Immunodiagnosis of Gnathostomiasis

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Human gnathostomiasis is a food-borne helminthic zoonosis caused by a tissue nematode, *Gnathostoma* spp.[1,2] The parasite was first discovered from the stomach wall of a tiger carcass in London in 1836.[3,4] However, the first human case was reported more than 50 years later in Thailand.[4-6] Nowadays, human gnathostomiasis is endemic causing relatively high prevalence in several countries in Asia such as Thailand, Japan, Indonesia, Philippines, Malaysia, Myanmar, and Indochina.[4,7-8] Human cases have been reported occasionally from Bangladesh, Canada, China, India, Korea, Sri Lanka, Australia, Latin America and Africa.[4,9-17] Also, there have been increasing cases of gnathostomiasis in non-endemic areas among travelers returning home and immigrants from the parasite endemic areas.[17-22] The larva migrans caused by advanced third stage larva(e) of *Gnathostoma* spp., may be regarded as a rare and neglected tropical disease in terms of the world prevalence rate; nevertheless it is recognized as an emerging public health problem in some parts of the world such as in the states of Sinaloa, Oaxaca, Veracruz, Tamaulipas, Guerrero, and Nayarit in Mexico because of the custom of eating raw freshwater fish (cebiche and callos which are traditional dishes, and sushi).[15]

Currently, up to 18 species of *Gnathostoma* (Family Gnathostomatidae Railliet, 1895; Blanchard, 1895, 1896; Subfamily Gnathostomatinae Railliet, 1895; Chabaud, 1975; diagnostic: Dey Sarkar, 1995) have been described.[23-26] They are: *Gnathostoma americanum* Travassos, 1925; *Gnathostoma binucleatum* Almeyda-Artigas, 1991; *Gnathostoma brasiliense* Ruiz, 1952; *Gnathostoma didelphis* Chandler, 1932; *Gnathostoma doloresi* Tubangui, 1925 (description: Shoho and Machida, 1979; diagnostic: Lin Xiumin, Chen Qingquan, Song Jiaosong, Li Xiunian, Wei Xin Mi, 1993); *Gnathostoma gracile* Diesing, 1839; *Gnathostoma hispidum* Fedstchenko, 1872; *Gnathostoma lamothei* Bertoni-Ruiz, Garcia-Prieto, Osorio-Sarabia, Leon-Regagnon, 2005; *Gnathostoma malaysiae* Miyazaki and Dunn, 1965 (redescription: Kamiya, Kamiya, Ohbayashi, Klongkamnuankarn, Vajrasthira, 1987); *Gnathostoma minutum* Schuurmans-Stekhoven, 1943; *Gnathostoma miyazakii* Anderson, 1964; *Gnathostoma neoprocyonis* nomen nudem; *Gnathostoma nipponicum* Yamaguchi, 1941; *Gnathostoma procyonis* Chandler, 1932; *Gnathostoma socialis* mustélidos, EUA; *G. spinigerum* Owen, 1836;

