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Short communication

Sequence conservation in the *Ancylostoma* secreted protein-2 of *Necator americanus* (Na-ASP-2) from hookworm infected individuals in Thailand

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ABSTRACT

The *Ancylostoma* secreted protein-2 of *Necator americanus* (Na-ASP-2) was one of the promising vaccine candidates against the most prevalent human hookworm species as adverse vaccine reaction has compromised further human vaccine trials. To elucidate the gene structure and the extent of sequence diversity, we determined the complete nucleotide sequence of the *Na-asp-2* gene of individual larvae from 32 infected subjects living in 3 different endemic areas of Thailand. Sequence analysis revealed that the gene encoding Na-ASP-2 comprised 8 exons. Of 3 nucleotide substitutions in these exons, only one causes an amino acid change from leucine to methionine. A consensus conserved GT and AG at the 5′ and the 3′ boundaries of each intron was observed akin to those found in other eukaryotic genes. Introns of *Na-asp-2* contained 23 nucleotide substitutions and 0–18 indels. The mean number of nucleotide substitutions per site (d) in introns was not significantly different from the mean number of synonymous substitutions per synonymous site (d_S) in exons whereas d in introns was significantly exceeded d_N (the mean number of nonsynonymous substitutions per nonsynonymous site) in exons ($p < 0.05$), suggesting that introns and synonymous sites in exons may evolve at a similar rate whereas functional constraints at the amino acid could limit amino acid substitutions in Na-ASP-2. A recombination site was identified in an intron near the 3′ portion of the gene. The positions of introns and the intron phases in the *Na-asp-2* gene comparing with those in other pathogenesis-related-1 proteins of *Loa loa*, *Onchocerca volvulus*, *Heterodera glycines*, *Caenorhabditis elegans* and human were relatively conserved, suggesting evolutionary conservation of these genes. Sequence conservation in Na-ASP-2 may not compromise further vaccine design if adverse vaccine effects could be resolved whereas microheterogeneity in introns of this locus may be useful for population genetics analysis of *N. americanus*.

1. Introduction

Approximately 600–700 million people are infected with hookworms, resulting in approximately 1.83 million disability adjusted life years (DALY) (Brooker et al., 2004). Although hookworm infection causes morbidity rather than mortality, approximately annual 65,000 fatal cases attributable to the infection have been estimated (WHO, 2002). Of the two major anthropophilic hookworm species, *Necator americanus* and *Ancylostoma duodenale*, the former is the most prevalent and widely distributed species worldwide. Intestinal blood and protein loss is the main pathological consequences caused by hookworm infections. In Thailand, *N. americanus* accounts for more than 98% of adult hookworms recovered from infected individuals after drug treatment (Radomyos and Saovakontha, 1968; Anantaphruti et al., 2002). The prevalence of hookworm infections in Thailand exhibits spatial variation with the highest prevalence approaching 70% in southern part of the country whereas a survey in a west-central province bordering Myanmar has shown that about half of rural school children were infected (Anantaphruti et al., 2007). Despite various strategies to control hookworms, high prevalence and rapid recurrence of infections after administration of anthelminthic drugs have precluded effective control measures whereas sanitary status in several remote communities remains to be improved. Alternatively, development of a vaccine against hookworm infection will substantially enhance other control measures (Hotez et al., 2010).

Like other helminthic infections, naturally acquired immune responses against hookworm infection do not confer protection upon subsequent parasite exposure. However, canine hookworm vaccines derived from irradiation-attenuated third stage larvae (irL3) (Miller, 1971) or live L3 of *Ancylostoma caninum* (Otto and Kerr, 1939) could protect vaccinated canines from anemia and reduce adult worm burden *albeit* the absence of sterile immunity. Alternatively, a subunit vaccine could be more practical for large scale production with ease of storage whereas its immunological mechanisms responsible for protection could be more feasible to be delineated (Diemert et al., 2008; Hotez et al., 2010). One of

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the attractive candidates for a hookworm vaccine is a protein member of the pathogenesis-related protein 1 (PR-1) superfamily expressed at the larval stage, designated *Ancylostoma* secreted protein-2 (ASP-2) (Goud et al., 2004). Experimental studies in dogs and hamsters revealed that ASP-2-derived immunogens of *A. ceylanicum* could induce specific antibodies that reduced adult worm burdens, fecal egg counts and host blood loss upon challenge infections with infective larvae (Goud et al., 2004; Mendez et al., 2005). Importantly, the protective effects conferred by vaccines derived from irL3 or live L3 of *A. caninum* were partly attributable to ASP-2 (Bethony et al., 2005; Fujiwara et al., 2006). Furthermore, anti-ASP-2 antibodies could elicit inhibitory effects on larval migration through tissue (Goud et al., 2005).

The *ASP-2* homolog has been identified in *N. americanus* designated *Na-asp-2*, encoding a 21.3 kDa secreted protein (Goud et al., 2005). The Na-ASP-2 protein contains 210 amino acids as inferred from the cDNA clone (Asojo et al., 2005). X-ray crystallography reveals that Na-ASP-2 is folded as a three-layer αβα sandwich flanked by an N-terminal loop and a cysteine-rich C-terminal portion. Structure-related function analysis suggests that Na-ASP-2 contains solvent-exposed, negatively charged putative binding cavity and charge mimicry of chemotaxins (Asojo et al., 2005). To date, only one cDNA sequence of *Na-asp-2* is available whereas the gene encoding this protein remains unknown. It is noteworthy that most eukaryotic genes contain introns that are removed from RNA transcripts by the spliceosome, a RNA-protein complex. Importantly, introns have been identified in the *Ancylostoma* secreted protein 1 gene (*ASP-1*) of *A. caninum* (Moser et al., 2007) and the *ASP-2* homolog of *Onchocerca volvulus* (Higazi et al., 2003). Sequence variation in introns has been successfully deployed for population genetics and phylogenetic studies of several organisms including *Schistosoma japonicum* (Zhao et al., 2011) and *Toxoplasma gondii* (Lehmann et al., 2000).

