

Diagnosis of gnathostomiasis by skin testing using partially purified specific antigen and total IgE levels

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Background: A finding of antibodies to *Gnathostoma spinigerum* 24-kDa antigen by immunoblot analysis is currently used to confirm a diagnosis of gnathostomiasis. A simple skin test for the diagnosis of gnathostomiasis was developed, and the results were evaluated and compared with the standard Western blot (WB) test.

Methods: This cross-sectional study was conducted at the Hospital for Tropical Diseases, Bangkok, Thailand, in 2008–2011. All eligible patients were tested with partially purified proteins of mAb-detected fractions pooled and sterilized by 0.2 µm diameter syringe filter, with a phenol saline solution of 1:10 w/v.

Results: A total of 69 cases, 39 gnathostomiasis cases and 30 controls, were enrolled into the study; the median age (IQR) was 40 (30.5–52.5) years. The most common presenting symptom was edema (56/69, 81%). Gnathostomiasis cases having strong cutaneous reactions to the intradermal test (81%) were also positive by immunoblot. A significant correlation between skin and immunoblot tests was detected ($p < 0.001$). The difference in total IgE levels between cases and controls was not statistically significant ($p = 0.51$). Logistic regression models showed that positive WB and skin-test results were significantly associated with gnathostomiasis ($p = 0.001$ and $p = 0.007$, respectively).

Conclusion: *Gnathostoma* skin testing, using prepared fractionated antigen solution of *Gnathostoma spinigerum*, yields good reactivity and significantly correlates with the results of immunoblot testing.

Keywords: Eosinophilia, IgE, Immunoblot, Gnathostomiasis, Skin test

Introduction

Gnathostomiasis is prevalent in Thailand and in other countries of Southeast Asia. Its manifestations involve soft-tissue inflammation with acquired eosinophilia following larval tracking in an infected body. The route of transmission is commonly via the ingestion of raw or insufficiently cooked meat of animal hosts (fish, eels, frogs, birds and reptiles).¹ Cutaneous gnathostomiasis is the most common form of the disease, which mostly presents with recurrent painless subcutaneous swelling. The swelling is due to both mechanical damage from the larva and the host's immunological response to the parasite and its secretions.

A highly sensitive and specific immunoblot (Western blot) test against the 24-kDa antigen of *Gnathostoma spinigerum* is currently used to confirm a diagnosis of gnathostomiasis.^{2–5} A simple and rapid test is needed to confirm the diagnosis at the bedside in

clinical practice, especially in remote, resource-limited areas. A skin test, using crude parasite extract, has been found useful in diagnosis, but lacking in specificity.^{2,4} In this study, we aimed to develop a simple alternative method to diagnose gnathostomiasis by skin testing with *Gnathostoma spinigerum* fractionated antigen solution and determine the association of total IgE levels with skin-test results among patients presenting with angioedema or eosinophilia.

Materials and methods

The study was conducted at the Hospital for Tropical Diseases, in Bangkok, Thailand, during the period April 2008 to September 2011. A diagnosis of possible gnathostomiasis was based on the following findings: cutaneous manifestations attributable to

gnathostomiasis, including creeping eruption, migratory swelling, nodule or plaque on any body part; eosinophilia with absolute eosinophil counts $\geq 500/\mu\text{L}$; and 'certitude case' or antibody specific to *Gnathostoma* antigen by immunoblot assay. Immunoblot assays were performed at the Department of Helminthology, Faculty of Tropical Medicine, Bangkok, using an antigen produced from infective-stage larvae of *Gnathostoma spinigerum*, as previously described.^{5,6} A diagnosis of certitude was defined as either a biopsy specimen of the cutaneous lesion that revealed a cross-section of the nematode, or removal of the nematode from the swollen area involved. The control group was selected from cases with other acquired eosinophilia, including other parasitic infections, allergy and autoimmune diseases.

All eligible patients signed for voluntary informed consent prior to any study-related procedure. An intradermal skin test with 0.05 mL of partially purified proteins of the mAb-detected fractions, pooled and sterilized by syringe filter with 0.2 μm diameter at a solution of 1:10 w/v phenol saline (Hollister-Stier, Spokane, WA, USA), or approximately 0.5 μg of *Gnathostoma* protein, was administered to the study participants. The partially purified antigen was prepared from the extract of digested infective *Gnathostoma* larvae collected from the livers of swamp eels and fractionated by Sephacryl S-200 modified syringe chromatography. The antigen per fraction was limited and selected with monoclonal antibody (mAb) to 24-kDa antigen using IgG-direct ELISA, as previously described.⁷ Also, 50% v/v glycerinated solution (Hollister-Stier) and histamine (0.01 mg/mL) were used as negative and positive controls for the skin test, respectively. The longest diameter was used as the assessment parameter for wheal response after skin testing. A skin test was considered positive when the mean wheal diameter was ≥ 5 mm, with no wheal at the negative control site, and mean wheal ≥ 3 mm at the positive control site (Figure 1).

