazinoisoquinoline derivative, it is a broad spectrum, safe and highly efficacious anti-schistosomal which is principally active against the adult stage of all the schistosome species (Gray et al., 2011; Sabah et al., 1986; Utzinger & Keiser, 2004; Utzinger et al., 2003; Vale et al., 2017). Due to its intensive large scale and repeated use of the same chemotherapeutic agent in the field, there is serious concern for the emerging of drug-resistant mutants of the parasite (Coles, 1989; Coles & Kinoti, 1997; Geerts et al., 1997). Effective vaccine is an alternative strategy to combat against schistosomiasis, which may use alone or in combination with chemotherapeutic drugs could provide effective and sustainable strategy for long term controlling the transmission of the disease (Botros et al., 2005; Doenhoff & Pica-Mattoccia, 2006; Fonseca et al., 2012).

A 23 kDa transmembrane membrane protein is one of tetraspanin proteins family (Gaugitsch et al., 1991). Its expressed in all stages of schistosome, including cercariae, lung-stage schistosomula, and adult worms (Cai et al., 2008; Harn et al., 1985; Jiang et al., 2010; Oligino et al., 1988; Tran et al., 2006). The 23-kDa membrane protein from *S. mansoni* (Sm23) and *S. japonicum* (Sj23) were shown to be promising immunogenic antigen for prophylaxis and immune-diagnosis. Sm23 is one of five major tegumental membrane proteins which elicits strong antibody response to schistosomiasis infection in mice, rats and human (Krautz-Peterson et al., 2017). The protein has been detected by all stages of human infected serum, especially the lung stage, and therefore this protein could be one of vaccine candidates (Reynolds et al., 1992b). Besides, the large hydrophilic domain (LHD) of recombinant Sj23 could induce protection against *S. japonicum* infection in various host including mice, sheep, cattle and buffalo (Shi et al., 2002a; Shi et al., 2001).

1.2 Goal objective of the study

1. To clone and characterize 23 kDa membrane protein gene from *S. mekongi*.
2. To predict the potential immunogenic epitope of 23 kDa membrane protein from *S. mekongi*.
3. To study the distribution of Smek23 encoding gene in parasite tissues by *In situ* Hybridization techniques.

1.3 Hypothesis of the study

Cloning, characterization and identification of 23kDa membrane protein gene from *Schistosoma mekongi* and predicted the potential immunogenic epitope of this molecule.

CHAPTER II

LITERATURE REVIEW

2.1 Schistosomiasis

Schistosomiasis is a chronic parasitic disease historically known as bilharzia caused by digenetic blood flukes that belong to the family Schistosomatoidae. World Health Organization (WHO) determines schistosomiasis as the second only to malaria in socioeconomic importance worldwide (Sarvel et al., 2011). There are five species of *Schistosoma* which epidemic in restricted geographic area. *S. mansoni* is the most prevalent in the Middle East, sub-Saharan Africa, South America and the Caribbean. *S. haematobium* infection is occurred in sub-Saharan Africa, North Africa, the Middle East and India. *S. japonicum* acquired only in Asia. *S. intercalatum* occurs in West and Central Africa while *S. mekongi* is restricted to Cambodia and Laos (Hagan & Gryseels, 1994). Only *S. haematobium* resides in the venous plexuses of the urinary bladder and are aetiologic agent of urinary schistosomiasis, the other four *Schistosoma* inhabit the mesenteric veins and are the causes of hepatosplenic and intestinal schistosomiasis (Kloos & Ethiopia, 2019).

Although, schistosomiasis is waterborne disease which endemic in rural area typically associated with poor rice farmers and fishermen in the tropical and subtropical zone, it is increasingly been reported among tourist people with a history of travel to endemic areas in Asia and Africa. Snail is an intermediate host which released the infective stage cercariae into water reservoir. Snails belonging to the species *Onchomelania*, *Biomphalaria*, *Bulinus*, and *Neotricula* are the vectors of *S. japonicum, S. mansoni, S. haematobium* and *S. mekongi* respectively. There are 240 million people are affected worldwide while an estimated 779 million people are at the risk of infection. There are 20 million people from 120 million infected people which develop severe clinical disease.

2.2 Epidemiology of Schistosoma species

S. mansoni is widespread in South America, the Eastern Mediterranean, the Caribbean, and Africa. Nearly 300,000 people die annually from schistosomiasis in Africa alone (van der Werf et al., 2003b). There is 90% of the 250 million people infected worldwide live in Africa where *S. mansoni* is the most prevalent species. And there is approximately 10 million pregnant women in Africa are infected (Furuhashi & Hotamisligil, 2008). *S. mansoni* is one of zoonotic schistosomiasis which infects human and wild rodents.

S. haematobium infection is a major cause of morbidity and disability in the endemic area of the Middle East and Africa, where nearly 110 million people are affected (Lara et al., 2011). In sub-Saharan Africa, *S. haematobium* is the cause of two-thirds schistosomiasis, which represents an important cause of severe urinary tract disease. Bladder wall pathology and hydronephrosis are the major causes of suffering in urinary tract schistosomiasis from *S. haematobium*. (van der Werf et al., 2003a).

S. intercalatum is a minor schistosomal species limited to a few western and central African countries (King, 2010).

Zoonotic *S. japonicum* infection are significant prevalence in China, Indonesia and The Philippines. Cattle and water buffalo contribute significant transmission role of this disease. There are approximately 60 million individuals' risk of infection and close to two million are currently infected in China and Philippines. A small focus of infection still persists on the island of Sulawesi in Indonesia. The disease has been eradicated in Japan through integrated multidisciplinary approach.

In the lower Mekong basin, *S. mekongi* is endemic along the area (Tanaka & Tsuji, 1997). Approximately, 140,000 people are at risk for infection with 80,000 found in Cambodia and a further 60,000 in Laos. While infection in animal reservoir hosts to the transmission of *S. mekongi* has not been established (Jacobson & Abel, 2007).

2.3 Life cycle of Schistosome species

The live cycle of schistosome need intermediate host snails and definite mammalian host. In the snail, this begins with the asexual reproduction development of miracidia into a sporocyst. Then sporocysts multiply and develop into infective stage cercariae. In the mammalian hosts, cercariae invade into human skin and shed their forked tail, forming schistosomula. The schistosomula migrate throughout the body's tissues through blood circulation. *Schistosomula* grow into adult worms, mate, and produce eggs.

2.3.1 Snail hosts

Schistosome eggs were released in fecae or urine from mammal hosts into the external environment. In the water, these eggs develop to miracidia, which hatch and infect intermediate host snails. *S. haematobium* infects snails of the genus *Bulinus. S. japonicum* infects snails of the genus *Oncomelania. S. mekongi* infects snails of the genus *Neotricula* and *S. mansoni* infects snails of the genus *Biomphalaria*. In the snail, the miracidium develops into a mother sporocyst, and then produces daughter sporocysts. Daughter sporocysts produce either cercaria or more daughter sporocysts. Snails can shed hundreds of cercariae daily; about 200 for *S. haematobium*, 15 to 160 for *S. japonicum*, and 250 to 600 for *S. mansoni*.

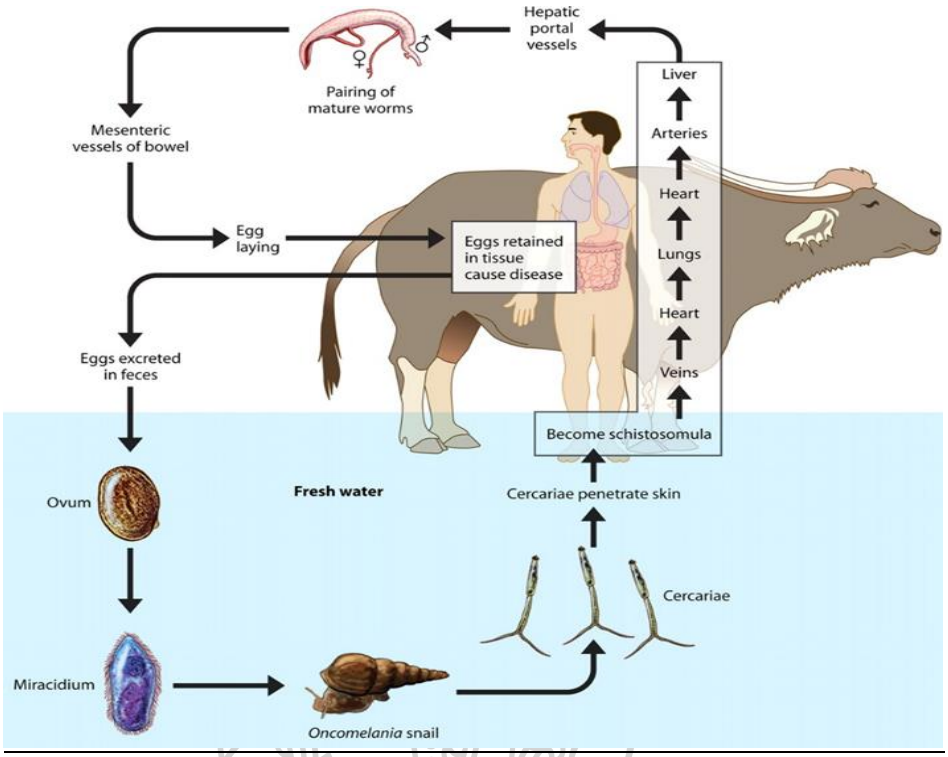

Figure 1. Schistosomiasis life cycle.
Asexual reproduction in snails and sexual reproduction in mammals.

From: McManus et al. (2010)

2.3.2 Mammalian hosts

Cercariae penetrate human skin and transform into young schistosomula by shedding their forked tail. After living two days in the subcutaneous, the parasites burrow through the dermis, penetrate a blood vessel wall, and gain access into the circulatory system. The schistosomula migrate through blood circulation to various site of body's tissue. Schistosomula become an adult schistosome worms.

Adult schistosome in humans host present in specific location to each species. *S. mansoni* worms live in small and large intestine. *S. japonicum* exists more frequently in the small intestine. *S. haematobium* survive in the bladder and ureters venules.

2.4 Pathology of schistosome species

After infective stage cercaria penetrate the skin of host, the cercariae lost their tails and turn to be schistosomula that enter circulartory system. Within 12 hours post cercaria penetration, people may develop skin rash, referred to as "swimers itch".

Schistosomula will go through blood stream to the heart. From the heart, they travel to the lung, and within 2-4 weeks they enter into liver, and become mature adult male and female worm. The male worm enwrap around the female and migrate to the mesenteric venule where eggs are produced. These sites trend to be specific for for *S. mansoni,*
S. intercalatum S. japonicum and *S. mekongi*. For *S. haematobium*, the eggs are trapped in the vesicle plexus. The pathology of schistosomiasis is caused by immunologic reactions to *Schistosoma* eggs trapped in tissues, by forming immune complexes of granuloma formation against tissue-deposited eggs.

The tissue reaction is manifested delayed type hypersensitivity through a T-cell mediated immune response against the soluble egg antigens (SEAGs). Schistosomal eggs are surrounded by white blood cell such as mononuclear phagocytes, eosinophils, lymphocytes, plasma cells and also fibroblast, which induced a granulomatous formation that lead to clinical signs of disease. The common pathologic lesion of schistosomiasis is due to granulomatous lesions which can obstruct vessels and fibro-inflammatory lesions around the trapped eggs. The number and location of eggs trapped in the tissues is a major caused of symptoms and clinical signs. In the early stage, the inflammatory reaction is readily reversible. In the chronic stages of the disease, the pathology is related to collagen deposition and fibrosis, resulting in organ damage that may be only partially reversible (Macalanda et al., 2019).

