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Sequence analysis of *Ancylostoma* secreted protein 2 of *Necator americanus* from Thai isolates

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Problem/background : *Hookworm infection is one of the major neglected tropical infectious diseases causing both overt and cryptic morbidity among ~600 million people living in tropical areas. As conventional hookworm control measures by improving sanitation and mass anthelminthic drug administration are not sustainable, development of hookworm vaccines is mandatory. One of the major vaccine candidates is Ancylostoma secreted protein-2 of Necator americanus (Na-ASP-2). To date, only one Na-ASP-2 sequence is available; therefore, it remains unknown whether the effectiveness of a vaccine derived from this molecule may be compromised by antigenic diversity in hookworm population.*

Objectives : *To determine the Na-ASP-2 sequences of filariform larvae isolated from infected Thais; and, to test whether the Na-ASP-2 is expressed in the adult stage of N. americanus.*

Design : *Descriptive study*

Setting : *Department of Parasitology, Faculty of Medicine, Chulalongkorn University*

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Materials and Methods : *The method deployed reverse transcription-polymerase chain reaction (RT-PCR) to amplify the Na-ASP-2 coding region using a randomly isolated single filariform larva from each of the 3 subjects and an adult female worm as the source of mRNA. The amplified products were used as templates for DNA sequencing.*

Results : *The Na-ASP-2 amplified products were obtained from all 3 filariform larvae but none from the adult worm. Of 699 nucleotides in the Na-ASP-2 gene, all were conserved among samples and identical with that in the GenBank,™ database accession number AY28808. Sequence analysis revealed no evidence of codon usage bias whereas a number of putative T cell epitopes were identified.*

Conclusion : *Sequence conservation in the Na-ASP-2 coding region suggests that efficacy of the vaccine would not be compromised by genetic diversity in hookworm population.*

Keywords : *hookworm, Necator americanus, Ancylostoma secreted protein-2, sequence conservation, T cell epitopes*

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เจริญชัย อึ๊งเจริญสุข, อุรัสยา พัฒนวงศ์, ทวีศักดิ์ แซ่เตีย, จตุรงค์ พุทธพรทิพย์, สมชาย จงวุฒิเวศย์. การวิเคราะห์ลำดับเบสของยีนที่สร้างโปรตีนคัดหลั่งชนิดที่ 2 ของแอนไซโลสโตมา ที่พบในพยาธิปากขอชนิดนีเคเตอร์อเมริกานัสจากตัวอย่างในประเทศไทย. จุฬาลงกรณ์เวชสาร 2556 มี.ค. – เม.ย.; 57(2): 145 – 60

เหตุผลของการทำวิจัย : *การติดเชื้อพยาธิปากขอเป็นสาเหตุหนึ่งของโรคติดเชื้อในเขตร้อนที่ถูกละเลย ซึ่งก่อให้เกิดภาวะการเจ็บป่วยที่ชัดเจนและที่ซ่อนเร้นในประชากรที่อาศัยในแถบร้อนประมาณ 600 ล้านคน เนื่องจากการควบคุมโรคพยาธิปากขอโดยการพัฒนาสุขอนามัยและการใช้ยารักษาในคนหมู่มากยังประสบปัญหา ดังนั้นการพัฒนาวัคซีนป้องกันโรคพยาธิปากขอจึงมีความจำเป็น ทั้งนี้โปรตีนที่มีศักยภาพหลักในการเป็นองค์ประกอบของวัคซีนคือโปรตีนคัดหลั่งชนิดที่ 2 ของแอนไซโลสโตมาที่พบในพยาธิปากขอชนิดนีเคเตอร์อเมริกานัส (Na-ASP-2) อย่างไรก็ตามปัจจุบันมีข้อมูลลำดับเบสของยีน Na-ASP-2 เพียงตัวอย่างเดียว ดังนั้นวัคซีนดังกล่าวจะประสบปัญหาในเชิงประสิทธิภาพจากความหลากหลายในรูปแบบของแอนติเจนที่อาจปรากฏในตัวอย่างธรรมชาติหรือไม่จึงยังไม่ทราบชัดเจน*