Gnathostoma turgidum Stossich, 1902; and *Gnathostoma vietnamicum* Le Van Hoa, 1965. Among these, 6 species are found in animals in Asia, *i.e.*, *G. doloresi*, *G. hispidum*, *G. malaysiae*, *G. nipponicum*, *G. spinigerum*, *and G. vietnamicum.* However, only 4 species, *G. spinigerum*, *G. dorolesi*, *G. hispidum*, and *G. nipponicum* infect humans.[25,27-29] The most prevalence cases in Asia especially Thailand are caused by *G. spinigerum*[30] while infections by the other 3 species occur frequently in Japan. Humans are incidental hosts of *Gnathostoma* spp., while both domestic and wild carnivores, *e.g.*, dogs, cats, tigers, golden cats, leopard cats and jungle cats are natural (definitive) hosts of this parasite.[23,31] Mature male and female worms live in the esophageal wall, stomach tumor or intestinal wall of the definitive hosts.[1,3,32] The fertilized parasite ova pass out with the host feces, hatch in water to first stage larvae, and are ingested by the first intermediate host, *i.e.*, some species of water cyclops.[33] Within the cyclops the larvae develop further to the second and early third stage larvae. The infected cyclops are prayed upon by fresh water fishes (*e.g.*, loaches, fresh water cat fish, serpentine fish), amphibians (*e.g.*, frogs), reptiles (snakes, swamp eels), chicken and other avian and mammals.[34] Within these second intermediate (paratenic) vertebrate hosts the parasite develops further to the infective (advanced third stage) larva (L3). The parasite completes its life cycle after the second intermediate hosts are eaten by the carnivorous definitive hosts where the L3 develop to adults within their stomach.[23,31] Humans get infection mostly by eating raw or undercooked meat or fresh water fish containing living L3. Prenatal infection has been reported in human newborns.[35] The infecting larva penetrates from the human gastrointestinal tract and roams about in the human body: skin and/or viscera, and may develop further to fourth stage larva, immature adult or sexually mature adult.[35,36] Migration of the parasite in dermal tissue produces cutaneous larva migrans characterized by recurrent/intermittent pruritic, migratory swellings (sometimes with local pain and low grade fever) from which occasionally the larva may emerge through the skin especially after albendazole stimulation.[37-40] Migration of the parasite in viscera, *i.e.*, visceral larva migrans, is a more serious form of gnathostomiasis. Vital organs of the host may be afflicted causing tissue damage and severe maladies. Frequently

the parasite wanders to the eyes causing pain, conjunctival erythema, opthalmitis, ocular hemorrhage or sometimes blindness. The parasite may invade the central nervous system causing subarachnoid hemorrhage, eosinophilic meningitis/myeloencephalitis, paresis/paralysis, and even death in some cases.[6,8,15,36,39,41-54] Patients with gnathostomiasis have peripheral blood eosinophilia.[39,52]

Diagnosis of gnathostomiasis

Gnathostomiasis can be indirectly diagnosed by using the patient's information, *e.g.*, inhabitant of-, immigrant from-, or traveler to- the parasite endemic area, history of eating raw/improperly cooked meat or fish, presentation of cutaneous intermittent/migratory swelling and high eosinophilia. However, accuracy of the clinical diagnosis depends on the awareness and experience of the attending physician as the clinical picture of gnathostomiasis is difficult to differentiate from those of other parasitoses such as angiostrongyliasis cantonensis, trichinellosis and cutaneous larva migrans caused by other parasites such as hookworms, or the occult form of lung fluke infection, *Paragonimus heterotremus*.[38,39,55-57] Eosinophilic meningitis caused by gnathostomiasis is indistinguishable from the malady caused by other (parasitic) etiology, especially angiostrongylus encephalitis.[58-60] Various modalities such as magnetic resonance imaging may be used to support the clinical diagnosis.[50,53] Definite diagnosis of gnathostomiasis is made in rare occasions by recovery of the larva from the infecting host such as when the parasite emerges through skin or eyeball or after surgical excision from tissue such as the brain.[40,53] DNA based-assays, *e.g.*, PCR and DNA hybridization, are not applicable for gnathostomiasis diagnosis, because there is no appropriate specimen containing the parasite or parasite DNA. Usually human gnathostomiasis is caused by a single larva infection (Harinasuta T, personal communication) and the infecting parasite does not confine itself to a particular anatomical site within the host. Similar to the DNA techniques, an antigen based-assay for human gnathostomiasis diagnosis by using specific antibody to the parasite as an antigen capture reagent is hampered by the lack of clinical specimens containing adequate amounts of the parasite antigen. Only a few studies reported the antigen based diagnosis for gnathostomiasis. Tuntipopipat *et al.*[61] performed a sandwich ELISA on cerebrospinal fluids (CSF) of 11 patients with central nervous system involvement using a biotin-streptavidin ELISA system. It was found that the antigen could be detected in CSF of only 3 patients. Nevertheless, a definite presence of the *Gnathostoma* antigen could be confirmed in only one case. Circulating antigen of *G. spinigerum* was detected by two-site ELISA in mice experimentally infected with 15 early third stage larvae of the parasite.[62]