Serum IgE was determined at the same time with monoclonal anti-human IgE antibody, using an ELISA kit (IBL International GMBH, Hamburg, Germany). The following demographic and clinical information was recorded for each patient: age, gender, place of residence, recent travel history, exposure to raw food, onset and

duration of clinical presentations, clinical signs, treatment and treatment outcome. The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University. Sample size was 35, assuming the skin test would yield 90% positive results, with acceptable CI $<10\%$. Subjects exhibiting a measured parameter from database in the case report form was expressed as a proportion or percentage of all subjects, and assessed by comparison of the case and control groups.

Demographic, clinical information, and laboratory data were analyzed using the Statistical Package for the Social Sciences version 18.0 (SPSS, Chicago, IL, USA). Quantitative data were tested for normality using the Kolmogorov-Smirnov test and reported as median (IQR) for non-normally distributed data. Qualitative data were reported as frequency (percentage) and analyzed by Pearson's χ^2 or Fisher's exact test, as appropriate. Univariate analysis was performed to determine possible risk factors for gnathostomiasis. Any variable with $p \leq 0.1$ by univariate logistic-regression analysis was included in the bivariate logistic regression analysis, using an enter method to determine independent factors associated with gnathostomiasis. All tests of significance were 2-sided; $p < 0.05$ indicated statistical significance.

Results

Of a total 69 cases, 39 gnathostomiasis (33 possible cases and 6 cases with certitude), and 30 control patients, were enrolled into the study. The control group included five cases of allergic rhinitis, five systemic lupus erythematosus (SLE), four chronic urticaria, four angiostrongyliasis meningitis, three HIV infection, two non-allergic rhinitis with eosinophilic syndrome (NARES), two hypereosinophilic syndrome (HES), one enteric fever, one eosinophilic fasciitis, one erythema nodosum (EN) with reactive arthritis, one rheumatoid arthritis, and one hepatitis B infection. Most of the study participants (62%, 43) were female, with a median age (IQR) of 40 (30.5–52.5) years. Most participants lived in northeastern Thailand (35%, 24), and the rest were from central Thailand and Bangkok (20%, 14 and 16%, 11, respectively). In addition, 9% (6) of the patients were from neighboring countries: Lao PDR and Myanmar. One third (23) of all cases had a history of allergy, including chronic rhinitis, food allergy, and drug allergy; however, the difference between the two groups was not significant.

Information on exposure risk was collected during the study. History of raw fish consumption was found to be significantly high among patients with gnathostomiasis ($p < 0.001$). The most common main presenting symptoms were edema (81%, 56), rash (6%, 4), itching (4%, 3), and skin nodule (3%, 2). However, the medical histories revealed that swelling was found in 86% (59) of cases; the sites involved were the upper limbs (55%, 38), lower limbs (41%, 28), and face (29%, 20). Median (IQR) duration of swelling was 42 (19–95) days. The most common swollen areas were the arm (41%, 28), leg (32%, 22), face (30%, 21), and hand (20%, 14). Swelling on the eyelid (10%, 7), lip (9%, 6), and cheek (9%, 6), were also noted. Demographic and clinical information are summarized in Table 1.

Most gnathostomiasis cases presented with swelling. In addition, migratory swelling of the limbs was significant among the patients ($p = 0.01$). However, the distribution of cutaneous swelling between cases and controls was not significantly different. Regarding eosinophilia, the median (IQR) of absolute eosinophil



Figure 1. A positive intradermal skin test with *Gnathostoma spinigerum* fractionated antigen solution (from upper to lowest levels: negative control, a solution of 1:100 [not evaluated] and 1:10 w/v phenol saline, and positive control).