วัตถุประสงค์ : *เพื่อตรวจสอบลำดับเบสของยีน Na-ASP-2 จากตัวอ่อนระยะติดต่อของพยาธิปากขอชนิดนีเคเตอร์อเมริกานัสในผู้ติดเชื้อชาวไทยและตรวจสอบการแสดงออกของยีนดังกล่าวในพยาธิระยะโตเต็มวัย*

รูปแบบการวิจัย : *การศึกษาเชิงพรรณนา*

สถานที่ทำการศึกษา : *ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*

ตัวอย่างและวิธีการศึกษา : *วิธีการศึกษาโดยใช้ปฏิกิริยาลูกโซ่โพลีเมอร์เรสชนิดรีเวอร์สทรานสคริปชั่นเพื่อเพิ่มปริมาณ mRNA ของ Na-ASP-2 ของตัวอ่อนระยะติดต่อของพยาธิปากขอชนิดนีเคเตอร์อเมริกานัสจำนวน 3 ตัวโดยแต่ละตัวได้จากการสุ่มจากจากผู้ติดเชื้อในแต่ละราย และพยาธิระยะโตเต็มวัย 1 ตัวอย่าง การหาลำดับเบสของยีนดังกล่าวทำโดยตรงจากผลิตผลจากปฏิกิริยาลูกโซ่โพลีเมอร์เรสดังกล่าว*

ผลการศึกษา : *ตัวอ่อนระยะติดต่อของพยาธิปากขอทั้ง 3 ตัวอย่างให้ผลบวกต่อการเพิ่มปริมาณ Na-ASP-2 แต่พยาธิตัวโตเต็มวัยให้ผลลบ ทั้งนี้ Na-ASP-2 ประกอบด้วยลำดับเบส 699 ตัวโดยพบว่าตัวอย่างทั้งหมดให้ผลเหมือนกันกับที่มีรายงานในฐานข้อมูล GenBank™ หมายเลข AY28808 จากการวิเคราะห์โคดอนของยีนนี้ไม่พบความเบี่ยงเบนจากการใช้โคดอนเพื่อสร้างกรดอะมิโน แต่พบว่ามีเอพิโทปของทีเซลล์หลายตำแหน่ง*

สรุป : *ประสิทธิภาพของวัคซีน Na-ASP-2 ไม่น่าจะประสบปัญหาในเรื่องความหลากหลายทางพันธุกรรมในประชากรของพยาธิปากขอในธรรมชาติ เนื่องจากไม่พบความหลากหลายของลำดับเบสในยีน Na-ASP-2 ในการศึกษานี้*

คำสำคัญ : *พยาธิปากขอ, นีเคเตอร์อเมริกานัส, โปรตีนคัดหลั่งชนิดที่ 2 ของแอนไซโลสโตมา, ความคงที่ของลำดับเบส, เอพิโทปของทีเซลล์.*

Although sanitary system following urbanization has important impact on the decrease in prevalence of a number of soil-transmitted helminthiasis, people in rural communities that occupy the majority of areas in developing countries remain vulnerable to these infections. In 2000, the Sixth Millennium Development Goals was launched by the United Nations aiming at improving the living status and reducing poverty among the poorest people.[1] As a consequence, a number of international initiatives for control of major infectious diseases including human acquired immunodeficiency virus (HIV)/AIDS, tuberculosis and malaria have been implemented. More importantly, it has been brought into concern that a number of other infectious diseases including helminthic, protozoal and some bacterial infections, when considered collectively, account for a great number of health burden worldwide; thereby, these diseases have been recognized as the neglected tropical diseases (NTDs). Characteristics of infections that meet the criteria of the NTDs are: (i) highly prevalent infections in the developing countries in tropical areas; (ii) biased distribution of the diseases towards people living in rural areas or among the poor people in urban areas; (iii) low mortality but high morbidity resulting in hidden or overt disabilities interfering childhood physical or cognitive development, pregnancy outcomes and workforce productivity; (iv) augmenting poverty and interfering with economic growth, and; (v) historically well-known diseases for mankind since ancient period.[1-3] Among helminthic NTDs, hookworm infections are accounted for 600 million cases worldwide and more than half of these occur in the South and Southeast Asia.[4]