2.5 Treatments for schistosomiasis – drugs

There are four chemotherapeutic drugs for treatment of schistosomiasis: praziquantel, metrifonate, oxamniquine and artemether (Conder & Campbell, 1995; Ross, 2002). All of these drugs are useful for treatment for both individual patient and for mass drug administration for communities in endemic area. Oxamniquine is only effective against schistosomiasis mansoni; resistance to this drug by the parasite was reported. The drug has side effects include mutagenic and carcinogenic effects, as well as effects on the central nervous system, its production and commercialization are controlled and reduced. Metrifonate can be used to treat against urinary

schistosomiasis, caused by *S. haematobium* (Organization, 1995). Its use is hindered by a complex administration schedule with multiple doses required over a 2 week. However, at present there is no longer commercially available (Ross, 2002). Artemether is a semisynthetic artemisinin derivative, a compound derived from the sweet wormwood Artemisia annual. It became active after being metabolized in the blood into dihydro-artemisinin (Liu et al., 2011). Artemisinin and its derivatives are highly effective against haematophagous parasites, notably malaria, but they have also proven effective against schistosome infection (Li et al., 2012). The drug acts through haeme-dependent reduction to sequentially generate free radicals: the haeme iron first attacks and breaks the endoperoxide linkage to artemisinin, producing an oxygen-free radical, which is then rearranged to produce a carbon-free radical that causes lethal damage through the alkylation of parasite proteins (Chaturvedi et al., 2010). A disadvantage of the artemisinin derivatives is their short in vivo half-life, typically ~2 h in humans (Djimdé & Lefèvre, 2009). The current drug of choice for treatment of schistosomiasis is praziquantel. It is a 2-cyclohexycarbonyl 1,2,3,6,7,11b-hexahydro-4H-pyrazino (2,1-a Isoquinolin-4one) compound. This drug has been deployed for mass drug administration in endemic regions and remains a primary tool in the war against the disease (Khaled, 2013). Praziquantel causes severe spasms and paralysis of the worms' muscles. The exact mechanisms of action of praziquantel is still poorly understood. Praziquantel is known to induce a rapid Ca 2+ influx inside the schistosome that distort the morphology and physiology of schistosome. Jeziorski and Greenberg (2006) showed that the B subunits of voltage-gated Ca2+ channels is the prime molecular target of PZQ. Despite its high efficacy, praziquantel has limitations. The drug is only effective against adult or pre-adult forms. Furthermore, praziquantel confers no protection against subsequent infection and people may become reinfected within days of treatment (Ross et al., 2002). Treatment failures for *S. mansoni* and *S. haematobium* infections have been observed, and the presence of resistant strains has been demonstrated experimentally. Although widespread resistance to praziquantel has not been observed clinically, the application of the drug in mass treatment campaigns may result in new resistant forms emerging and new replacement drugs and formulations are needed.

2.6 Prevention of schistosomiasis – vaccines

At present, the control measure of schistosomiasis still depends on a single drug praziquantel (PZQ), which used as individual treatment and as a mass control strategy. Praziquantel-based chemotherapy effectively reduces both infections and intensity; however, it is time consuming, expensive, and cannot prevent re-infection. Furthermore, there is an increasing concern about praziquantel resistance following long-term repeated use of the drug in endemic areas. Therefore, a more sustainable option would be development of an integrated control program that, in addition to praziquantel treatment. Development of schistosomiasis vaccine, is one of strategies to prevent and control schistosomiasis. The combination strategy between vaccine and chemotherapy would decrease both morbidity and reduce the cases of re-infection. Such a novel control program for schistosomiasis would improve significantly on the current strategy, which is based on chemotherapy alone.

The alternative, a subunit vaccine, has thus been promoted as an important alternative strategy for the control and elimination of schistosomiasis (Bergquist & Colley, 1998; Khaled, 2013; Liu et al., 2011; McManus & Loukas, 2008b).

Therefore, efforts have been directed to discover and identify suitable protective antigens from schistosomes, leading to the development of recombinant vaccines, DNA vaccines, peptide–epitope-based vaccines, multivalent vaccines and chimeric vaccines (McManus and Loukas, 2008). Of the vaccines trialed, a number have been promoted for human trials, including the Bilvax vaccine based on a 28 kDa *S. haematobium* glutathione-S-transferase, which has entered phase 3 clinical trials, and a *S. mansoni* tetraspanin (Sm-TSP-2) (Tran et al., 2006), which has entered phase 1 trials. Other vaccines presented at a recent vaccine discovery workshop sponsored by the Bill and Melinda Gates Foundation in the United States identified additional vaccines still in experimental development, including Sm14, a fatty acid binding protein, a calpain (Smp80) from *S. mansoni*, and Sj23, a TSP, a triose-phosphate isomerase, an insulin receptor, and paramyosin from *S. japonicum* (Y. Zhu et al., 2004; Z. Zhu et al., 2012). An advantage of vaccination strategies against the zoonotic *S. japonicum* is that the parasite is found in a variety of domesticated animals, including water buffalo and goats in China. Researchers involved in controlling this

species in China and the Philippines have developed vaccines for use in animals as transmission-blocking vaccines, based on modelling of transmission dynamics in endemic regions (McManus et al., 2009). Antigen discovery studies are still progressing using a variety of immunomics and proteomic approaches. It is now widely appreciated that targeted approaches are required for antigen discovery, and there is continuing interest in considering fundamental cell biological and developmental understanding with molecular advances.

2.7 Schistosome tegument

The tegument of schistosomes the tegument, or body wall, of schistosomes is a dynamic host-adapted interface between the parasite and its vascular environment. The tegument is a highly polarized syncytium and possesses functional analogy with transporting epithelia, including the gut lining or the syncytiotrophoblasts of the human placenta. The tegument plays significant roles in nutrient uptake, immune evasion and modulation, excretion, osmoregulation, sensory reception, and signal transduction (Jones et al., 2004). Given the importance of the schistosome tegument in nutrition and immune evasion, proteins of this surface layer are recognized as prime candidates to target for vaccine and therapeutic drug development.

2.8 Schistosome 23 kDa protein

The schistosome tegument is dynamic host-interactive layer that is vital for parasite survival involving: immune evasion, nutrition, excretion, osmoregulation and signal transduction (Van Hellemond et al., 2006). Proteins exposed to the host on the tegumental surface are important for completion of the parasitic life cycle. 23 kDa protein is one of member of tetraspanin family. Among the tegumental parasite antigens that interplay with the host, the tetraspanin (TSP) family is of particular interest.

TSPs are membrane-spanning proteins characterized by four transmembrane domains (TM1-4) and two extracytoplasmic loop (EC1 and EC2). There are three motif structures (-CCG-,-PXSC-,and-GC-) in EC2 which contains four conserved cysteine residues and are evolutionally conserved from primitive organisms such as

schistosome to human (Huang et al., 2005; Kitadokoro et al., 2001; Mark & Rigau-Pérez, 2009). TSPs act as “molecular facilitators” with functions involved in cell proliferation, activation, motility, and metastasis.

The 23 kDa molecules of *S. mansoni* and *S. haematobium* were the first identified members of the transmember-4-superfamily (TM4SF, TSP) in schistosomes (Tran et al., 2006; Wright et al., 2012). The Sj23 of *S. japonicum* was originally identified using a monoclonal antibody that recognized the surface of *S. japonicum* (Davern et al., 1991). It was reported to be immunogenic and more than 90% of patients infected with S. japonicum had antibodies to this antigen; however, vaccination trials with this antigen generated various levels of protection (Da’Dara et al., 2008; McManus & Loukas, 2008a).

2.8.1 The 23 kDa proteins and development of vaccine

There is currently no vaccine available for using against parasitic infections in both human and animal, including schistosomiasis. Despite several researchers working to achieve this goal, one of the focuses has been on identifying new candidate immunoprophylactic targets. In 1990, World Health Organization (WHO) was set the bar to achieve protective efficacy in humans at a consistent induction of 40% protection, and although this is a modest goal, it is yet to be reached with the six most promising schistosomiasis vaccine candidates (Sm28GST, IrV5, Sm14, paramyosin, TPI, and Sm23) (Ismail, 2011). Sj23 from *S. japonicum* is one of proteins candidate that was shown to be immunogenic and is a promising antigen for diagnosis and immuno-prophylaxis. large hydrophilic domain (LHD) of *S. japonicum* 23 kDa could induce protection against *S. japonicum* infection in mice, as well as in schistosome native hosts: sheep, cattle and buffalo (Shi et al., 2002a; Shi et al., 2001). 23 kDa was originally of interest because of its diagnostic that it is related to 23 kDa protein from *S. mansoni* (Sm23), but the finding that the epitope in large hydrophilic domain of Sm23 is recognized by a passively protective monoclonal antibody, made it also a vaccine candidate (Bellacosa et al., 1991). It has been reported that Sm23, induces 40-50% protective (Bergquist, 1995), and Sm23 plasmid DNA vaccine induces 30-43% protection against infection in mice. Large hydrophilic domain of 23 kDa from

S. japonicum Chinese strain (Sj23C HD) have been cloned, expressed in *Escherichia coli* and showed the vaccine efficacy against infection in Sheep at 51.2-66.1% protection (Y. Zhu et al., 2004). The 23 kDa (Sj23) and Triose phosphate isomerase (SjTPI) from Schistosoma japonicum were evaluated as DNA vaccines in combination with IL-12 plasmid DNA adjuvant against *S. japonicum* infection in water buffalo, the result showed protection at 50% (Da'Dara et al., 2008; Yu et al., 2006).

DNA vaccines encoding the tetraspanin 23 kDa integral membrane protein (SjC23), alone or fused to bovine heat shock protein 70 (Hsp70) reduced worm burdens of *S. japonicum* infection in buffaloes by 50.9% and 45.5%, respectively, and fecal miracidial hatching by 52.0% and 47.4% (Da'Dara et al., 2008). In mice, the DNA vaccines was also determined the efficacies against. *S. japonicum*. Mice vaccinated with cocktail DNA vaccines encoding the 23-kDa tetraspanin membrane protein (SjC23) and triose phosphate isomerase (SjCTPI) showed a significant worm reduction of 32.88% ($P < 0.01$) and egg reduction of 36.20% ($P < 0.01$). The vaccine efficacy was enhanced when animals were boosted with cocktail protein vaccines; adult worm and liver egg burdens were reduced 45.35% and 48.54%, respectively. (Mark & Rigau-Pérez, 2009). Chen et al. (2011), observed that mice immunized with *Salmonella typhimurium* harboring Sj23LHD-GST promoted protection 41.69-50% in worm burden and elicited remarkable activation of antigen specific CD4 + and CD8 + T cells and high levels IL-2, IL-12 and IFN-gamma. Z. Zhu et al. (2012) showed that mice immunized with purified recombinant protein LHD-Sj23-GST (large hydrophilic domain of 23 kDa antigen of *S. japonicum* fused with Sj26GST) with Freund's adjuvant (FA), Montanide ISA 206 and Montanide ISA 70M, induced a significant protection at 58.8, 26.3% and 54.3% ($P < 0.05$) respectively, compared to those mice treated with respective adjuvant only. All three adjuvants induced increasing of the titer of total IgG and IgG subtypes (IgG1, IgG2a, IgG2b, IgG3) significantly. Which IgG antibodies, especially IgG1 and IgG3 isotypes, were confirmed to be the main humoral component that corresponds with protection against parasite (Maizels & Yazdanbakhsh, 2003; Unkeless et al., 1988). X. Wang et al. (2014) showed that SjC23 DNA vaccine could induced protection against *S. japonicum* at 46.44 % when used in combination with Lysine-

modified PAMAM-Lys. The PAMAM-Lys vector elicited a predominantly IgG2a antibody response and a tremendously increase in the production of IL-2 and IFN-gamma which increased the protective effect of the SjC23 DNA vaccine against *S. japonicum* infection (X. Wang et al., 2014). A. Da'Dara et al. (2019a) demonstrated that DNA vaccine of SjC23 could induced 30% protection against *S. japonicum* infection in buffalo. There was higher protection when using SjC23 DNA vaccine in combination with IL-12, the protection was 46%. However, they observed that the highest levels of efficacy was observed in buffalo which immunized with two-dose regimen trial by priming with the combination of SjC23-Hsp70 and SjTPI-Hsp70 plasmid DNAs followed by boosting with a combination of recSjC23 and recSjTPI. This combination trial yielded the significant level of protection by reduce worm burdens at 55.1% and eggs/gram liver at 53.2% which compared to levels in control vaccinated buffalo.

