

# A DOT-ELISA TEST USING A *GNATHOSTOMA SPINIGERUM* RECOMBINANT MATRIX METALLOPROTEINASE PROTEIN FOR THE SERODIAGNOSIS OF HUMAN GNATHOSTOMIASIS

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**Abstract.** Gnathostomiasis caused by *Gnathostoma spinigerum*, is a hazardous food-borne helminthic zoonosis, and is endemic especially in developing countries in Asia. Definitive diagnosis, relying upon identification of worms from human tissues or body, is rarely accomplished. Consequently, sensitive supporting tools such as serological tests have been used widely. But these methods are time consuming, need sophisticated equipment and are impractical in some settings. In the present study a dot enzyme-linked immunosorbent assay (dot-ELISA), using *G. spinigerum* recombinant matrix metalloproteinase protein as the antigen, was developed and assessed using sera of gnathostomiasis and other parasitosis patients as well as healthy controls. The accuracy, sensitivity, specificity, positive and negative predictive values were 97.4%, 100%, 96.1%, 92.9%, and 100%, respectively. The dot-ELISA appears to be a suitable rapid test for diagnostic purpose as well as epidemiological studies.

**Keywords:** *Gnathostoma spinigerum*, dot-ELISA, human gnathostomiasis, recombinant matrix metalloproteinase

## INTRODUCTION

Gnathostomiasis is an important food-borne helminthic zoonosis caused by spirurid round worms of the genus *Gnathostoma*. The disease is endemic in Asia and the Americas (Waikagul and

Diaz Camacho, 2007) and often reported in travelers returning from those areas (Moore *et al*, 2003; Katchanov *et al*, 2011). *Gnathostoma spinigerum* is a major causative species in Asian countries, *ie* Japan, Thailand, Vietnam, etc (Daengsvang, 1981; Nawa, 1991; Xuan le *et al*, 2002; Herman and Chiodini, 2009). Humans become infected by eating raw or undercooked flesh of a wide range of animals including freshwater fish, chicken, frogs which contains *Gnathostoma* advanced third-stage

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larvae (AL3). Ingested larvae migrate aimlessly in the human body and produce various signs and symptoms depending on the organs involved. Involvement of vital organs, *ie*, central nervous system, eye, etc, may lead to severe disease and death (Daengsvang, 1981; Nawa, 1991; Katchanov *et al*, 2011).

Parasitological diagnosis of human gnathostomiasis is usually done by identifying larvae recovered from the human body. Worms can be recovered after drug treatment, surgery, or after they have spontaneously exited the body. However, direct recovery of larvae is rare. Consequently, diagnosis of gnathostomiasis is more commonly reached by using clinical features, history of consuming risky foods, blood eosinophilia and serological test results. To date, a number of serological diagnostic tests, including enzyme-linked immunosorbent assays (ELISA) (Maleewong *et al*, 1988; Diaz Camacho *et al*, 1998) and immunoblotting using *Gnathostoma* AL3 extract (Tapchaisri *et al*, 1991; Wongkham *et al*, 2000; Anantaphruti *et al*, 2005; Laummaunwai *et al*, 2007), have been evaluated and reported. Recently, a recombinant matrix metalloproteinase (rMMP) protein of *G. spinigerum* AL3 has been produced and can be used as the sensitive and specific antigen for serodiagnosis of human gnathostomiasis by immunoblotting (Janwan *et al*, 2013a,b). The rMMP protein can be used as a diagnostic antigen and potentially replace native parasite antigens for development of a gnathostomiasis diagnostic kit.

However, standard ELISA and immunoblotting need sophisticated equipment and are too complicated to be performed routinely under field conditions. A dot enzyme-linked immunosorbent assay (dot-ELISA) using rMMP protein as the antigen provides a simple, rapid and practical test.

Consequently, the aim of the present study is to develop and test the performance of rMMP based dot-ELISA for serodiagnosis of human gnathostomiasis.

## MATERIALS AND METHODS

### Human sera

All serum samples were retrieved from the serum bank of the Faculty of Medicine, Khon Kaen University. They were anonymized before use: (i) negative control-healthy adult volunteer group ( $n = 21$ ), which included samples from subjects visiting Srinagarind Hospital, Faculty of Medicine, Khon Kaen University whose stool examination by formalin ethyl acetate concentration technique (Elkins *et al*, 1986) revealed no parasites at the time of blood collection. Pooled sera from all of the healthy individuals was also used as negative control for individual assays; (ii) gnathostomiasis group consisting of samples taken from parasitologically confirmed gnathostomiasis patients ( $n = 9$ ) and from patients who presented with clinical signs and symptoms indicative of cutaneous and visceral gnathostomiasis as well as neurognathostomiasis ( $n = 30$ ) (Daengsvang, 1981; Punyagupta *et al*, 1968; Boongird *et al*, 1977; Intapan *et al*, 2010) and who had produced IgG antibody specifically against the 21/24 kDa *G. spinigerum* larval antigen, as detected by immunoblotting; (iii) serum samples from other parasitosis patients ( $n = 56$ ). Infections in this last group were identified by parasitological methods except cysticercosis cases, which were diagnosed via computerized tomography scan and were serological positive (Intapan *et al*, 2008) (Table 1). Informed consents were obtained from all adult participants and from parents or legal guardians in the case of minors. The study protocol was

Table 1  
Type of serum and dot enzyme-linked immunosorbent assay results against the recombinant matrix metalloproteinase protein.