Hookworm infections in humans are mainly caused by *Necator americanus* and *Ancylostoma duodenale*; the former is responsible for more than 85% of all infected cases globally.[5] Humans acquire the infection mainly by skin penetration of filariform larvae (third stage larvae, L3). After reaching the circulation, the larvae migrate to the lungs and are subsequently carried to the upper respiratory tract where they are swallowed into the gastrointestinal tract. After several molts, the adult human hookworms attach themselves to the mucosa of the upper small intestine by using their buccal capsule comprising teeth or cutting plates; thereby mechanical and lytic destruction of tissues ensues. Intestinal blood loss caused by hookworm infections occurs as a result of bleeding at the site of worm attachment and blood meal taken from the parasites. Therefore, iron deficiency anemia and protein malnutrition are the major ailments caused by severe hookworm infections, especially among childhood and pregnant women. It has been demonstrated that the severity of hookworm infections is positively correlated with the adult worm burden in the intestine.[6-8] Although the majority of hookworm infected individuals do not have overt morbidity, several lines of evidence suggest a causal role of hookworm infections and adverse health impacts such as cognitive impairment (i.e. poor memory, inappropriate reasoning ability and poor reading comprehension) and physical growth retardation in children compromising educational achievement.[9] Furthermore, when a parameter known as disability-adjusted life year (DALY), or the numbers of life years lost from premature mortality or disability, was applied to the burden of hookworm infections at the global scale, it has been estimated that approximately 22.1 million DALYs lost is due to this helminth *per se*.[10]

Despite the fact that hookworm infections can be controlled by anthelminthic drugs, improvement of sanitary system in each endemic community, avoidance of exposure to infective hookworm larvae and other control measures, these interventions do not reach significant achievement in several endemic areas of the Sub-Saharan Africa.[11] Importantly, controls of hookworm infections by mass anthelminthic administration are unsustainable because the emergence of drug resistant parasites in the endemic areas and rapid recurrent infections are common. Therefore, an alternative control strategy is urgently required such as development of a hookworm vaccine.[1-4]

It has been known that naturally acquired hookworm infection neither induces immunity against re-infection nor reduces number of worm burden upon subsequent exposure.[12] However, immunization studies in dogs and hamsters with irradiated L3 of *Ancylostoma caninum* have shown remarkable reduction in adult worm burden, host blood loss and eggs per gram of feces upon subsequent challenge infections.[13, 14] Analysis of canine hookworm antigens involved in inducing such protective immunity has identified a number of secreted proteins. One of these antigens is cysteine-rich secretory protein from L3 with a molecular weight of 21.3 kDa, designated *ancylostoma* secreted protein-2 (ASP-2).[15, 16] Experimental evidence from *in vitro* models of tissue invasion has shown that antibodies induced by ASP-2 can elicit reduction in L3 penetration akin to those induced by immunization with irradiated L3.[4,17] Consistent results have been achieved when the homologue of this protein from L3 of *A. ceylanicum* was used as an immunogen.[18, 19] Likewise, this homologous protein has been identified in *N. americanus,* designated Na-ASP-2.[20] Importantly, antibodies raised against the recombinant Na-ASP-2 could inhibit tissue invasion by filariform larvae *in vitro*. A phase I clinical trial reveals safety and immunogenicity of the Na-ASP-2 derived vaccine.[21] To date, it is unknown whether antigenic diversity occurs in Na-ASP-2 because only one Na-ASP-2 sequence is available. Importantly, antigenic diversity in natural parasite population could compromise the efficacy of a vaccine containing variant antigens.[22] Therefore, in the present study, we analyzed the nucleotide sequence encoding this protein from randomly selected *N. americanus* isolated from infected individuals.