Table 1. Demographic and clinical manifestations of gnathostomiasis case and control groups

Characteristics	Gnathostomiasis (n=39) n (%)	Control (n=30) n (%)	p-value
Demographic			
Gender: Female	25 (64)	18 (60)	NS
Age (years): Median (IQR)	39 (30–52)	42 (34–54.5)	NS ^a
Residency area			
Northeastern area	16 (41)	8 (27)	NS
Bangkok and nearby areas	6 (15)	12 (40)	0.02
History of raw fish consumption	34 (87)	12 (40)	<0.001
History of eating raw meat	35 (90)	13 (43)	<0.001
History of eating frog	8 (21)	3 (10)	NS
History of allergy	15 (38)	8 (27)	NS
Clinical manifestations			
Presentation with cutaneous swelling:	38 (97)	18 (60)	<0.001
Arm swelling	18 (46)	10 (33)	NS
Leg swelling	13 (33)	9 (30)	NS
Swelling on the face	12 (31)	9 (30)	NS
Migratory swelling	23 (59)	7 (23)	0.01
Intermittent swelling	26 (67)	14 (47)	NS
Duration of illness (days): Median (IQR)	40 (20–84)	42 (17–142.5)	NS ^a

NS: not significant.

^a Comparison using Mann-Whitney U test.**Table 2.** Comparison of skin test and laboratory results between gnathostomiasis cases and control group (non-gnathostomiasis cases)

Laboratory investigations	Gnathostomiasis (n=39)	Control (n=30)	p-value
Absolute eosinophil count (cells/ μ L): median (IQR)	869 (546–2218)	541.5 (146.25–1207)	0.01 ^a
<i>Gnathostoma</i> immunoblot positive	85% (33)	3% (1)	<0.001
Skin test positive with <i>Gnathostoma</i> extract	87% (34)	20% (6)	<0.001
Diameter of skin test reaction (mm): median (IQR)	14 (10–18)	0 (0–0)	<0.001 ^a
Total IgE: median (IQR)	88.97 (22.74–250.09)	24.99 (2.11–310.03)	NS ^a
IgE $\geq$ 100 kU/L	49% (19)	30% (9)	NS

NS: not significant.

^a Comparison using Mann-Whitney U test.

count and its percentages were 693 (315.5–1392) cells/ μ L and 10 (6.45–18)%, respectively; eosinophil counts were significantly higher for gnathostomiasis patients than controls (median [IQR]: 869 [546–2218] vs. 541.5 [146.25–1207] cells/ μ L, $p=0.01$; Table 2). Logistic regression was used to examine demographic and clinical parameters associated with gnathostomiasis; no associated factors were found.

Association of skin testing with partially purified *Gnathostoma spinigerum* antigen and 24-kDa detection by immunoblot test

Skin reactions were observed for 15 minutes after skin testing. No severe or systemic reaction was noted among the study

participants. Most localized skin reactions resolved spontaneously within 30 minutes; all disappeared in the first hour post-injection. The median longest diameter (IQR) of the skin test, using 1:10 w/v partially purified protein solution, was 10 (0–15) mm in all cases. When the cases and controls were compared, the longest diameters were significantly different (Table 2). Among the patients with gnathostomiasis, 85% (33) had *Gnathostoma* IgG antibody by immunoblot test, and 87% (34) showed wheal reactions from skin test using 1:10 w/v partially purified protein solution (Table 2). The specificities of the gnathostomiasis serology and skin tests were 96% and 80%, respectively. Regarding the cross-reactivity of skin testing in tissue-dwelling heterologous parasitic infections, one of the angiostrongyliasis patients had a positive test result, with the longest wheal diameter of 10 mm (75%