Type of serum	Group <sup>a</sup>	No. positive/no. total (%)
Healthy control	i	0/21 (0%)
Confirmed gnathostomiasis	ii	9/9 (100%)
Suspected gnathostomiasis	ii	30/30 (100%)
Cysticercosis	iii	0/5 (0%)
Taeniasis	iii	0/5 (0%)
Opisthorchiasis viverrini	iii	1/5 (20%)
Fascioliasis	iii	0/5 (0%)
Paragonimiasis	iii	0/4 (0%)
Angiostrongyliasis	iii	0/5 (0%)
Strongyloidiasis	iii	0/6 (0%)
Hookworm infection	iii	0/5 (0%)
Capillariasis	iii	1/6 (16.7%)
Ascariasis	iii	0/4 (0%)
Trichinosis	iii	1/6 (16.7%)

<sup>a</sup> See materials and methods.

approved by the Khon Kaen University Ethics Committee for Human Research (HE561477).

#### Dot-ELISA

The rMMP protein was constructed from a cDNA encoding the MMP protein of *G. spinigerum* AL3, cloned and expressed as previously described (Janwan *et al*, 2013a) with some modifications using pQE-31 expression vector and *Escherichia coli* XL-1Blue (Qiagen, Germany) as the expression system. The rMMP protein was solubilized using solubilizing solution (8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M Tris-Cl, pH 8.0) and collected. The recombinant protein fused with 6-Histidine (6-His)-tagged residues, and was purified using Ni-NTA His Bind Resin (Novagen, Darmstadt, Germany). The optimum concentration of the rMMP protein, 0.5 µg, was previously determined by checkerboard titration and used as antigen in

this study. Ten microliters (0.5 µg) of the antigen diluted in solubilizing solution, were spotted separately onto a nitrocellulose (NC) membrane (GE Healthcare, Piscataway, NJ), air-dried for 15 minutes at room temperature (RT) and incubated at 37°C for 2 hours. The unoccupied sites of the NC membrane were then blocked by immersion of the membrane for 30 minutes in 3% skimmed milk in phosphate buffered saline (PBS), pH 7.5, containing 0.1% Tween-20 (PBST). Next, the NC membrane was washed with 1% skimmed milk in PBST (2 times) and was cut into 8x25 mm strips (2 spots of antigen/strip) and stored at -20°C until use. Each strip was incubated with individual human serum samples (diluted 1:100 in 1% skimmed milk in PBS) absorbed with *E. coli* lysate for 30 minutes at RT. The strips were washed with 1% skimmed milk in PBST (5 times) and incubated with goat anti-human IgG (H+L) HRP conjugate

(Invitrogen, Carlsbad, CA) at a dilution of 1:20,000 (in 1% skimmed milk in PBS) for 1 hour at RT. After a further 5 washes with 1% skimmed milk in PBST, the precipitate dots on strips were rendered visible in 3, 3'-diaminobenzidine-tetrahydrochloride solution. The reaction was stopped after 5 minutes by washing strips with distilled water. The developed color dots were read with the naked eye after the blot had dried and the results were interpreted arbitrarily, according to color intensity, as positive (a color with a clear contrast to the background), weakly positive (there is a color but lower intensity) and negative (no color development was observed).

#### Reading and data analysis

The results were independently read, on the same day but 30 minutes apart, by two independent observers, to estimate inter-observer variability. The precision of the dot-ELISA was also investigated by performing the test on different days using the same pooled positive and negative sera, the same batch of antigens, and the same conditions. Similar results were shown from all, which revealed that day to day variation was minimal. The level of cross-reaction was estimated independently for individual parasitosis sera. The diagnostic accuracy, sensitivity, specificity, and positive and negative predictive values were calculated as previously described (Galen, 1980).

#### RESULTS

For clinical interpretation, readings of "positive" and "weakly positive" were both regarded as indicating a positive result (the one patient returning a weakly positive result – Fig 1 - had clinical symptoms indicative of gnathostomiasis). The sera were scored as positive when each of the two observers gave the same inter-

pretation. In fact, both observers agreed on the readings in every case. Dot-ELISA employing the rMMP protein for diagnosis of human gnathostomiasis was evaluated using individual sera from healthy control, gnathostomiasis patients and patients with other parasitic diseases (Fig 1). The results are summarized in Table 1. The calculated values for accuracy, sensitivity, specificity, positive and negative predictive values are 97.4%, 100%, 96.1%, 92.9%, and 100%, respectively. Sera from opisthorchiasis (1/5), capillariasis (1/6), and trichinosis (1/6) patients appeared to cross-react (data not shown) in the dot-ELISA (Table 1).