Materials and Methods

Parasite samples

Three stool samples containing hookworm ova were obtained from patients attending the Outpatient Department of King Chulalongkorn Memorial Hospital in Bangkok. Diagnosis of hookworm ova in the stool samples was performed by direct smear examination of a single stool sample from each subject. The remaining parts of these fresh stool samples were subject to polyethylene tube culture. After 5 days of incubation at ambient temperature (ranged from 25 to 35^{o}C, hookworm larvae were taken from the aqueous portion at the bottom of the tube and examined by light microscope (Olympus BX50). Individual larvae from each sample were isolated by micromanipulation using inverted microscope. Identification of filariform larva of *N. amaericanus* was based on characteristic conspicuous buccal spears, wide esophageal bulb, transverse striations on sheath

until tail region.[5] A single larva was randomly isolated from each isolate and transferred in a 1.5 ml RNase-free microcentrifuge tube for subsequent RNA isolation. Meanwhile, an adult female *N. americanus* collected from a patient during endoscopy was included for analysis.

RNA extraction

Individual larva was mechanically disrupted using a sterile plastic homogenizer. mRNA extraction was performed by using QIAamp RNeasy Mini kit (Qiagen, Germany) following the manufacturer's recommendations.

cDNA synthesis

cDNA was generated from mRNA extract from each larval by using Takara RNA PCR kit (AMV) version 3.0 (Takara, Japan). In brief, synthesis of the first strand cDNA was performed in a 0.2 ml PCR tube by adding the following components: Avian Myeloblastosis Virus (AMV) reverse transcriptase (1 mM), RNase inhibitor (1unit/μL), oligo dT-adapter primer (0.125 μM), dNTP mixture (1 mM), 10X RT buffer (1 μL), $MgCl_2$ (5 mM) and RNase free water to make a final reaction volume of 10 μL. The reaction mixture was placed in a thermal cycler using a single cycle of 60 ^{o}C, 30 min, 99 ^{o}C, 5 min and 5 ^{o}C, 5 min.

Amplification the *Na-ASP-2* coding sequence

Amplification of the *Na-ASP-2* cDNA was performed in a total volume of 50 μL containing 2 μL first strand cDNA sample, 5x PCR buffer (10 μL), 0.25 μL Takara ExTaq™ HS (0.025 unit/μL), 0.5 μL (0.2 μM) each of forward PCR primer (NMASP2-F1: 5'-GTCTTCTATCACATGTTTGG-3') and reverse primer (NMASP2-R1: 5'-TTAAAGCAACT ACAGATCA CTAC-3'), and sterile distilled water 36.75 μL. Thermal cycler profile for DNA amplification consisted of 94 ^{o}C, 2 min and 30 cycles of 94 ^{o}C, 30 sec, 60 ^{o}C, 30 sec and 72 ^{o}C, 1.5 min. The amplified PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transilluminator.

DNA sequencing

DNA sequences were obtained directly from the PCR-amplified products and determined from both directions using the Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI3100 Genetic Analyzer (Applied Biosystems, USA). Overlapping sequences were obtained by using sequencing primers.

Ethical aspect

The ethical approval of this study has been obtained from the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB No. 324/53).

Data analysis

Alignment of DNA sequences was done comparing with the *Na-ASP-2* sequence available in the GenBank™ database accession number AY28808 by using the CLUSTAL_X program.[23] Prediction of HLA-I binding peptides followed the method taken into account proteasomal C terminal cleavage and transporter associated with antigen processing (TAP) transport efficiency as developed by Larsen and colleagues.[24] Threshold for epitope identification was set at $\geq$ 0.75 using weight on C-terminal cleavage at $\geq$ 0.15 and weight on transporter associated with

antigen processing transport efficiency at ≥ 0.05. Hydrophobicity and hydrophilicity profiles of the amino acids followed the Hopp and Woods scale.[25]

Results

Detection of *Na-ASP-2* from clinical samples

Amplification of the purified cDNA from 3 individual larvae, each derived from *N. americanus*-infected individuals revealed single band PCR products measured ~700 bp, compatible with the expected size of the *Na-ASP-2* coding sequence. However, cDNA isolated from a female adult worm gave no PCR product as well as negative control using sterile water (Figure 1). The intensity of positive PCR products varied between samples, probably from difference in the mRNA content of each larva or simply from difference in mRNA yield during preparation.