Like most other infections, humans infected with *Gnathostoma* spp., develop a serum antibody response to the parasite antigens. Detection of these antibodies is a sensitive presumptive diagnosis of gnathostomiasis.[63-68] Several immunological tests have been developed for the immunodiagnosis of gnathostomiasis. These include various versions of immunoprecipitation tests,[63,69-71] skin test for immediate hypersensitivity reaction,[72-75] radioimmunoassay,[76] enzyme linked immunosorbent assay (ELISA or IgG-ELISA),[55,64,67,68,71,74,77-79] Western blot analysis,[66,79,80-82] indirect hemagglutination test,[67] indirect immunofluorescent antibody test,[83] and circumoval and larval microprecipitation tests.[84] Most of the serological methods used a crude extract of either adult parasite or larvae (*G. spinigerum* or *G. dorolesi*) as a diagnostic antigen. Most studies reported high diagnostic sensitivity compared to the clinical and/or parasitologically confirmed diagnosis. Nevertheless, most of the tests suffer relatively low diagnostic specificity owing to common antigenic components shared by *Gnathostoma* spp., with other parasites. Immune response (especially anti-bodies) in the sera of individuals (particularly the inha-bitants of parasite endemic areas), induced from past exposure to several other parasites, bind to one or more components in the crude *Gnathostoma* extract rendering false positive results of most antibody based assays as well as the skin test.[55,71,73,76,77,85,86] Therefore, a specific antigen of the *Gnathostoma*, should be sought. Such an antigen in pure form (or pure enough to eliminate the cross reacting components) or the parasite recombinant protein of similar antigenicity to the native antigenic counterpart should increase the specificity of the antibody based-tests for gnathostomiasis diagnosis.

By using SDS-PAGE, it was found that a crude water extract of advanced third stage larvae of *G. spinigerum* obtained from the livers of naturally infected swamp eels contained more than 40 proteins.[66] Among them more than 20 proteins ranged in molecular masses from 13 to 150 kDa, were immunogenic in the infected hosts, *i.e.*, parasitologically confirmed patients.[66] In 1991, the sera of patients with parasitologically confirmed gnathostomiasis, clinically diagnosed gnathostomiasis, and patients with various other parasitic infections, and sera of healthy, parasite free subjects were studied for the antibody reactivity to SDS-PAGE separated crude water extract of L3 of *G. spinigerum* by Western blot analysis.[81] The complexity of the parasite crude extract confirmed that more than 20 antigenic bands were revealed by the sera of parasitologically confirmed gnathostomiasis patients. Among them, one prominent antigenic band located at 24 kDa consistently reacted with the antibody in sera of all confirmed gnathostomiasis cases.[81] The *G. spinigerum* specific component was subsequently purified by using gel filtration, isoelectricfocusing and ion exchange chromatographies and used in an indirect ELISA for the diagnosis of human gnathostomiasis. This specific antigenic component had pI of 8.5 and comprised 0.23% of the total proteins in the crude water extract of *G. spinigerum* L3. The specific antigen gave 100% diagnostic sensitivity and specificity for human gnathostomiasis diagnosis.[79] By using two dimensional polyacrylamide gel electrophoresis and Western blotting, Wongkham *et al.*[87] confirmed that the antigenic components of *G. spinigerum* infective larvae with molecular masses of 23-25 kDa and pI of 8.3-8.5 were uniquely recognized by the sera of patients with parasite confirmed gnathostomiasis. Tuntipopipat *et al.*[88] used a low molecular weight (<29 kDa) fraction of partially purified *G. spinigerum* L3 extract in the diagnosis of gnathostomiasis and demonstrated high diagnostic specificity, sensitivity and accuracy of the assay. Slight differences in the molecular masses of the specific components in different studies is likely to be due to the different protein markers and conditions used in the Western blotting. Luammuanwai *et al.*[89] detected specific IgG subclasses in the sera of patients with proven and clinically diagnosed gnathostomiasis by immunoblotting technique and confirmed that the 24