#### DISCUSSION

The dot-ELISA is a versatile solid-phase immunoassay for antigen or antibody detection. The method uses small volumes of reagent dotted onto solid surfaces such as nitrocellulose and other membranes which strongly bind proteins. After reaction with antigen-specific antibody and enzyme-conjugated anti-antibody, the addition of a chromogenic substrate produces a colored precipitate dot on the membrane which is read by the naked eye (Pappas, 1988). The dot-ELISA has been used widely in the serodiagnosis of human helminthiases, including fascioliasis (Intapan *et al*, 2003), paragonimiasis (Maleewong *et al*, 1997), cysticercosis (Piña *et al*, 2011), toxocariasis (Bojanich *et al*, 2012), trichinosis (Mahmoud and Moustafa, 2003). For serodiagnosis of human gnathostomiasis, dot-ELISA using adult-worm extracts of *G. spinigerum*, *G. hispidum* and *G. doloresi* has been reported (Ishiwata *et al*, 2003; Sakamoto *et al*, 2004). Here, the dot-ELISA using rMMP protein of *G. spinigerum* as antigen was developed. The results demonstrated

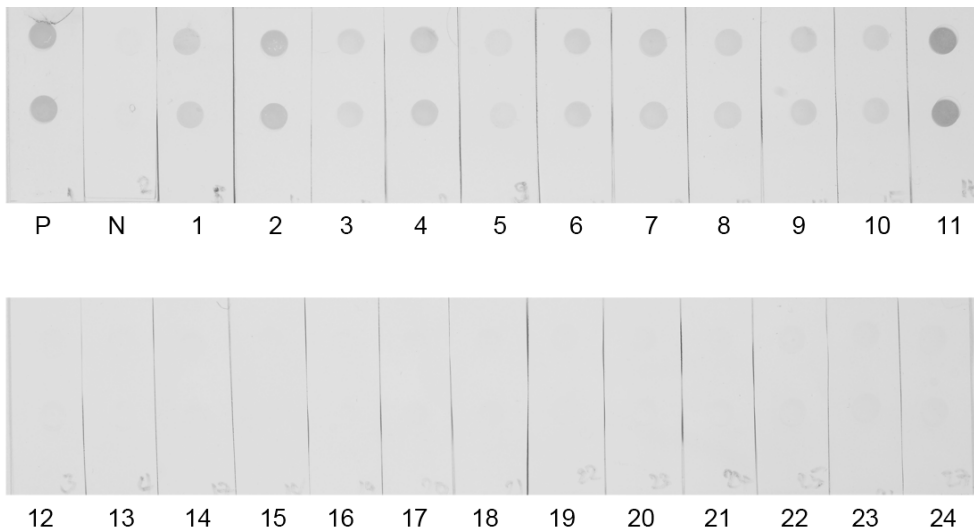


Fig 1—Representative dot enzyme-linked immunosorbent assay using recombinant matrix metalloproteinase protein as the antigen probed with 1:100 diluted human sera. Pooled human gnathostomiasis patients (P), pooled negative controls (N), parasitologically confirmed cutaneous gnathostomiasis (1-3), ocular gnathostomiasis (4-6), clinically suspected gnathostomiasis (7-11), healthy control (12, 13), trichinosis (14), paragonimiasis (15), hookworm infection (16), capillariasis (17), fascioliasis (18), ascariasis (19), cysticercosis (20), opisthorchiasis viverrini (21), taeniasis (22), strongyloidiasis (23), angiostrongyliasis (24). Lanes P, 1-4 and 6-11 were scored by both observers as positive. Lane 5 was scored as weakly positive and lanes N and 12-24 were scored as negative.

high accuracy, sensitivity and specificity. The recombinant protein can be reliably mass-produced and the antigen strips are stable for at least three months at  $-20^{\circ}\text{C}$  (data not shown).

Some cross-reactions with opisthorchiasis (1 of 5), capillariasis (1 of 6), and trichinosis (1 of 6) sera were found. It is possible that these patients might have had previous subclinical infection with *G. spinigerum*. Findings of such a cross reaction does not pose a problem in differential diagnosis because these parasitic infections usually present with clinical features distinguishable from those of gnathostomiasis. However, this test needs to be evaluated with more samples. Results will need to be interpreted cautiously if it is applied in an area endemic for opis-

thorchiasis, capillariasis and trichinosis.

The dot-ELISA method is specific, sensitive, rapid, reagent-conservative, cost effective, easy to perform and to interpret, portable and does not require sophisticated equipment that is often unavailable in developing countries. Moreover, the dot-ELISA could be adapted to employ microtiter plates for large-scale serological surveys or dipsticks for small numbers of sera in a local hospital.

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