Comparison of the *Na-ASP-2* sequences

Determination of DNA sequences from the PCR amplified products revealed sharp peak with little or no background noise and no superimposed signals in electroperogram. The *Na-ASP-2* sequence of each larva contained 699 bp that was identical in all 3 samples (isolates A, B and C) examined. Comparison of these nucleotide sequences with AY28808 has shown perfectly conserved sequences among these isolates (Figure 2). Figure 2 also depicts the conserved cysteine residues that are characteristic of the pathogenicity related

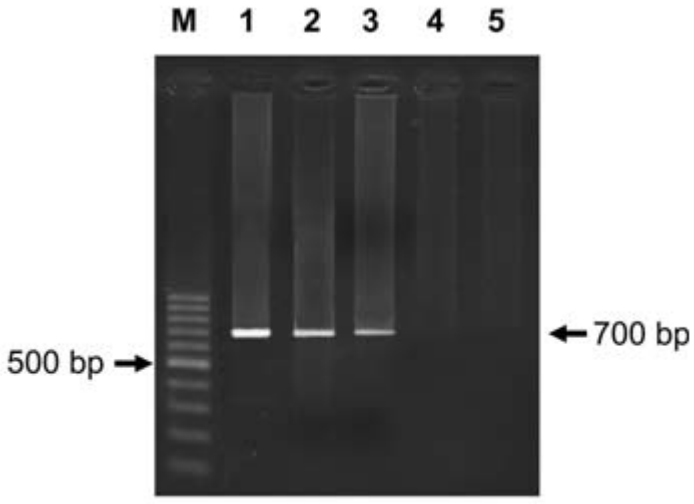

Figure 1. Amplified products of *Na-ASP-2* generated from RT-PCR using cDNA templates from filariform larva A (lane 1), larva B (lane 2), larva C (lane 3) and mature adult female (lane 4) of *Necator americanus*. Lanes M and 5 are 100 bp-ladder marker and water negative control, respectively. Product size is shown on the right of the gel. Samples were analyzed in 2% agarose gel electrophoresis.