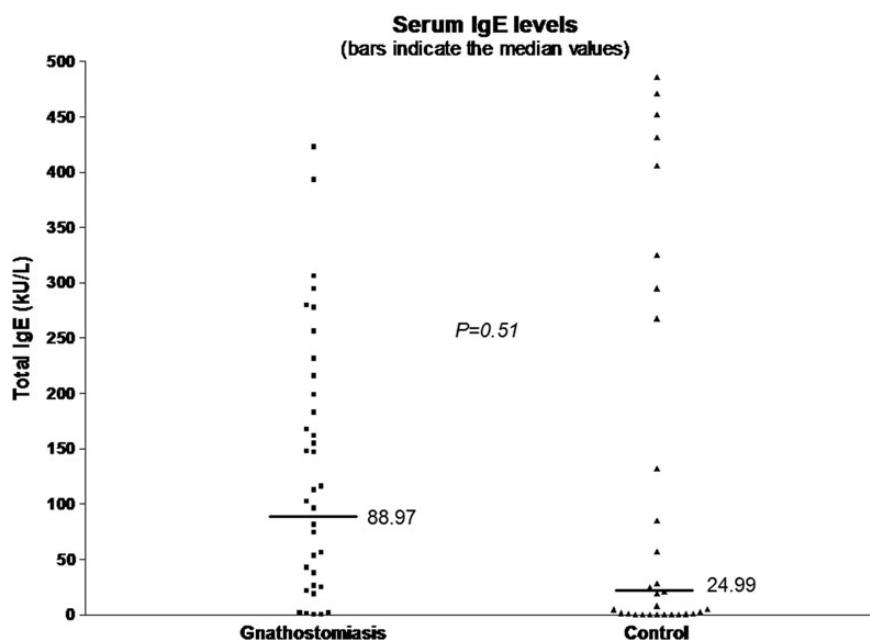


Figure 2. Distribution of total IgE (kU/L) between the gnathostomiasis cases and the control group.

specificity). The associations of the test results were significant for both tests ($p < 0.001$). These two diagnostic tests were further evaluated using logistic regression. A positive immunoblot test against 24-kDa antigen to *Gnathostoma spinigerum*, and skin testing with partially purified *Gnathostoma* antigen, were significant predictive factors for gnathostomiasis ($p = 0.001$ and $p = 0.007$, respectively) with 95% CI OR of 48.7 (4.47–530.67) and 15.9 (2.14–118.18), respectively.

Serum total IgE and results of skin testing with partially purified *Gnathostoma spinigerum* antigen and 24-kDa detection by immunoblot test

The median (IQR) IgE level was 57.1 (4.97–272.76) kU/L for all cases, and 89 (22.7–250.1) kU/L for gnathostomiasis cases. However, the difference in total IgE levels between cases and controls ($p = 0.51$) was not significant (Figure 2). Of cases having strong cutaneous reactions to 1:10 w/v antigen solution, 81% were positive by *Gnathostoma* immunoblot. Patients with positive immunoblot tests were likely to have higher eosinophil counts than those who had negative immunoblot tests (median [IQR]: 813 [543–1312] vs 390 [138–863], $p = 0.01$). Furthermore, patients with strong skin-test reactions were likely to have statistically higher absolute eosinophil counts (849.5 vs 550 cells/ μ L, $p = 0.03$) than those with negative skin tests. However, there was no significant difference in total IgE levels between patients with positive and negative skin tests (116 vs 23 kU/L, $p = 0.08$). There was a statistically significant association between high levels of IgE (≥ 100 kU/L) and positive immunoblot and skin tests ($p = 0.03$ and 0.04 , respectively).

Discussion

Gnathostomiasis is a common food-borne zoonosis in Thailand. As a referral center for tropical diseases, the Hospital for Tropical

Diseases in Bangkok sees many presumptive cases of gnathostomiasis from different areas of Thailand. Significantly, the patients with gnathostomiasis in this study came from areas outside Bangkok ($p = 0.021$), and had a history of consuming raw or inadequately cooked fish ($p < 0.001$). In urban areas, gnathostomiasis is an important health problem because of changing food preferences and eating habits, particularly the increased availability of ready-to-eat fresh foods and emerging Japanese/Korean food trends.⁸ Changes in dietary cultures and practices in rural areas are key to reducing the transmission of gnathostomiasis. While health campaigns have been conducted for populations at risk, more effective preventive campaigns and continued public health education are required to address the problem. Apart from exposure history, significantly higher eosinophil counts were detected among patients with gnathostomiasis and patients with positive immunoblot or skin tests. However, eosinophilia was not associated with gnathostomiasis by multivariate analysis.⁹ This finding might be a result of using eosinophilia as an inclusion criterion in this study. In addition, most tissue-dwelling parasitic infections can cause eosinophilia, which may not be helpful in the diagnosis of gnathostomiasis.