2.8.2 The 23kDa protein and development of diagnostic tools.

In 1991, the monoclonal antibody against Sj23, the 23-kDa protein from *Schistosoma japonicum* has been successfully used in immunodiagnostic assays to detect *S. japonicum* infection in Philippine patients. The sequence analysis has shown that Sj23 is the homologue, with 84% amino acid identity, of Sm23, a 23-kDa molecule from *S. mansoni*. The domain structures of Sj23 and Sm23 are strikingly similar to the human membrane proteins ME491, CD37, CD53 and TAPA- 1, which may suggest a functional role for the schistosome cellular proliferation (Davern et al., 1991). Besides, Sm23 is one of five tegumental membrane proteins (SmTsp2, Sm23, Sm29, SmLy6B and SmLy6), that recognized in *S. mansoni* infection of both permissive (mouse) and non-permissive (rat) rodent models, as well as humans. (Krautz-Peterson et al., 2017). Compared the levels of antibody raised against the hydrophilic domain (HD) of the 23-kDa membrane protein (Sj23HD) and soluble egg antigen (SEA) of Schistosome japonicum for early detection over the course of 42 days post-schistosome infection in mice. This results demonstrated that antibody raises against Sj23HD could be detected early infection in mice and immunoblotting showed greater specificity and sensitivity than ELISA for detection Sj23HD antibodies (J. Wang et al., 2011). Li et al. (2012) demonstrated that rLHD-Sj23-GST

iELISA have been used to detect the infection of *S. japonicum* in Cattle with positive rate 3-44-18.4%. Jin et al. (2010) demonstrated that the recombinant antigen which constructed an epitope of recombinant protein Sj23 and SjGCP (Gynecophoral Canal protein) was a promising diagnostic antigen for detecting *S. japonicum* in water buffalo better than signal recombinant protein Sj23 and SjGCP.

Cai et al. (2017), developed ELISA assays for diagnosis of *S. japonicum* infection based on the detection of antibodies against the large hydrophilic domain of the 23 kDa Sj23 tegumental protein (Sj23-LHD), two saposin proteins (SjSAP4 and SjSAP5) and two combinations (SjSAP4 + Sj23-LHD and SjSAP5 + Sj23-LHD. They found that a combination SjSAP4 Sj23-LHD provided the best diagnosis efficacy at 87.0% sensitivity and 96.67% specificity. Elisa based on using this combination could provide an important component for monitoring integrated control measures for schistosomiasis in the Philippines, in the future.

2.9 Prediction of B-cell antigenic epitope

The delineation define epitopes on protein antigens has attracted the attention of several scientists in recent years. Identification of epitopes on proteins would be useful for diagnostic purposes and also in the development of peptide vaccines (Schmidt, 1989). To aid experimental workers, Hopp and Woods have developed a method for prediction of antigenic determinants (Hopp & Woods, 1981). The approach of Hopp and Woods has been modified to take into account the fact that antigenic sites are on the surface of the protein and most surface residues are antigenic. Recently, Parker et al., have used three parameters - hydrophilicity, accessibility and flexibility - to predict B cell epitopes using a composite plot. This method has improved the prediction of antigenic determinants as compared to Hopp and Woods' method but misses some of the experimentally observed determinants. On the other hand, Welling et al. have calculated the antigenicity value for each amino acid from its frequency of occurrence in epitopes and used these values to predict epitopes (G. Welling et al.). The Kolaskar and Tongaonkar antigenicity calculations are an epitope analysis that is developed from Hopp and Woods and has 75% greater accuracy. For Bepipred Linear Epitope Prediction is also an analysis of

the epitope position, which is based on the relationship between sensitivity and specificity of binding with B-cells (Lapoile et al., 2006).

Therefore, this study focuses on predicting Smek23 B cell epitope which may provide a promising vaccine or immunodiagnostic target for future solution to eliminate schistosomiasis in endemic areas.

CHAPTER III

MATERIALS AND METHODS

3.1 Cloning, identification and expression of Smek23

3.1.1 Cloning identification and Characterization of Smek23 cDNA

Adult worm cDNA of *S.mekongi* from research project “Development of Serodiagnosis for *Schistosoma mekongi* in Mice by Sandwich ELISA and Localization of Cathepsin B on the Worm” which was approved by Animal Care and

Use Committee of Faculty of Tropical Medicine, Mahidol University (FTM-ACUC 023/2017). Briefl, total RNA was isolated from the whole adult *S. mekongi* worms by TRIzol reagent (Molecular Research Center, Inc.) using the protocol provided by the manufacturer, and stored at −80 ◦C. The partial cDNA sequence of the Smek23 was amplified by RT-PCR using the following set of primers: forward primer (5'-ATG GCG ACT TTG GGT ACT GGG ATG A-3') and reverse primer (5'-GAG TAG TCC ACA CAC ACT ACA CTC-3'). The primer was designed from 23 kDa protein of *S. japonicum* mRNA (GenBank: M63706.1). The PCR was performed in 35 cycles at 94 ◦C for 30 s, 55 ◦C for 30 s and 72 ◦C for 90 s. The PCR product was ligated into the pGEM-T easy vector (Promega, USA) and used for sequencing (Macrogen, South Korea).

Smek23 DNA sequences generated form cDNA clones and the deduced protein sequences were subjected to search the nucleic acid and protein database using the basic local alignment search tool (BLAST) (http://ww.ncbi.nih.gov/BLAST) and swiss-Prot database (https://swissmodel.expasy.org/). Multiple alignment of homologous sequences from closely related *Schistosoma* species were carried out by Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). Using this alignment, neighbor-joining (Saitou & Nei, 1987) analysis was performed using the Mega X (Kumar et al., 2018) with bootstrap resampling using 1000 repetitions (Felsenstein, 1985). The prediction of 3-D model was visualized the I-TASSER program (Roy et al., 2010; Yang & Zhang, 2015; C. Zhang et al., 2017; Y. Zhang, 2008) and SignalP (Petersen et al., 2011). The Smek23 protein structural analysis was done by using TMpred, a program to predict transmembrane regions and their orientation (Hofmann, 1993).

3.1.2. Expression of recombinant Smek23 in *E. coli*

A full DNA sequence of Smek23 with hexahisditine tag at C-terminus was amplified from cDNA of *S. mekongi* adult worm using the forward primer:5'Smek23NdelF:5'-CAT ATG GCG ACT TTG GGT ACT GGG ATG A-3' and the reverse primer:3'Smek23XholR:5'CTC GAG TTA ATG ATG ATG ATG ATG ATG AAC ATT CTC ATA TTC TTT TAT TTG TCG-3'. The PCR product was ligated into PET17b vector with Ndel and Xhol digestion prior to transforming

into *E. coli* (BL21). The correct Smek23 DNA sequencing was verified by DNA sequencing. To produce rSmek23, the bacteria was inoculated into LB-broth containing 1 µg/ml ampicillin and grown at 37 ◦C until the OD_{600} reading of 0.6-0.8, then 1 mM IPTG was added to the culture medium and incubated at 30 ◦C for 6 h. recombinant Smek23 (rSmek23) were obtained by lysing bacteria cells with the lysis buffer (50 mM NaH2PO4. 300 mM NaCl, 8 M urea, 6 M GuHCL and 20 mM Imidazole pH 8.0), and the protein was purified with washing buffer containing 20 mM Imidazole and eluted from the resin with elution buffer containing 250 mM Imidazole. The purified rSmek23 was dialysis tubing which excludes MW 12 kDa or greater (SIGMA D-9777) in cold-PBS for 3 hrs before uses.

3.2 Prediction of antigenic sites

In this study, the potential hydrophilic antigenic epitopes of Smek23 were found out in order to identify the antigenic determinants. Antigenic epitopes are determined using several prediction methods, for example, Hopp and Woods (Hopp & Woods, 1981); Welling (G. W. Welling et al., 1985); HPLC/Parker & al (Parker et al., 1986); Kolaskar & Tongaonkar antigenicity (Kolaskar & Tongaonkar, 1990) and B-EpiPred Server (Lapoile et al., 2006).

3.3 The distribution of Smek23 in parasitic tissues by in situ hybridization techniques

Gene-specific primers (Smek23_F and Smek23_R for gene) were used for localization of expression of Smek23 mRNA by performing hybridization. The adult worms were collected and fixed in 2% paraformaldehyde at 4 ◦C for 18 h. the tissues were then processed through routine tissue processing before being embedded in paraffine blocks. Tissue sections (5 µm-thickness) were cut and mounted onto silane-coated slides. The gene specific primers (Smek23_F and Smek23_R gene) were used to amplify DIG-oligonucleotide labeling kit (Roche, Germany) from cDNA of *S. mekongi*. DIG-labeled sense or antisense RNA probe was added to the hybridization solution containing the *S. mekongi* sections, and then rotated at 50 ◦C

for 12 h. after hybridization, the *S. mekongi* were stained and photographs were taken with Leica compound microscope equipped with a digital camera (Leica, Germany).

CHAPTER IV

RESULT

4.1 Cloning and sequencing of 23 kDa membrane protein from S. mekongi (Smek23)

The cDNA encoding Smek23 of adult *S. mekongi* was cloned by RT-PCR showed the specific band of Smek23 cDNA fragment (Figure 2). The PCR product of Smek23 DNA sequencing was verified by DNA sequencing. The nucleotide sequence of Smek23 was 686 bp in length. The nucleotide sequence of Smek23 showed an open reading frame encoding 23 kDa integral membrane protein containing 218 amino acids (Figure 3). The expected molecular weight of Smek23 determined from its constituent amino acids is 23.62 kDa.

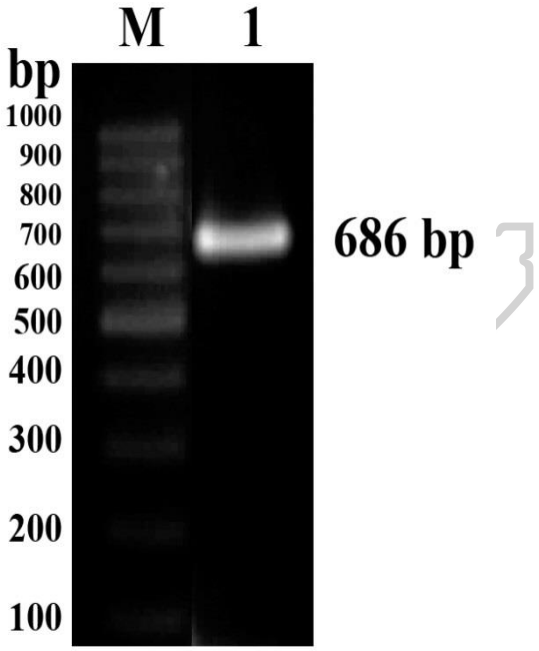

Figure 2. The PCR product specific band of Smek23 cDNA fragment.
Lane M: 100 bp molecular marker and Lane 1: Smek23 cDNA fragment

The Smek23 has four transmembrane domains (TM) and other transmembrane 4 superfamily (TM4SF) characteristics. When the primary amino acid sequence of Smek23 was examined for the presence of functional sites, the following motifs were indicated: GAGAYVEVK, tyrosine kinase phosphorylation; ATLGTGMRC, threonine kinase phosphorylation; and EVKFSQYGA, DYKGSVPDS, and SVPDSCKEG, Serine kinase phosphorylation (Figure 3). The SignalP tool which incorporates a prediction of cleavage sites and signal/non-signal peptide predicted no presence of signal peptide in the protein (Figure 4).