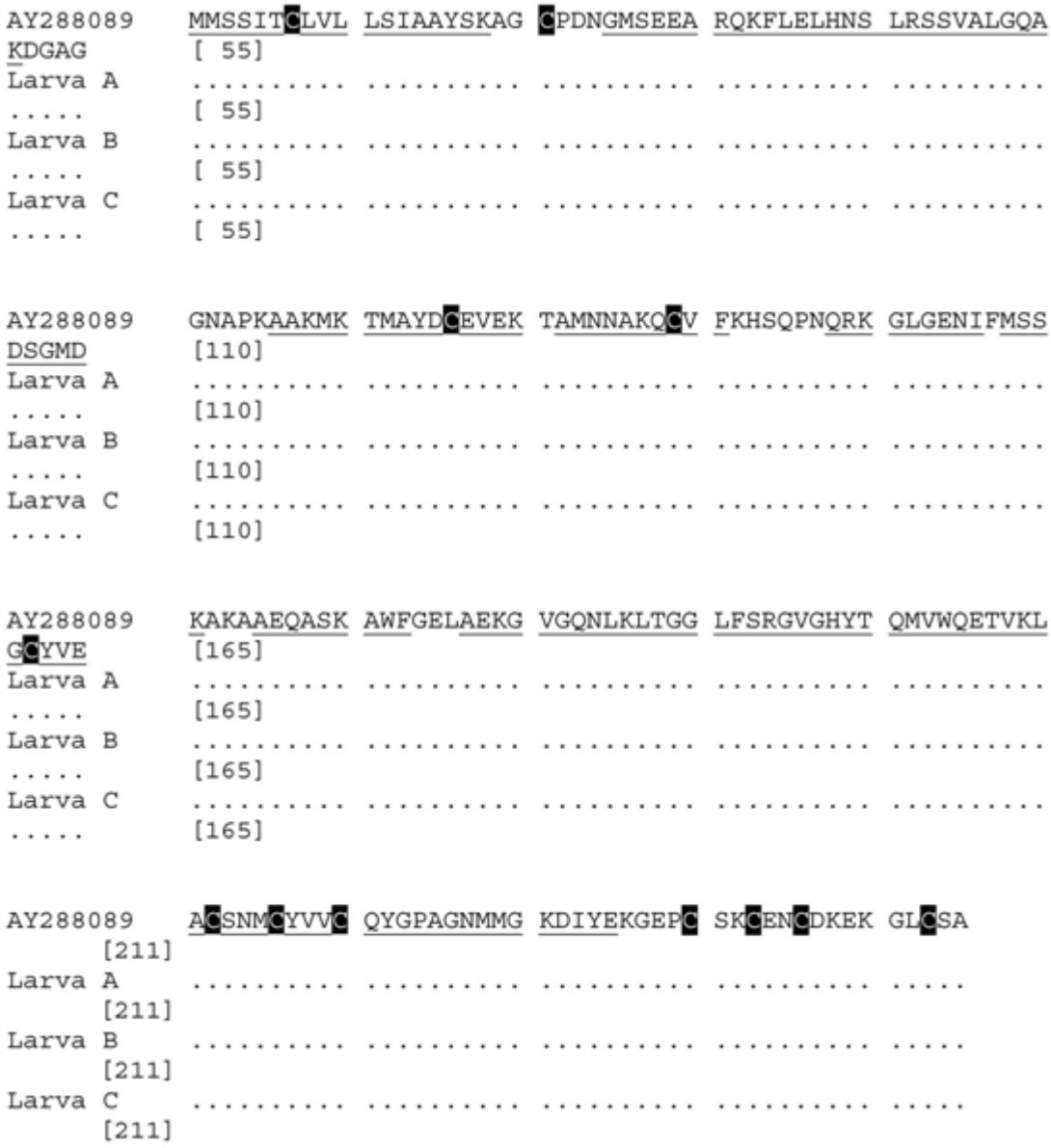

Figure 2. Alignment of deduced amino acid sequences of *Na-ASP-2* among 3 Thai samples and the GenBank™ accession number AY288089. Dots represent identical residues. Conserved cystein residues are highlighted. Locations of putative T cell epitopes are underlined. Residue numbers are shown on the right of the alignment.

Codons in *Na-ASP-2*

The gene encoding *Na-ASP-2* contained 46.3% G+C content. There was no biased distribution of G+C occurred at each of the three nucleotide positions in the codons; thereby, the codon bias index (CBI) or a measure of the deviation from the equal use of synonymous codons for the *Na-ASP-2* locus was 0.345. CBI values have a range from 0 indicating uniform use of synonymous codons to 1 indicating maximum codon bias. In terms of hydrophobicity and hydrophilicity following Hopp and Woods scale, the *Na-ASP-2* protein contained 47 hydrophilic (D, E, K, R) and 67 hydrophobic (C, F, I, L, M, V, W, and Y) residues accounting for 22.3% and 31.9% of total amino acid residues, respectively.[25]

Putative T cell epitopes in Na-ASP-2 protein

A total of 52 peptides containing 9 amino acids that had predicted HLA binding scores (more than 0.75) were identified in Na-ASP-2. These peptides are predicted to be recognized by at least 5 HLA-A supertypes and 7 HLA-B supertypes that include A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58 and B62 (Table 1). Of these, 9 peptides were recognized by two different HLA supertypes whereas peptides LVLLSIAAY and AAKMKTMAY were predicted to bind 4 and 3 different HLA supertypes, respectively. It is noteworthy that these predicted T cell epitopes were found to be scattering across the Na-ASP-2 and accounted for 77% of the protein (Figure 2).

Table 1. Putative T cell epitopes in the Na-ASP-2 protein recognized by some HLA supertypes.