Tissue parasites can induce adaptive immune responses, which selectively evoke T helper 2 (Th2) cells driving an IgE response. Skin testing is a bioassay to detect binding of specific *Gnathostoma* antigen to IgE cross-linked Fc ϵ RI on cutaneous mast cells or activated eosinophils causing a release of chemical mediators, which leads to the development of a wheal-and-flare reaction.¹⁰ A positive skin reaction was shown by gnathostomiasis cases where a significant association was demonstrated between high eosinophil counts and positive skin tests. Although skin testing is considered a safe procedure commonly performed in the allergy/immunology field of clinical practice, it is not without risk. The rare, yet potentially life-threatening anaphylactic event means that full emergency equipment and epinephrine should be readily available in any clinic if required. The *Gnathostoma*

antigen used in this study was obtained almost exclusively from natural sources, and was generally unacceptably unhygienic for injection through the human skin. In a large scale study of clinical practice, sterilization techniques are required to prepare the antigen extract for future use. In addition, the reliance on proteins derived from natural sources introduces immense variability into the commercial preparation of the product. The antigen content of infective *Gnathostoma* larvae may vary by season, growth conditions, genetic differences between geographic regions, or other unpredictable variables. For this reason, antigen extracts need to validate the protein biological quality and potency in producing wheal reactions among definite cases of gnathostomiasis, and manufactured lots should be uniform for large scale evaluations.

In this study, a test kit for intradermal skin testing was found preliminarily safe and showed significant correlations with standard immunoblot results. The positive and negative predictive values (PPV and NPV) of the skin test were 85% and 83%, respectively, which is comparable to the immunoblot test (NPV=82%) in this study. With higher sensitivity (87% vs 85%), the skin test may be a good diagnostic tool for screening suspected cases of gnathostomiasis. Nevertheless, skin testing had lower degrees of specificity among angiostrongyliasis cases with eosinophilic meningitis, for whom the immunoblot test was more specific, with fewer cross-reactive bands.^{5,11,12} The precise sensitivity and specificity of skin testing depend on the antigen extraction method used, as well as the different variables inherent in the pathophysiologic mechanism of this bioassay, including different populations of IgE antibodies, concomitant medications, physiologic characteristics of the patient, and the device used. The non-standardized product used in the study was labeled as protein weight by volume. In standardized extracts, the important *Gnathostoma* antigenic proteins have been identified. The preparation of verified quantitative 24-kDa specific *Gnathostoma* protein can yield higher degrees of specificity for skin testing, and in the future, can be developed into a commercial kit.

Increases in total serum IgE among gnathostomiasis patients, and increases in antigen specific IgE antibody levels, have been shown in previous studies.^{13,14} High serum IgE levels were associated with positive skin testing using prepared fractionated antigen solution of *Gnathostoma spinigerum* and positive immunoblot results, despite the absence of significantly different IgE levels in the case and control groups. The cut-off serum IgE level of ≥ 100 kU/L was significantly associated with positive skin and immunoblot tests. However, IgE levels could not predict and support the diagnosis of gnathostomiasis in a presumptive case. In this study, both specific immunoblot and skin test using *Gnathostoma spinigerum* fractionated antigen solution yielded acceptable PPV and NPVs. In addition, a positive result can be used to screen for gnathostomiasis in hospital-based patients. Thorough investigations and reviews of exposure history and risk behaviors can exclude other possible causes.

Our findings support the hypothesis that skin testing helps diagnose gnathostomiasis by the demonstration of in-vivo IgE specific to *Gnathostoma* antigens. However, conflicting results for the differences in specific IgE against *Gnathostoma* antigens, between the gnathostomiasis patients and the healthy volunteers, were detected in in-vitro studies.^{14,15} Also, there are some limitations to the study due to the study methods, including small sample size, information (observer) and recall biases. This study provided insights into potential challenges and opportunities for

further study; the findings will inform and support the conduct of further much-needed research in this area including the correlations of 24-kDa specific *Gnathostoma* protein specific IgE and skin-test responses, and anti-IgE treatment for cutaneous gnathostomiasis. In conclusion, skin testing using the fractionated antigen solution of *Gnathostoma spinigerum* was safe and could yield good reactivity. It also correlated well with results by immunoblot in a hospital-based population of suspected gnathostomiasis cases. To verify the test accuracy and validate this simple diagnostic tool, a standardized manufacturing process is required to develop specific *Gnathostoma* antigen extract for a large-scale clinical trial.

Authors' contributions: WM, VB, and BP collaborated in writing the manuscript. WM, VB, and PD were also involved in the design and preparation of the study. WP, SN, PD, and WM prepared the partially purified antigen for skin testing. WP, SN, and PD conducted the immunoblot analysis. BP conducted the ELISA testing for serum total IgE. WM and VB were involved in patient recruitment, carried out the clinical assessment, and performed the skin testing. WM and BP performed the database extraction and statistical analyses. WM chose the main foci for data analysis and BP participated in interpreting the results. WM, VB, BP, and SN drafted the manuscript. All authors read and approved the final manuscript. WM is guarantor of the paper.

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