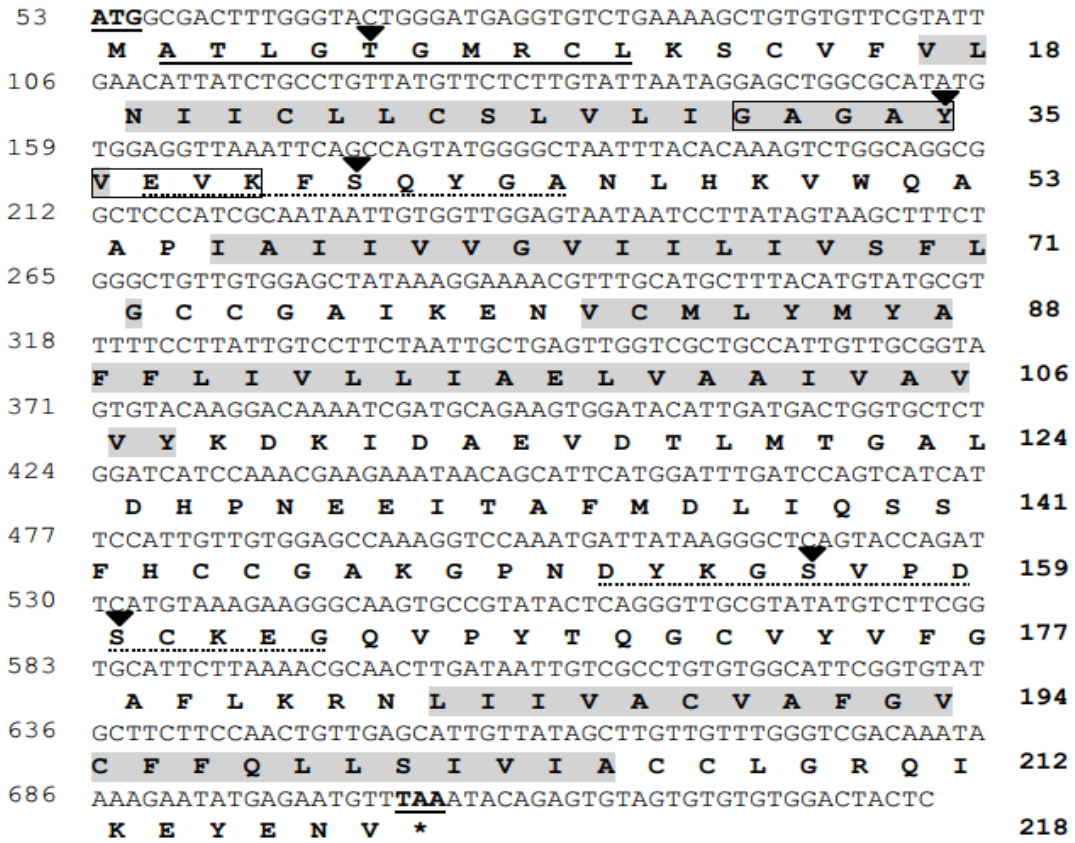

Figure 3. Nucleotide and the deduced amino acid sequence of Smek23. The predicted start ATG and stop TAA codons are shown in underlined and bold face. Putative functional sites are threonine kinase phosphorylation (underlined); tyrosine kinase phosphorylation (boxed) and Serine kinase phosphorylation (dash line).

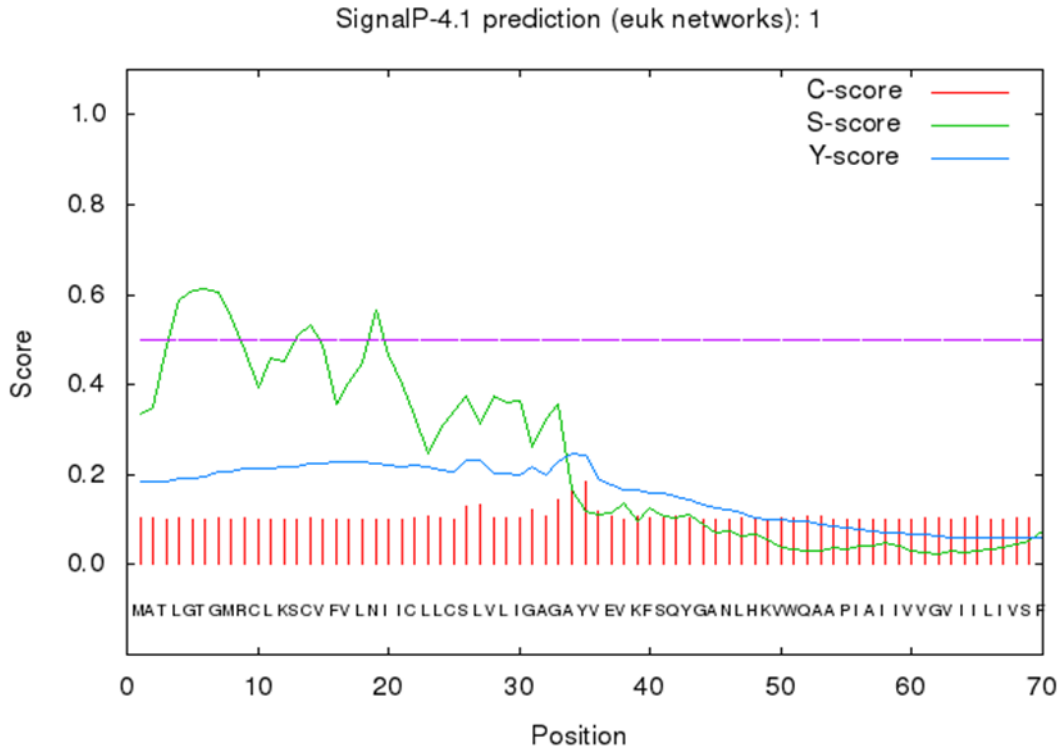

Figure 4. SignalP result for the signal peptide of Smek23 protein

The SignalP using several artificial neural networks and Hidden Markov Models. No signal peptide was detected and it was predicted to be an integral membrane protein. Score C, S and Y represent cleavage site score, signal peptide score and combination score (derived from C and S scores), respectively.

4.2 Bioinformatics identification and characterization of Smek23

The transmembrane topology of Smek23 was predicted by using TMpred program (Figure 5). The putative domains of Smek23 fall within the transmembrane 4 superfamily (TM4SF) as follows: cytoplasmic domains, CYT 1 (position 1-16) 16 amino acids, CYT 2 (position 73-80) 8 residues, CYT 3 (position 206-218) 13 residues; extracellular domains, EXT 1 (position 37-55) 19 residues, EXT 2 (position 109-183) 75 residues; transmembrane domains, TM 1 (position 17-36) 20 residues, TM 2 (position 56-72) 17 residues, TM 3 (position 81-108) 20 residues, and TM 4 (position 184-205) 22 residues. The conservation of this domain structure with other schistosome 23 kDa proteins showed in Figure 5.

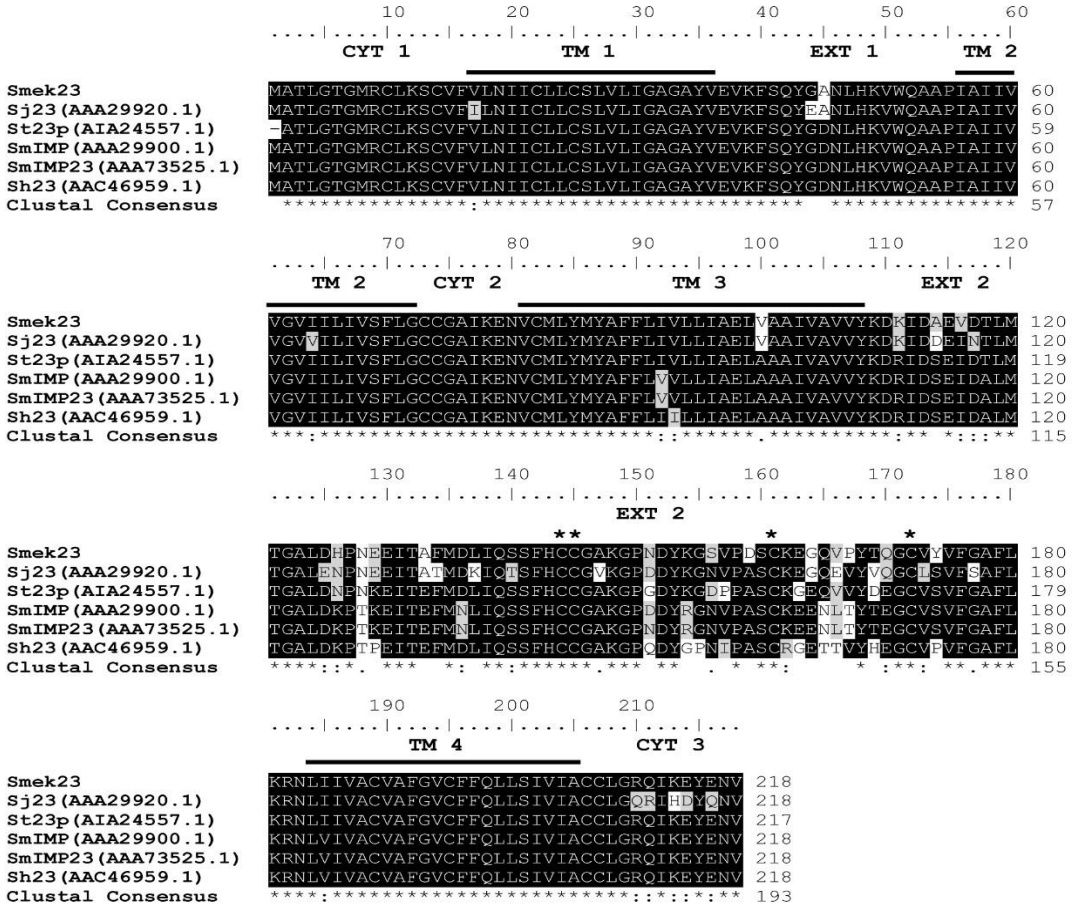

Figure 5. Multiple sequences alignment of the deduced amino acid sequences of Smek23 with other Schistosome 23 kDa protein.

The amino acid sequences of Smek23 with other 23 kDa protein sequences showing highly conserved amino acids by grey and black outlines. The asterisk (*) indicates identical amino acids, two dots (:) indicates conserved amino acid substitutions and dot (.) indicate semi-conserved amino acid substitutions. The cysteine residues of the large extracellular domain of TM4SF involved in disulfide bonding is indicated by asterisk (*). The TM4SF-like domains of Smek23 are indicated as follows: CYT 1 to 3, cytoplasmic domains; TM 1 to 4, transmembrane domains; EXT I and 2, extracellular domains. Smek: *Schistosoma mekongi*; Sm: *S. mansoni*; Sj: *S. japonicum*; Sh: *S. haematobium*; St: *S. turkestanicum*. Database accession numbers for the proteins aligned here: Sm23: AAA29900.1, AAA73525.1; Sh23: AAC46959.1, Sj23: AAA29920.1; St23: AIA24557.1.