HLA Supertype	Predicted epitope	Binding score*
A1	NMCYVVCQY	1.4196
	MMSSITCLV	0.9788
	AAKMKTMAY	0.9062
	MSEEARQKF	0.8761
	MSSDSGMDK	0.8293
	LVLLSIAAY	0.791
A2	MMSSITCLV	1.4344
	KTMAYDCEV	1.2939
	TQMVWQETV	1.1435
	MVWQETVKL	1.0525
	AMNNAKQCV	0.8095
A3	KLTGGLFSR	1.3305
	LLSIAAYSK	1.1778
	MSSDSGMDK	1.1317
	MMGKDIYEK	1.0842
	QMVWQETVK	0.9508
	GMSEEARQK	0.9112
	FLELHNSLR	0.8595
	MAYDCEVEK	0.8434
	LVLLSIAAY	0.8413
	SSVALGQAK	0.7781
	GLFSRGVGH	0.7677
A4	KFLELHNSL	1.1164
	QYGPAGNMM	0.8497
A26	ETVKLGCYV	1.596
	LVLLSIAAY	1.1159
	EACSNMCYV	0.8322

Table 1. Putative T cell epitopes in the Na-ASP-2 protein recognized by some HLA supertypes. (Continued)

HLA Supertype	Predicted epitope	Binding score*
B7	MVWQETVKL	0.9761
	EARQKFLEL	0.957
B8	EARQKFLEL	1.3763
	NLKLTGGLF	1.0109
	AAKMKTMAY	0.87
	NSLRSSVAL	0.8245
B27	QRKGLGENI	0.8641
B39	NSLRSSVAL	1.3994
	MSSITCLVL	0.88
B44	AEKGVGQNL	1.7827
	AEQASKAWF	1.3057
	EEARQKFL	1.1143
B58	VGHYTQMVW	1.36
	MSSITCLVL	0.9547
	KTMAYDCEV	0.9373
	SEEARQKF	0.7554
B62	LVLLSIAAY	1.4019
	AAKMKTMAY	1.2943
	NLKLTGGLF	1.0856
	LFSRGVGHY	1.0465
	MNNAKQCVF	0.9269
	NMCYVVCQY	0.9133
	CQYGPAGNM	0.8598
	TQMVWQETV	0.8252
	VEACSNMCY	0.7949

Discussion

To date, several potential vaccine targets for human hookworm vaccine have been identified. Most of these candidate antigens mainly are derived from hookworm proteins or enzymes involving in nutritional and metabolic requirements of the adult worms. As for hookworm to acquire nutrients, host red blood cells are lysed by a cascade of enzymes involving in hemoglobin degradation, collectively called hemoglobinases, such as aspartic protease (APR1), cysteine proteases and metalloproteases that are expressed on the brush border membrane of hookworm's digestive tract.[26] The by products of hemoglobin degradation are heme and hematin that are toxic for hookworm; thereby, hookworm derived glutathione S-transferase (GST) is essential for

detoxifying the oxidative damage caused by these substances. It has been anticipated that immunization with these enzyme-derived immunogens would elicit neutralizing antibodies that would interfere with hookworm blood feeding process and consequently result in impairment of adult parasite survival in the human intestine.[26] The foreseeable goals would be reduction in morbidity caused by adult worms, reduction in worm burden and indirectly interfering with hookworm transmission because of the decrease in number of eggs excreted in feces. On the other hand, other vaccine targets are those involving in the process of hookworm infection during skin penetration.[21, 26] Although molecules attributable to skin penetration may need to be further identified, the secretory protein Na-ASP-2 has been unequivocally demonstrated to play important role in skin penetration by filariform larvae. Na-ASP-2 shares several structural motifs with pathogenesis-related (PR) protein superfamily comprising characteristic conserved cysteine domains.[16] The PR proteins have been identified in a variety of organisms ranging from prokaryotes to multicellular eukaryotes of both animal and plant kingdoms.[16] These proteins mainly involve in cellular defense and cell proliferation that are essential for survival. After the success of phase I clinical trial using recombinant Na-ASP-2 adjuvanated with Alhygrogel as immunogens, a larger scale of study has been conducted in the Brazilian subjects living in hookworm endemic areas.[21]