To date, the extent of antigenic polymorphism in Na-ASP-2 has not been fully explored among natural parasite isolates, an issue that could compromise vaccine efficacy if strain-specific immunity ensues. The aims of this study are to determine the *Na-asp-2* gene structure and to explore the extent of sequence variation in this locus among *N. americanus*-infected individuals in Thailand.

2. Materials and methods

2.1. Sample collection

In 2010, a cross-sectional survey of hookworm infection was performed among individuals residing in Tak (n = 148), Ratchaburi (n = 65) and Nakhon Si Thammarat (n = 42) Provinces in Thailand, located 426 km northwestern (GPS 16° 1′ 0″ N, 98° 51′ 46″ E), 100 km western (GPS 13° 32′ 36″ N, 99° 20′ 24″ E) and 780 km southern (GPS 8° 40′ 0″ N, 99° 55′ 54″ E) of Bangkok, respectively. Fresh stool samples were collected from these subjects after obtaining informed consent. Stool examinations were done by both direct smear and formalin–ethylacetate sedimentation technique showing that 52 individuals harbored hookworm ova. Unpreserved stool samples containing characteristic hookworm ova were subject to polyethylene tube culture until filariform larvae of hookworm appeared in the aqueous phase at the bottom of the tubes (Sasa et al., 1965). In total, hookworm larvae were obtained from polyethylene tube culture in 32 asymptomatic subjects comprising 17 isolates from Tak, 8 from Nakhon Si Thammarat and 7 from Ratchaburi. Isolates were named using the initial of each province followed by number. All these samples harbored characteristic filariform larvae of *N. americanus* based on characteristic features as described (Beaver et al., 1984). The ethical aspects of this study have been approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

2.2. DNA and RNA extraction

Both DNA and RNA were extracted from individual larvae of each isolate for comparative analysis of the gene structure and coding region of *Na-asp-2*, respectively. To avoid mixed DNA templates from heterogenous larval population in each stool sample, a single larva from each culture was isolated by micromanipulation. Prior to DNA purification, disruption of the larva was performed by using a homogenizer. DNA was subsequently extracted by using innuPREP DNA Mini kit (Analytik Jena AG, Germany). RNA extraction was performed by using QIAamp RNeasy Mini kit (Qiagen, Germany). cDNA was generated from a larval sample and amplified by using Takara RNA PCR kit (AMV) version 3.0 (Takara, Japan).

2.3. Polymerase chain reaction and sequencing

The *Na-asp-2* gene was amplified using a forward primer NMASP2F0 (5′-GAAAATCACAATGATGTCTTCTATCAC-3′) and a reverse primer NMASP2R0 (5′-GCTGGCATCATGTTTATTTGAATATT AAAG -3′) that were derived from a Chinese strain of *N. americanus* (GenBank™ accession number AY288089)(Goud et al., 2005). Amplification of the target DNA was carried out in a total volume of 30 μl of the reaction mixture containing template DNA, 2.5 mM $MgCl_2$, 300 mM each deoxynucleoside triphosphate, 3 μl of 10X ExTaq PCR buffer, 0.3 μM of each primer and 1.25 units of ExTaq DNA polymerase (Takara, Seta, Japan). Thermal cycling profile contained preamplification denaturation at 94 °C for 1 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and a final extension at 72 °C for 5 min. The PCR was performed in a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). The PCR product was examined by electrophoresis in a 1% agarose gel and visualized under a UV transilluminator. The PCR product was purified by using QIAquick PCR purification kit (Qiagen, Germany). DNA sequences were determined directly and bi-directionally from PCR-purified templates. Primers used for sequencing of PCR products were NMASP2F1 (5′-GTCTTCTATCACATGTTTGG-3′), NMASP2F2 (5′-ATG TTTGGTTCTTCTCTCGATTG-3′), NMASP2F3 (5′-GCCAAGGATGGAGC TGGTGGA-3′), NMASP2F4 (5′-AACCAAAGGAAAGGATTGG-3′), NM ASP2R1 (5′-TTAAAGCAACTACAGATCACTAC-3′), NMASP2R2 (5′-CC TATTCTAACGTTTGCTTTAGTCGT-3′), NMASP2R3 (5′-CAACCTGAAG ATTTTCGAATTAAA-3′) and NMASP2R4 (5′-GCTTGCAAAATTATTAA GTTAGAC-3′). Sequencing was performed on an ABI3100 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequences reported in this study have been available in the GenBank™ database under the accession numbers JX046759–JX046790.

2.4. Data analysis

Sequences were aligned using the CLUSTAL X program with manual adjustment (Thompson et al., 1997). The nucleotide position at which the alignment postulated a gap was excluded from the following analyses. Haplotype diversity (h) including its standard deviation (Nei, 1987) and the minimum number of recombination events in the history of sequences (Rm) (Hudson and Kaplan, 1985) were determined as implemented in the DnaSP version 5.10.01 software (Librado and Rozas, 2009). A recombination event in the parameter Rm is inferred to have occurred between two polymorphic sites when all four possible gamete types involving the two sites are found in the sequences analyzed (Hudson and Kaplan, 1985). Detection of recombination by phylogenetic approach was also performed by using Genetic Algorithm