The multiple alignment of deduced amino acid sequences of Smek23 with 23 kDa membrane protein, tetraspanin protein, and CD63 protein from related schistosome species (AAA29900.1, AAA73525.1, AAN17276.1 from *S. mansoni*; AAC46959.1 from *S. haematobium*; AAA29920.1, AEG74369.1, CAX75552.1 from *S. japonicum*; and AIA24557.1 from *S. turkestanicum*), 23 kDa integral membrane protein from *Fasciola* spp. (TPP57640.1 from *F. gigantica* and THD26539.1 from *F. hepatica*), CD63 and CD81 antigen from animal host and human (AAD43135.1, NP_598416.1 from mouse; XP_003639366.1, XP_022261237.1 from dog; QHY93513.1, ABK63185.1 from pig; AAH02349.1, pdb_5TCXA from human showed in Figure 6.

The Smek23 amino acid sequences showed the highest degree of identity with *S. turkestanicum* 23 kDa (GenBank: AIA24557.1) at 91.2 %. The identity of Smek23 amino acid sequences with integral membrane protein 23 from *S. mansoni* (Genbank: AAA73525.1 and AAA29900.1) showed at 89.9 and 89.4, respectively. The identity of Smek23 amino acid sequences with 23 kDa protein from *S. japonicum* (Genbank: AAA29920.1) and *S. haematobium* (Genbank: AAC46959.1) showed at 88.0 and 87.6%, respectively (Table 1). The identity of Smek23 amino acid sequences with tetraspanin 2 from *S. mansoni* (Genbank: AAN17276.1) and *S. japonicum* (Genbank: AEG74369.1) showed identity at 27 and 27.2%, respectively. The identity of Smek23 amino acid sequences with CD63 antigen from *S. japonicum* (Genbank: CAX72085.1) showed at 27.2% (Table 1).

The identity of Smek23 amino acid sequences with CD63 antigen from *Mus musculus* (Genbank: AAD43135.1), *Canis lupus familiaris* (Genbank: XP_003639366.1), *Sus scrofa* (Genbank: QHY93513.1) and *Homo sapiens* (Genbank: AAH02349.1) showed at 28.8, 30.9, 30.6, and 30.9%, respectively (Table 1). The identity of Smek23 amino acid sequences with CD81 antigen from *Mus musculus* (Genbank: NP_598416.1), *Canis lupus familiaris* (Genbank: XP_022261237.1), *Sus scrofa* (Genbank: ABK63185.1), *Homo sapiens* (PDB: 5TCXA) showed at 20, 17.9, 19.6, and 18.2%, respectively (Table 1).

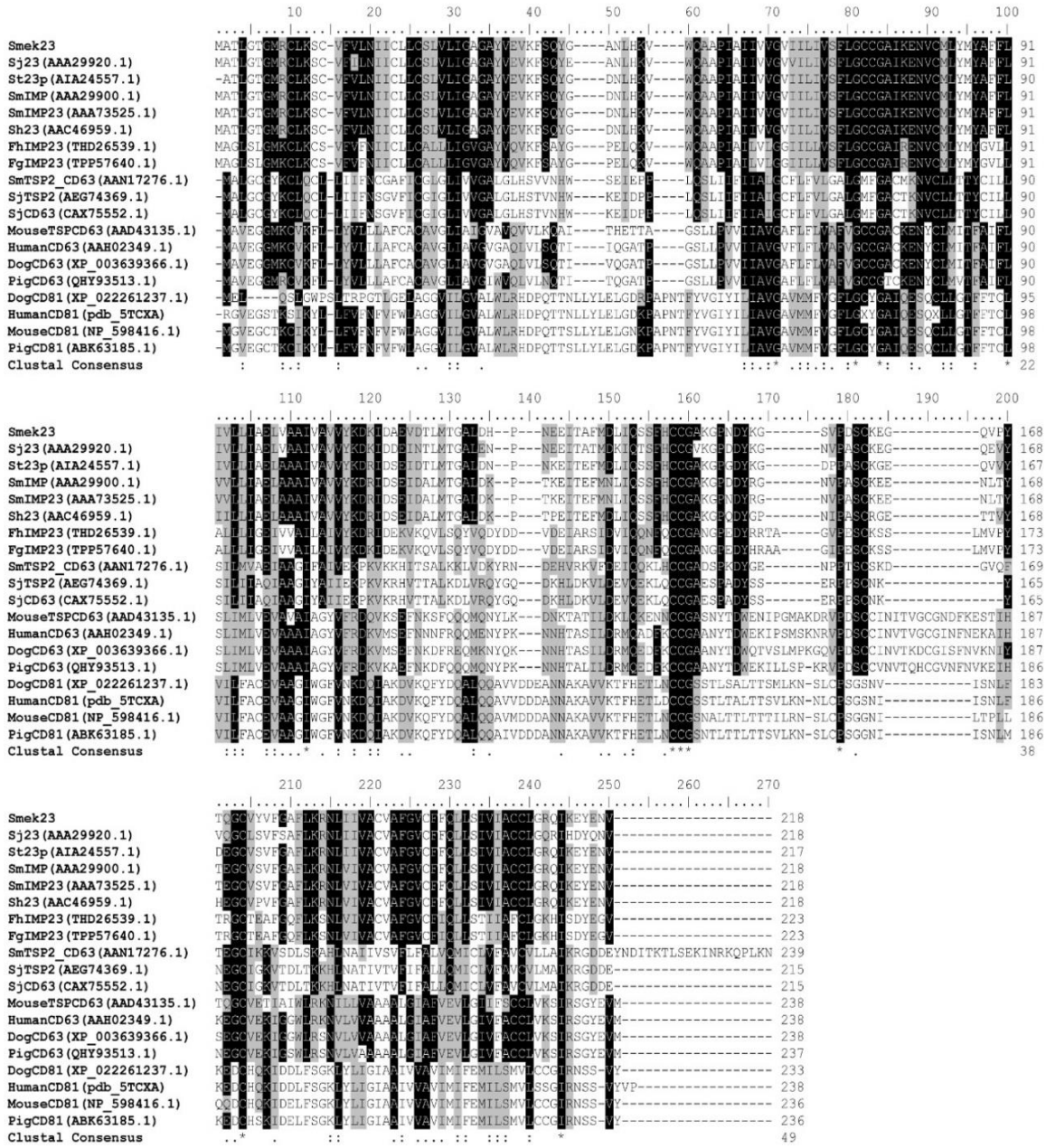

Figure 6. Multiple sequences alignment of the deduced amino acid sequences of Smek23 with 23 kDa protein, tetraspanin, CD63 and CD81 antigens.

The amino acid sequences of Smek23 with other protein sequences showing highly conserved amino acids by grey and black outlines. The asterisk (*) indicates identical amino acids, two dots (:) indicates conserved amino acid substitutions and dot (.) indicate semi-conserved amino acid substitutions. Smek: *S. mekongi*; Sm: *S. mansoni*; Sj: *S. japonicum*; Sh: *S. haematobium*; St: *S. turkestanicum*; Fh: *F. hepatica*; Fg: *F.gigantica.*

Table 1. Sequence identity matrix of Smek23 amino acid sequences with 23 kDa protein, CD63 and CD81

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Smek23	**ID**																		
Sj23(AAA29920.1)	**0.88**	ID																	
St23p(AIA24557.1)	**0.912**	0.857	ID																
SmIMP(AAA29900.1)	**0.894**	0.839	0.926	ID															
SmIMP23(AAA73525.1)	**0.899**	0.834	0.926	0.995	ID														
Sh23(AAC46959.1)	**0.876**	0.821	0.922	0.931	0.931	ID													
FhIMP23(THD26539.1)	0.623	0.582	0.6	0.614	0.614	0.6	ID												
FgIMP23(TPP57640.1)	0.623	0.582	0.596	0.6	0.6	0.6	0.982	ID											
SmTSP2_CD63(AAN17276.1)	0.27	0.266	0.276	0.275	0.275	0.283	0.279	0.283	ID										
SjTSP2(AEG74369.1)	0.272	0.263	0.278	0.268	0.268	0.272	0.286	0.291	0.702	ID									
SjCD63(CAX75552.1)	0.272	0.263	0.278	0.268	0.268	0.272	0.286	0.291	0.702	1	ID								
MouseTSPCD63(AAD43135.1)	0.288	0.28	0.285	0.284	0.284	0.284	0.288	0.284	0.229	0.235	0.235	ID							
HumanCD63(AAH02349.1)	0.309	0.301	0.315	0.305	0.305	0.305	0.284	0.284	0.252	0.264	0.264	0.794	ID						
DogCD63(XP_003639366.1)	0.309	0.297	0.315	0.305	0.305	0.305	0.288	0.288	0.252	0.268	0.268	0.76	0.899	ID					
PigCD63(QHY93513.1)	0.306	0.294	0.303	0.294	0.294	0.294	0.281	0.281	0.25	0.265	0.265	0.764	0.861	0.861	ID				
DogCD81(XP_022261237.1)	0.179	0.179	0.179	0.183	0.183	0.183	0.179	0.179	0.189	0.201	0.201	0.196	0.192	0.204	0.208	ID			
HumanCD81(pdb_5TCXA)	0.182	0.178	0.183	0.186	0.186	0.186	0.195	0.195	0.19	0.2	0.2	0.219	0.223	0.231	0.224	0.857	ID		
MouseCD81(NP_598416.1)	0.2	0.196	0.193	0.196	0.196	0.2	0.221	0.217	0.19	0.202	0.202	0.236	0.228	0.24	0.237	0.822	0.882	ID	
PigCD81(ABK63185.1)	0.196	0.196	0.201	0.205	0.205	0.2	0.213	0.213	0.194	0.21	0.21	0.236	0.236	0.248	0.241	0.856	0.92	0.94	ID

Phylogenetic tree constructed using amino acid sequences of 23 kDa protein, tetraspanin, CD63 and CD81 antigen by Neighbor-joining method with the MEGA X program (Bootstrap 1,000 replicates) (Figure 7). The study showed that 59 amino acid sequences can be classified into 4 groups. The first group is CD81 antigen that group is supported by a bootstrap value of 96% and divided into 2 clades; CD81 antigen from host (cattle, buffalo, sheep, goat, pig, cat, dog, mouse, rat, and human) supported by a bootstrap value of 100% and CD81 from *S. haematobium*. The second group is CD63 antigen that supported by a bootstrap value of 67% and divided into 2 clades; CD63 antigen from host (cattle, buffalo, sheep, goat, pig, cat, dog, mouse, and human) supported by a bootstrap value of 100% and CD63 antigen from *Trichuris trichiura*. The third group is CD63 antigen and tetraspanin protein from parasites that supported by a bootstrap value of 99% and divided into 3 clade; the first clade including *Paragonimus westermani* CD63, *Opisthorchis viverrini* tetraspanin 2B, *Clonorchis sinensis* 23 kDa integral membrane protein and *F. gigantica* tetraspanin was supported by a bootstrap value of 90%, the second clade including *C. sinensis* CD63, *O. viverrini* tetraspanin 2A and *F. hepatica* tetraspanin was supported by a bootstrap value of 88% and the last clade CD63 antigen and tetraspanin from *Schistosoma* species (including *S. mansoni*, *S. japonicum*, *S. haematobium*, and *S. bovis*) was supported by a bootstrap value of 88%. The fourth group is 23 kDa membrane protein and tetraspanin 23 protein from *Schistosoma* spp., and 23 kDa membrane protein from *Fasciola* spp. and *C. sinensis* that supported by a bootstrap value of 100% and divided into 2 clade; 23 kDa integral membrane protein from *F. gigantica*, *F. hepatica* and *C. sinensis* was supported by a bootstrap value of 98% and the second clade including 23 kDa membrane protein (*S. mekongi*, *S. mansoni*, *S. japonicum*, *S. haematobium*, and *S. turkestanicum*) and tetraspanin 23 protein from *S. mansoni*, *S. haematobium*, *S. bovis*, *S. curassoni*, *S. guineensis*, *S. intercalatum*, *S. margrebowiei*, and *S. rodhaini* was supported by a bootstrap value of 100%. The result showed that Smek23 was closely related to *S. japonicum* 23 kDa (Genbank: AAA29920.1) supported by a bootstrap value of 93%. (Figure 7).