To achieve effective vaccine efficacy, immunogens in any given vaccines need to mount immunity directing against target antigens that exist in natural populations of pathogens. In general, several antigens of eukaryotic pathogens, whose life history composes of sexual stages and several developmental forms, are complex and exhibit antigenic polymorphism among isolates as a result of interallelic recombination. For examples, antigenic polymorphism in surface antigens of *Plasmodium falciparum* and *P. vivax* that have been considered prime vaccine targets such as merozoite surface protein-1 (MSP-1) and circumsporozoite protein (CSP) exhibit extensive sequence variations among field isolates.[27 - 32] Polymorphism in these loci is mainly generated by intragenic recombination during sexual stages in mosquito vectors and is shaped by selective pressure exerted by host immune responses. Importantly, the C-terminal part of MSP-1 can induce either inhibitory or blocking antibodies whereas the C-terminal part of CSP contains polymorphic T cell epitopes that may cause immune escape mechanism through altered peptide ligand antagonism that could compromise vaccine efficacy.[29, 32 - 35] However, our analysis of sequence variation in the Na-ASP-2 coding region from 3 unrelated filariform larvae of *N. americanus* from infected Thais has shown perfect sequence identity among these isolates. It is, therefore, conceivable that the problem on vaccine escape variants naturally occurring prior to the implementation of Na-ASP-2 vaccine would not be a major concern for hookworm vaccine albeit a larger sample size are required to reaffirm this finding.

During the manufacturing process, immunogens may be purified directly from pathogens after large scale cultivation. However, for human parasites such as hookworm and malaria, it would not be practically effective in terms of time and production cost to make a vaccine through such conventional approach. By contrast, production of recombinant

proteins as immunogens would be a more efficient alternative.[22] However, recombinant protein expression in heterologous host system could be hindered by several factors, one of which is codon usage bias in the target gene. The extreme A+T richness (~80%) in *P. falciparum* genome has affected feasibility and efficiency of recombinant protein production.[36- 39] On the other hand, our sequence analysis of *Na-ASP-2* has shown that codon bias index was 0.345, approaching the pattern of uniform use of synonymous codons; thereby, codon usage in this gene would not a compromising factor for recombinant DNA technology-based hookworm vaccine development. Furthermore, using algorithm for T cell epitope prediction, a number of peptides spanning ~77% of the Na-ASP-2 sequence possess high binding scores to various HLA-A and HLA-B supertypes, suggesting that this protein is highly immunogenic and reaffirming the immunogenicity of this protein in experimental vaccine trials in animals and humans.

Although several proteins are expressed consistently throughout parasite developmental cycle, many of them are found exclusively for each developmental stage. Herein, we have reaffirmed that the Na-ASP-2 transcripts were found exclusively at L3 stage and none were expressed at adult stage because no cDNA of *Na-ASP-2* from the adult hookworm could be amplified by PCR. Stage-specific expression of parasite proteins that are immunogenic to hosts might be both functionally vital for parasite survival and beneficial for immune evasion by the parasite. It has been well recognized that immunity against antigens expressed at one stage of parasite usually is not cross-reactive to those expressed at other developmental stages. Therefore, combining immunogens derived from various developmental stages of the parasite would provide a better rationale design of a hookworm vaccine. Importantly, our finding that Na-ASP-2 is conserved among clinical isolates of hookworm would encourage vaccine incorporation with other vaccine candidate immunogens. However, the limited number of parasite samples in this study undoubtedly requires further larger scale study.

Conclusion

The Na-ASP-2 coding sequences from Thai isolates display perfect identity with that in the GenBank database. Expression of this protein did not occur in the adult worm, suggesting stage-specific expression. No apparent codon usage bias in the Na-ASP-2 gene may enhance recombinant protein production whereas several putative T cell epitopes in this protein support its immunogenicity. Sequence conservation in the Na-ASP-2 coding region suggests that efficacy of the vaccine would not be compromised by genetic diversity in hookworm population.

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