kDa component of *G. spinigerum* infective larvae gave the best sensitivity and specificity in detecting specific IgG4 in the sera of the patients. Subsequently, the cDNA library of the *G. spinigerum* L3 was cloned. Full length DNA sequence coding for the diagnostic 24 kDa which was recognized by gnathostomiasis patients' sera and a specific monoclonal antibody to the 24 kDa component (clone GN6/24[90]) was found to contain 732 nucleotides. The deduced amino acid sequence revealed 33-39% similarity to the matrix metalloprotease (MMP) of *Caenorhabditis elegans* and several vertebrates.[91] The protein possesses the catalytic domain but lacks propeptide and hemopexin-like domains found in other MMPs.[91] The 24 kDa protein had a 23 amino acid signal peptide at the amino terminus indicating that it is a secreted protein.[91] This speculation was confirmed by the presence of the diagnostic protein in excretory-secretory (ES) products of L3.[88,91,92] Anatomically, the 24 kDa component was found in the esophagus and intestine of the *G. spinigerum* infective larvae collected from the livers of naturally infected swamp eels and from experimentally infected mice.[92] Distilled water was found be the best extraction solution for the protein.[92] The 24 kDa component was N-glycosylated; nevertheless both the patients' sera and the specific monoclonal antibody of clone GN6/24 readily recognized the immunogenic epitope in the deglycosylated protein.[93] Recently, by using gel based-proteomics, it was revealed that the 24 kDa component of *G. spinigerum* L3 exists naturally in several immunogenic isoforms (data to be published).

Currently, the finding of a serum antibody to the specific component, ~ 24 kDa protein of the *G. spinigerum* L3, by Western blot analysis together with the relevant epidemiologic and clinical pictures of the patient are diagnostic criteria for human gnathostomiasis. However, the serological method is performed by only few laboratories, *i.e.*, Department of Parasitology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700; Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400; and Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand. Clinical samples (serum, CSF or pleural effusion) from suspected gnathostomiasis patients are sent for the presumptive testing from various health settings within the country and also from overseas. The limitation of the diagnostic service is mainly due to an inadequate supply of the L3 extract. The L3 must be collected individually under a dissecting microscope from viscera of the naturally infected second intermediate hosts such as livers of the naturally infected swamp eels and frogs. Not only the L3 collection is laborious and unpleasant, but also there has been a difficulty in finding the infected second intermediate hosts. Moreover, the specific *G. spinigerum* component was less than 0.23% of the total L3 proteins.[79] In the SDS-PAGE for the Western blot diagnosis, a high amount of the crude L3 extract must be loaded into the gel slot. The obstacles have been solved recently by using the recombinant antigen (which reacted specifically and readily to the antibody in the sera of patients with gnathostomiasis) instead of the native 24 kDa *G. spinigerum* L3 protein as an antigen in the serodiagnosis of human gnathostomiasis (data to be published).

CONCLUSION

Gnathostomiasis is a foodborne parasitosis caused by a nematode of *Gnathostoma* spp., Currently 18 species of the parasite have been described, but only 4 species infect humans, *i.e.*, *G. spinigerum* (especially cases in Thailand), *G. dorolesi*, *G. hispidum*, and *G. nipponicum*. Humans are incidental hosts of *Gnathostoma* spp., while domestic and wild carnivores are natural (definitive) hosts. Humans get infected by eating raw or improperly cooked meat or fish (the parasite second intermediate/ paratenic hosts) containing living infective (advanced third stage; L3) larva(e). Clinical features of gnathostomiasis are cutaneous and/or visceral larva migrans, and peripheral blood eosinophilia. Occasionally, gnathostomiasis causes serious clinical manifestations such as opthalmitis, blindness, ocular or subarachnoid hemorrhage, eosinophilic meningitis/myeloencephalitis, paresis, paralysis or even death because the parasite likes to invade the eye and the central nervous system. Definite diagnosis of gnathostomiasis is rarely made by recovery of the parasite from the patient's skin, eyeball or surgical excision. Patient's history (inhabit in-, migrated from- or traveled to- the endemic area, and consumed raw/undercooked meat or freshwater fish), clinical pictures (*e.g.*, intermittent, pruritic, migratory cutaneous swelling and eosinophilia) and the presence of antibody to a diagnostic protein of L3, *i.e.*, native 24 kDa component or its recombinant surrogate, are the presumptive diagnosis for human gnathostomiasis.

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