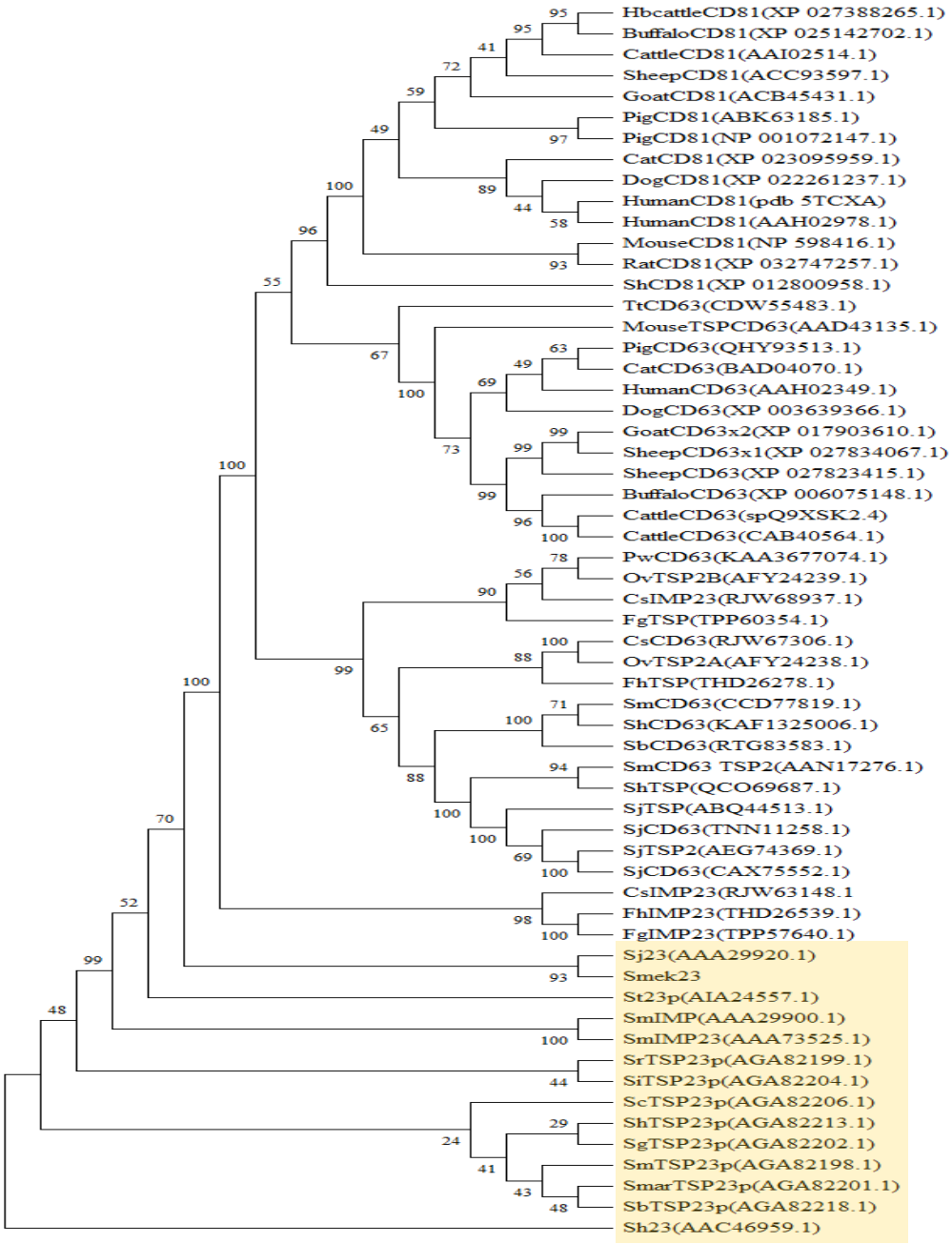

Figure 7. The phylogenetic analysis of Smek23 with 23 kDa membrane protein, tetraspanin protein, and CD63 protein.

The phylogenetic tree was constructed by neighbor-joining method (Saitou and Nei, 1987) in MEGA X (Kumar et al., 2018). Saitou and Nei (1987) the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The numbers at the branching point indicate percent bootstrap value. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling, 1965).

The secondary and tertiary protein structure of Smek23 was predicted by iterative implementation of the Threading ASSEmbly Refinement (I-TESSER) program (Roy et al., 2010; Yang & Zhang, 2015; C. Zhang et al., 2017; Y. Zhang, 2008). This software proposed five 3D model structures based on C-score which is a confidence score used for estimating the quality of predicted models (Table 2).

Table 2. The score of different properties related to five Smek23 tertiary structure models were predicted

Name	C-score	Exp. TM-score	Exp. RMSD	No. of decoys	Cluster density
Model 1	0.47	0.78±0.10	4.6±3.0Å	8631	0.4108
Model 2	-1.71			1100	0.0465
Model 3	0.24			5958	0.3282
Model 4	-0.96			1865	0.0988
Model 5	-2.25			590	0.0270

Ultimately, 5 possible tertiary structures were predicted by I-TASSER tool. According to C-scores calculated by this software, model 1, with a C- score of 0.47, had the highest confidence between the other four models. Data are shown in Figure 8 and it contained seven α-helixes were shown in pink, and blue represents turns of the protein (TM-score = 0.78±0.10, RMSD = 4.6±3.0Å). The predicted structures suggest that Smek23 contained 27.06% coil, 65.14% helix and 7.8% strands Figure 9.

Figure 8. Predicted Tertiary structure of Smek23 protein.
A probabilistic structural model 1(C- score of 0.47; TM-score = 0.78±0.10; RMSD = 4.6±3.0Å) for Smek23 chimeric protein by I-TASSER server. Seven α-helixes in pink. The blue represents turns in the peptide backbone.

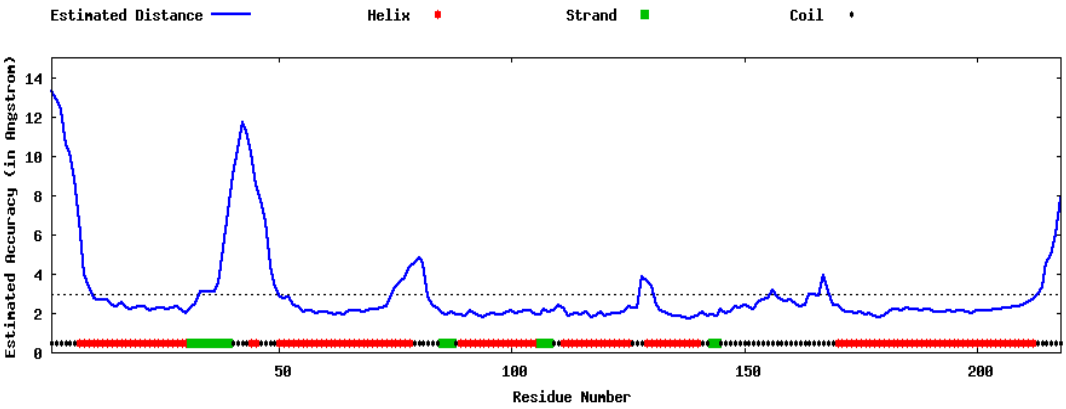

Figure 9. The local accuracy estimation for the first model of Smek23. The local accuracy was defined as the distance deviation (in Angstrom) between residue positions in the model and the native structure

4.3 Production of Smek23 protein

The rSmek23 with hexahistidine at C-terminus was expressed and purified from *E. coli* under denaturing conditions. The band with molecular masses of 24 kDa of purified rSmek23, as analyzed by SDS-PAGE, are shown in Figure 10.

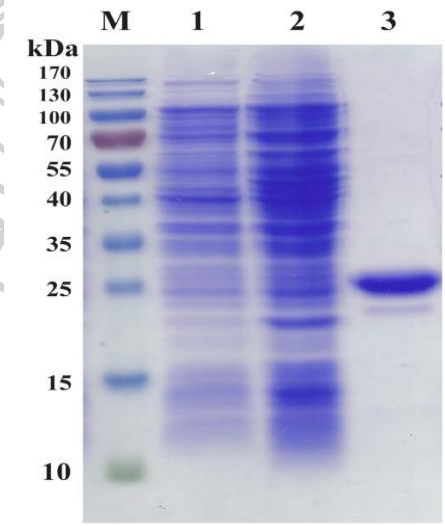

Figure 10. Electrophoretic pattern of *S. mekongi* expressed from the bacterial system. Each fraction was analyses by 12.5% SDS-PAGE stained with Coomassie Blue. Lane M: PageRuler Prestained Protein Ladder; Lane 1: Total protein from *E. coli* BL21/pET17b-Smek23 before induction; Lane 2: Total protein from *E. coli* BL21/pET17b-Smek23 after induction; Lane 3: rSmek23 purified with Ni-NTA Column.

4.4 Determination of antigenic peptides

In the present study, Hoppand Woods, Welling, Parker, Kolaskar and Tongaonkar (1990) and B-EpiPred Server, antigenicity scales were designed to predict the locations of antigenic epitope region in 23 kDa protein of the human blood fluke

S. mekongi. By analyzing graphical and numerical data, it was found that according to Hopp and Woods (1981) scale the regions 9-10, 76-79, 109-119, 125-130, 132-133, 147-166, 209-214 contained the potential hydrophilic regions (Hydrophilicity: score > 0).The analysis found high in position between 109-119 (Maximum Score 1.578) in a protein, which present in extracellular domain 2 (EXT 2), assuming that this antigenic region would be presented on the protein surface and thus would be placed in hydrophilic regions (Figure 11).

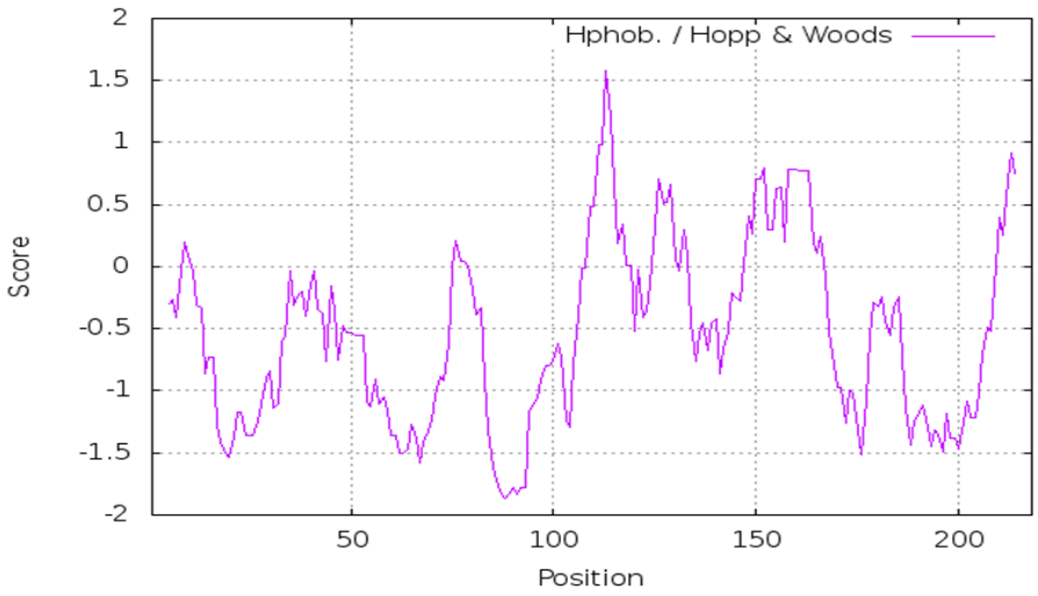

Figure 11. Graphical representation of antigenic peptide evaluation by Hopp & Wood

In the Welling et al. 1985 antigenicity plot gives value as the log of the quotient between percentage in average proteins and percentage in a sample of known antigenic regions, the predicted hydrophilic regions were 36, 38, 44-53, 97-98, 101-108, 109-115, 125-127,150-151, 156, 158. The data found high in extracellular domain 1 (EXT 1) in position 44-53 (Maximum Score 0.702) (Figure 12).

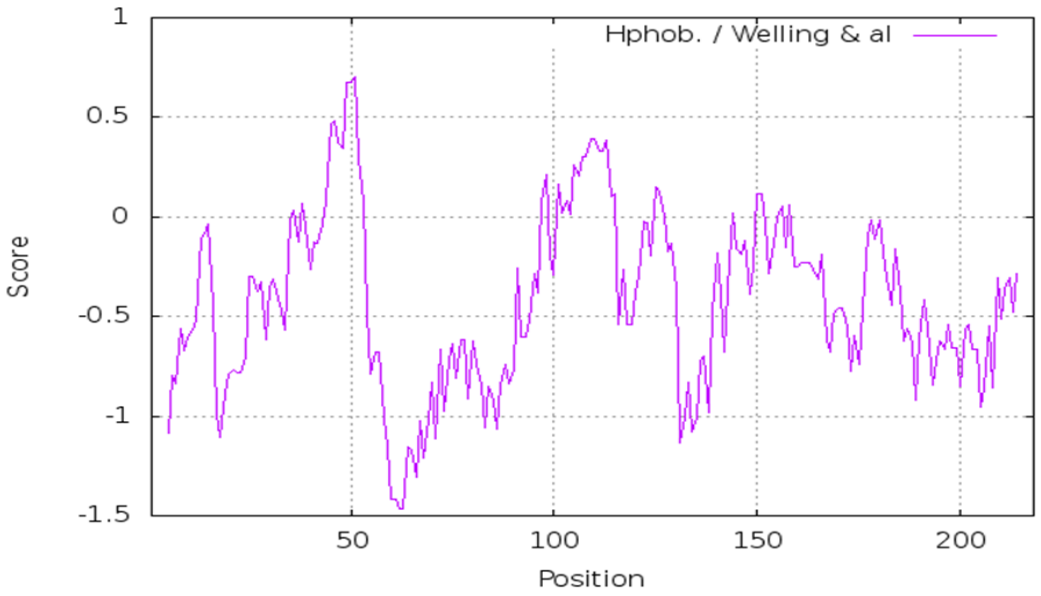

Figure 12. Graphical representation of antigenic peptide evaluation by Welling & al

We also study the hydrophobicity plot of HPLC / Parker and the predicted hydrophilic regions were 5-11, 33-36, 37-46, 48-50, 72, 75-79, 107-108, 109-119, 120-133, 137, 139-140, 142-171, 173-174, 209-214. The highest peak is found high in extracellular domain 2 (EXT 2) in position between 143-171 (Maximum Score 5.157) (Figure 13).

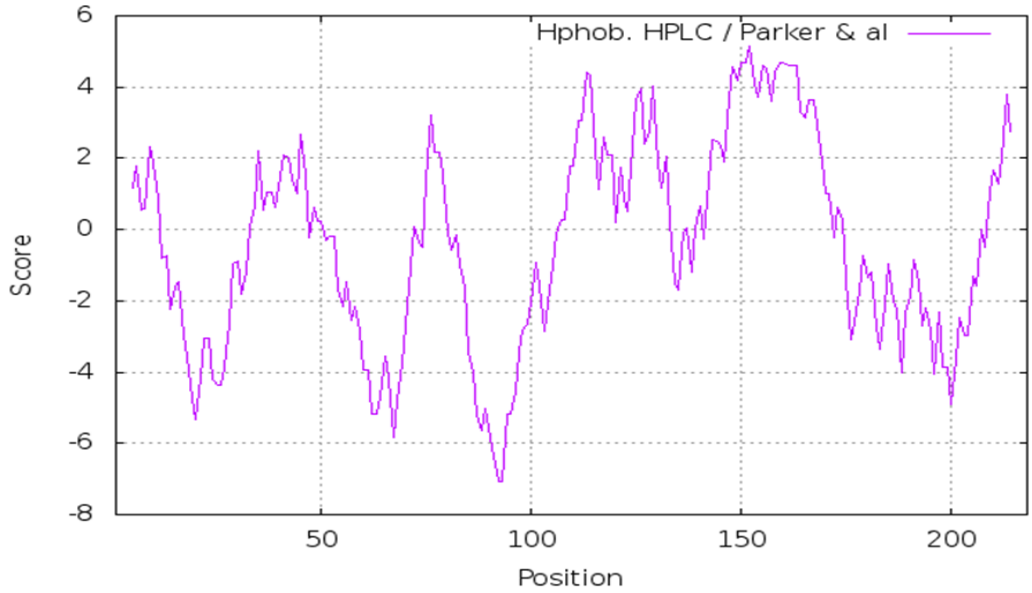

Figure 13. Graphical representation of antigenic peptide evaluation by HPLC/Parker & al

According to Kolaskar and Tangaonkar (1990) antigenicity scale, at 1.0 as the threshold level, the most likely antigenic determinants were at 11-LKSCVFVLNIICLLCSLVLI-30, 56-IAIIVVGVIILIVSFLGCCGA-76, 90-FLIVLLIAELVAAIVAVVYK-109, 169- TQGCVYVFG-177, 186-IVACVAFGVCFFQLLSIVIACCLG-209 (Figure 14).

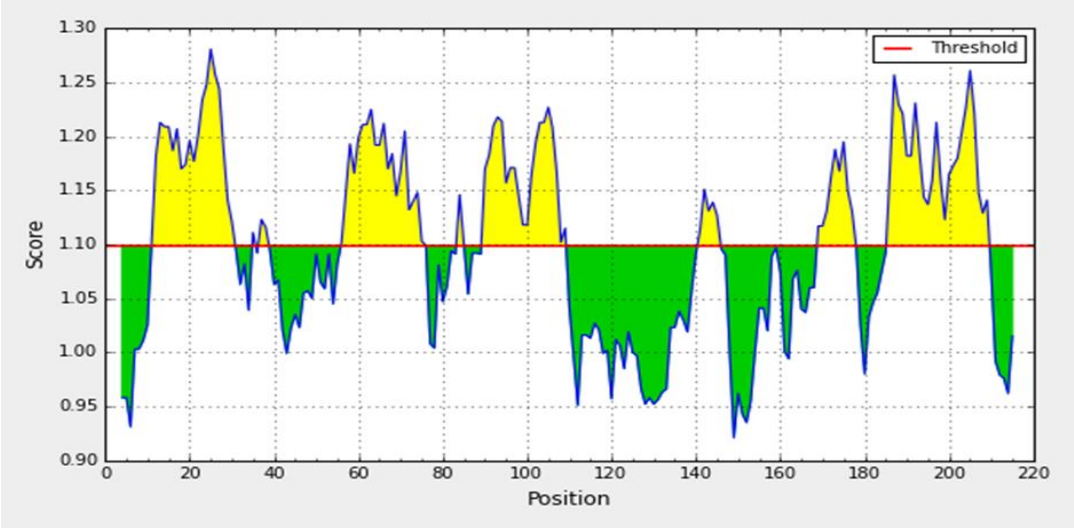

Figure 14. Kolaskar and Tongaonkar Antigenicity for Smek23

BepiPred predicts the location of linear B-cell epitopes result found that between 147-169 is 147-AKGPNDYKGSVPDSCKEGQVPYT-169 and the maximum score (1.999) is found at the position 152 (Figure 15).

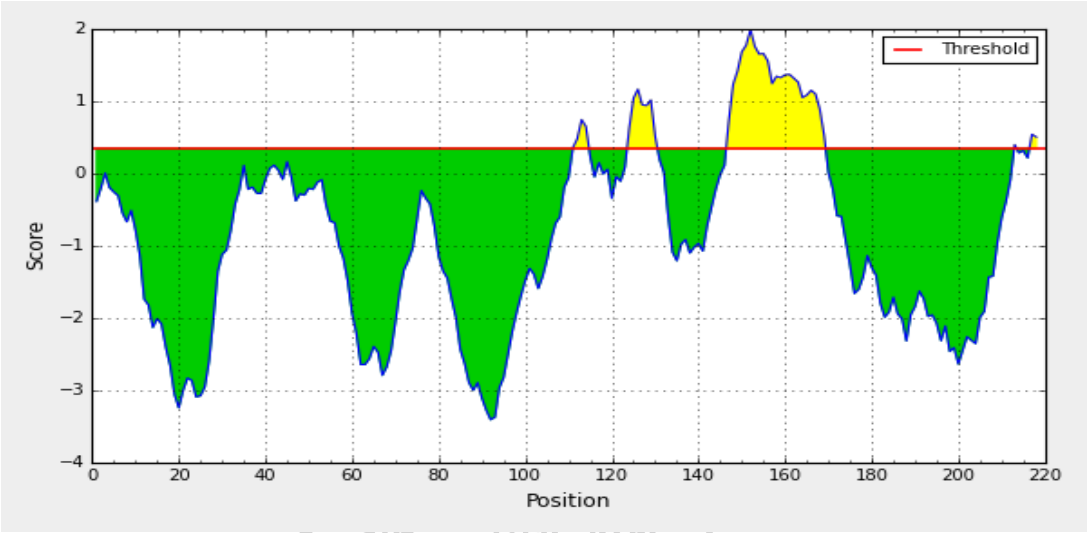

Figure 15. Graphical representation of antigenic peptide evaluation by Bepipred Linear Epitope Prediction for Smek23

Prediction of immunogenic region which exposed on the surface of the protein is a necessary step for epitope-based vaccine design. In this study, the hydrophilic regions of Smek23 proteins which are supposed to be antigenic and exposed to the surface of the protein were identified for antigenic determinants. The overlapping sequences of prediction epitopes have been summarized in Table 3. There were three major overlapping regions which were hit by all programs (Hopp & Woods (1981); Welling, Parker and B-EpiPred). These regions were 111-KIDA-114, 125-DHP-127 150-PNDYKGSVPDSCKEGQVPYT-169. All of these three regions were located in extracellular domain 2 (EXT 2), which is a large hydrophilic domain (LHD) of Smek23 molecule.

Table 3. The potential hydrophilic 6egions and epitope prediction sites from Hopp & Woods, Welling & al, Parker & al Kolaskar & Tongaonkar and B-EpiPred programs.

TM4SP	Hopp & Woods	Welling & al	Parker & al	Kolaskar & Tongaonkar	Bepipred
CYT 1 (1-16)	9-10		5-11	11-30	
TM 1 (17-36)		36	33-36		
EXT 1 (37-55)		38, 44-53	37-46, 48-50		
TM 2 (56-72)			72	56-76	
CYT 2 (73-80)	76-79		75-79		
TM 3 (81-108)		97-98, 101-108	107-108	90-109	
EXT 2 (109-183)	109-119,	109-115	109-119		111-114
	125-130, 132-133	125-127	120-133		124-130
			137, 139-140		
	147-166	150-151, 156, 158	142-171, 173-174	169-177	147-169
TM 4 (184-205)				186-209	
CYT 3 (206-218)	209-214		209-214		213

4.5 The distribution of Smek23 in parasitic tissues by in situ hybridization techniques

Paraffin longitudinal sections of adult *S. mekongi* were hybridized with an antisense Smek23 RNA probe. Positive hybridization signals were detected in tegumental cells (Figure 16). Hybridization signals were not observed in control section.

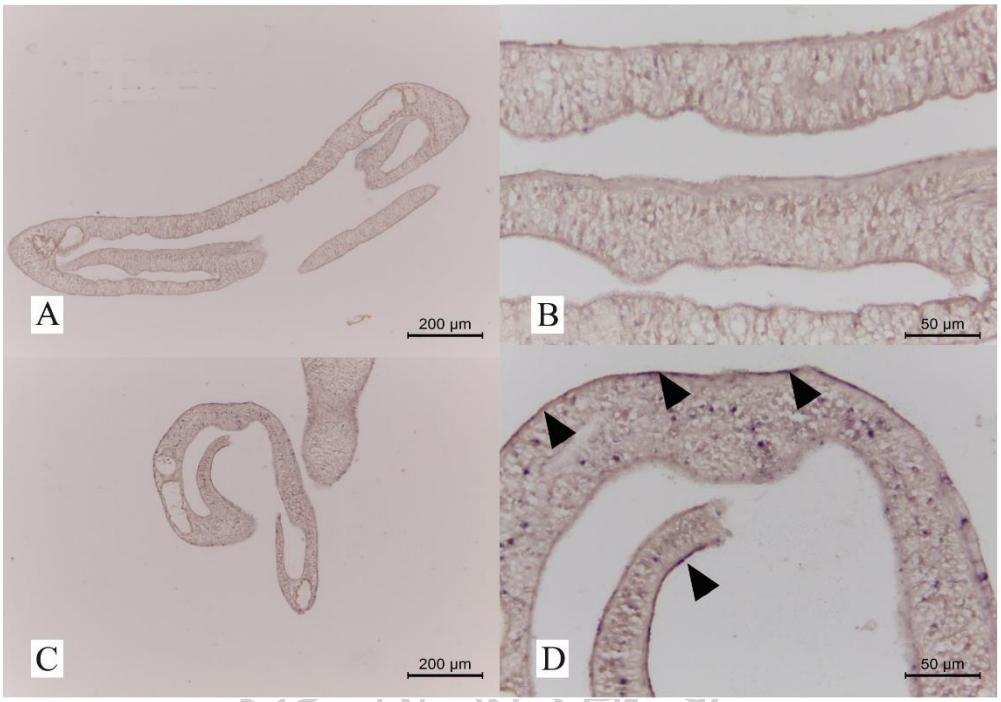

Figure 16. *In situ* hybridization of *S. mekongi* adult stage longitudinal-sections with Smek23 RNA probes.

A. Control section probed with Bismarck brown Y for counter stain. No labelling in any part of the worm is seen. (Scale bar = 200 µm)

B. Control section probed with Bismarck brown Y for counter stain. No labelling in any part of the worm is seen. (Scale bar = 50 µm)

C. Medium magnification micrograph showing intense staining in the tegument (Scale bar = 200 µm)

D. Medium magnification micrograph showing intense staining in the tegument (arrow) (Scale bar = 50 µm)

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DISCUSSION

5.1 Cloning, identification and expression of Smek23

From the results of comparing the identity of amino acid sequences through Clastal W sequence analysis and Sequence Identity Matrix Alignment (BioEdit). The high level of identities in amino acid sequences indicate the close relationship between *S. mekongi* and those Trematode parasite species as expected. The Smek23 amino acid sequences showed the highest degree of identity with the *Schistosoma* species of red deer that found in Hungary and central Asia, *S. turkestanicum* 23 kDa (GenBank: AIA24557.1) at 91.2 %. The identity of Smek23 amino acid sequences with other schistosome species (*S. mansoni*, *S. japonicum* and *S. haematobium* showed at 87.6-89.9% respectively. By contrast, the amino acid sequences for the homologous genes of mammalian hosts, CD81 showed low identity with Smek23 kDa which ranged from 0.17-0.2%. According to the phylogenetic analysis in this study, it revealed that Smek23 exhibited distant evolutionary relationship from the Tetraspanin of mammalian host species. The low degree of conservation observed from amino acid sequences of mammalian hosts could reveal its applicable for using as the vaccine candidate against the schistosome infection which may not interfere with the hosts' Tetraspanin molecule during the vaccination.

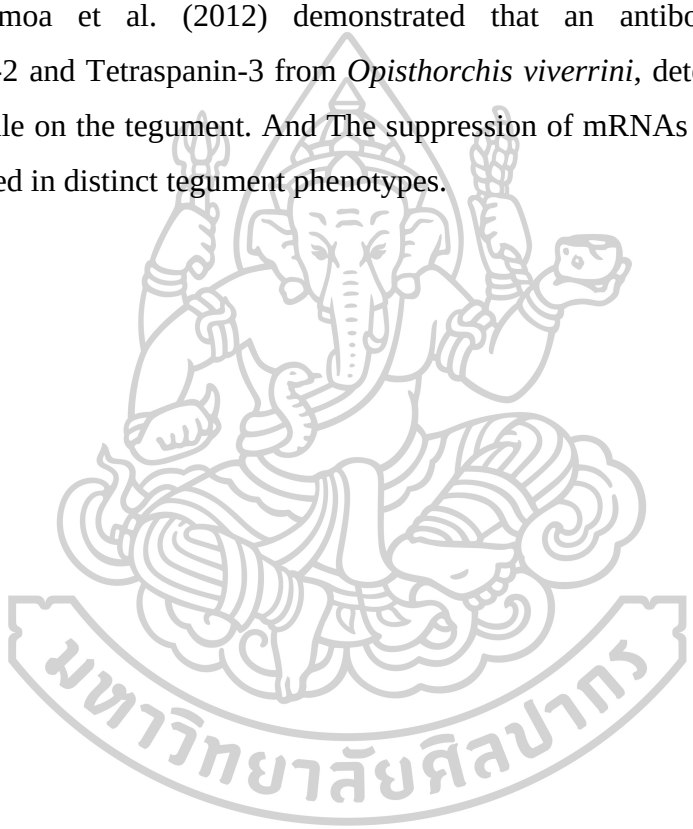
5.2 Prediction of antigenic sites Prediction of B-cell antigenic epitope

Successful vaccine is a key strategy to eliminate and eradicate schistosomiasis, but currently no such vaccine exists. Smek23 is one of the proteins that exposed to the host environment and are antigenic in nature so this protein could be estimated as a vaccine candidate that are capable of inducing a significant immune response into the host. B cell epitopes are part of proteins which prime the antigenicity and recognized by the human antibodies. Identification of B cell epitopes are useful for the development of a novel vaccine. This study uses bioinformation approach to predict the immunogenic epitope on the molecule of Smek23. We choose 5 programs to

predicted the candidate epitopes. The First, B-EpiPred program (Lapoile et al., 2006), predicted B cell epitope which B-cells are considered a core component of the adaptive immune system, as they have the ability to recognize and provide long-term protection against infectious pathogens. The second and third, Hopp and Woods (Hopp & Woods, 1981) and Parker (Parker et al., 1986), respectively predicted potential hydrophilic region. The fourth and fifth, Welling (G. Welling et al.), and Kolaskar and Tongaonkar (Kolaskar & Tongaonkar, 1990), predicted antigenic epitope. The result showed that there are three candidate immunogenic epitopes which were selected by all of four programs (Hopp and Woods, Welling, Parker and B-EpiPred). These regions were 111-KIDA-114, 125-DHP-127 150-PNDYKGSVPDSCKEGQVPYT-169. All of these three regions were located in extracellular domain 2 (EXT 2), which is a large hydrophilic domain (LHD) of Smek23 molecule. Large hydrophilic domain portion of 23 kDa protein from *S. mansoni* appeared to be highly immunogenic molecule in mice. The protein was recognized by mice and human *S. mansoni*-infected serum (Reynolds et al., 1992a). J. Wang et al. (2011) demonstrated that antibody responses to the Sj23HD antigen could be monitored for early detection of schistosome infection in mice by immunoblotting and ELISA. Li et al. (2012) also demonstrated that large hydrophilic domain (LHD) of Sj23 molecule could use as antigen for immunodiagnosis by ELISA for *S. japonicum* infection in cattle. Furthermore, the large hydrophilic domain from *S. japonicum* has been reported to be one of potential candidate vaccine antigens. Z. Zhu et al. (2012) demonstrated that large hydrophilic domain of Sj23 induced 54.3-58.8% protection against the infection in mice (Z. Zhu et al., 2012). Similar results have been reported by (Shi et al., 2002b; Shi et al., 2001), they showed that the recombinant protein of *S. japonicum* 23 kDa and its large hydrophilic domain (LHD) could produce protection against *S. japonicum* infection in mice, sheep, cattle and buffalo. A. A. Da'Dara et al. (2019b) demonstrated that vaccination of water buffalo with combination vaccine of 23 kDa and TPI from *S. japonicum* (Sj23, SjTPI) plasmid DNA vaccines, induced 50% efficacy to challenge infection.

5.3 The distribution of Smek23 in parasitic tissues by in situ hybridization techniques

This preliminary study demonstrated that the Smek23 RNA was present at the tegument. This result related with previous study demonstrated that Tetraspanin-2 protein from *S. japonicum* was immunolocalized on tegument of lung-stage schistosomula and adult worms. In *S. mansoni*, the molecule of tetraspanin 2 also localized to tegument membrane compartments (Schulte et al., 2013). Changklungmoa et al. (2012) demonstrated that an antibody raised against Tetraspanin-2 and Tetraspanin-3 from *Opisthorchis viverrini*, detected the present of both molecule on the tegument. And The suppression of mRNAs encoding these two genes resulted in distinct tegument phenotypes.



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CONCLUSIONS

6.1 Cloning, identification and expression of Smek23

1. The cDNA encoding Smek23 of adult *S. mekongi* was cloned and sequenced. The nucleotide sequence of Smek23 was 686 bp in length. The nucleotide sequence of Smek23 showed an open reading frame encoding 23 kDa integral membrane protein containing 218 amino acids. The expected molecular weight of Smek23 determined from its constituent amino acids is 23.62 kDa.

2. Smek23 has four transmembrane domains (TM) and other transmembrane 4 superfamily (TM4SF) characteristics. When the primary amino acid sequence of Smek23 was examined for the presence of functional sites, the following motifs were indicated: GAGAYVEVK, tyrosine kinase phosphorylation; ATLGTGMRC, threonine kinase phosphorylation; and EVKFSQYGA, DYKGSVPDS, and SVPDSCKEG, Serine kinase phosphorylation.

3. The Smek23 amino acid sequences showed the highest degree of identity with the *Schistosoma* species of red deer that found in Hungary and central Asia, *S. turkestanicum* 23 kDa. The identity of Smek23 amino acid sequences with other schistosome species (*S. mansoni*, *S. japonicum* and *S. haematobium* showed at 87.6-89.9% respectively.

4. Phylogenetic analysis in this study, it revealed that Smek23 exhibited distant evolutionary relationship from the Tetraspanin of mammalian host species (CD63, CD81). The low degree of conservation observed from amino acid sequences of mammalian hosts could reveal its applicable for using as the vaccine candidate against the schistosome infection which may not interfere with the hosts' Tetraspanin molecule during the vaccination.

6.2 Prediction of antigenic sites Prediction of B-cell antigenic epitope

Three candidate B cell immunogenic epitopes were predicted by four programs (Hopp and Woods, Welling, Parker and B-EpiPred). These regions were

111-KIDA-114, 125-DHP-127 150-PNDYKGSVPDSCKEGQVPYT-169. All of these three regions were located in extracellular domain 2 (EXT 2), which is a large hydrophilic domain (LHD) of Smek23 molecule.

6.3 The distribution of Smek23 in parasitic tissues by in situ hybridization techniques

Smek23 from *Schistosoma mekongi* was expressed in the tegument of adult worms.

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