■ ORIGINAL ARTICLE

Evaluation of Rapid IgG4 Test for Diagnosis of Gnathostomiasis

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Abstract: Human gnathostomiasis is a parasitic disease caused by *Gnathostoma* nematode infection. A rapid, reliable, and practical immunoassay, named dot immuno-gold filtration assay (DIGFA), was developed to supporting clinical diagnosis of gnathostomiasis. The practical tool detected anti-*Gnathostoma*-specific IgG4 in human serum using crude extract of third-stage larvae as antigen. The result of the test was shown by anti-human IgG4 monoclonal antibody conjugated colloidal gold. The sensitivity and specificity of the test were both 100% for detection in human sera from patients with gnathostomiasis (13/13) and from healthy negative controls (50/50), respectively. Cross-reactivity with heterogonous serum samples from patients with other helminthiases ranged from 0 (trichinosis, paragonimiasis, clonorchiasis, schistosomiasis, and cysticercosis) to 25.0% (sparganosis), with an average of 6.3% (7/112). Moreover, specific IgG4 antibodies diminished at 6 months after treatment. This study showed that DIGFA for the detection of specific IgG4 in human sera could be a promising tool for the diagnosis of gnathostomiasis and useful for evaluating therapeutic effects.

Key words: Gnathostomiasis, diagnosis, DIGFA, IgG4

INTRODUCTION

Gnathostomiasis is a food-borne zoonosis caused by the infection of third-stage larvae (L3) of *Gnathostoma* spp. nematodes, which is commonly found in second-intermediate or paratenic hosts, such as freshwater fish, shellfish, frogs, and poultry [1,2]. The genus comprises 13 well-known species, 6 of which have been recorded for humans [3]. Gnathostomiasis is mainly endemic in Southeast Asia, where people consume raw or undercooked fish. In the past decade, increasing number of travellers returning from endemic areas and nonendemic natives who have eaten imported raw fish infected with *Gnathostoma*, developed gnathostomiasis.

Although isolation of *Gnathostoma* worms from the lesions they caused can make definitive diagnosis, it is rarely successful even in biopsies of cutaneous lesions due to larva's migration properties. In addition, biopsy is impractical for visceral infection. Alternatively, clinical symptoms, exposure history, and serological tests are being applied in combination in clinical diagnosis of human gnathostomiasis. Several serological test techniques have been utilized in different laboratories and immunoblot for detection through reaction with a *Gnathostoma*- specific 24 kDa band is commonly used to confirm clinical diagnosis, with the highest specificity [4-7]. However, the immunoblot is labour-intensive and time-consuming, and requires sophisticated instruments and well-trained operators. Therefore, there is an urgent need for a rapid, reliable, and practical immunoassay for supporting clinical diagnosis of gnathostomiasis.

Specific antigens purified from native crude extracts or generated by genetic engineering methods, have been investigated for the diagnosis of gnathostomiasis [8-13]. Nevertheless, the preparation of these specific antigens is difficult for institutions without adequate health laboratory facilities in remote areas. In this study, we present a novel rapid immunoassay, dot immunogold filtration assay (DIGFA), to detect *Gnathostoma*-specific IgG4 in human serum using crude extract of L3 as antigen. In the present study, a preliminary evaluation for diagnostic sensitivity and specificity of this immunoassay was made, using serum samples from patients with gnathostomiasis, other helminthiases and normal healthy individuals.

MATERIALS AND METHODS

Ethics declarations

All human serum samples were collected with written informed consent of the subjects or their guardians after verbal explanation of the study. The present study was approved by the Ethics Committee of Zhejiang Academy of Medical Sciences under the approval no. 2018-018 and National Institute of Parasitic Diseases, Chinese Centre for Disease Control and Prevention under the approval no. NIPD2011-009.

Serum samples

In the present study, 13 serum samples from patients with gnathostomiasis were collected from 2011 to 2015 in Mainland China, among whom 2 patients have received parasitological diagnosis and the other 11 patients were diagnosed based on the triad of clinical symptoms, exposure risk and positive reaction against *Gnathostoma*-specific 24-kDa band by immunoblot. In addition, 50 healthy negative controls were collected from healthy donors with no history of exposure to parasites and no indication of helminth infection according to a stool examination at the time of blood collection. Details on the serum samples used in the current study are provided in Table 1.

For assays on cross-reactivity, 112 sera were obtained from 3 groups of patients with other helminthiases. The first group consisted of 50 sera from nematodiasis: angiostrongyliasis cantonensis (20), trichinosis spiralis (15) and intestinal nematodiasis (15). The second group included 30 sera from trematodiasis: paragonimiasis westermani (10), schistosomiasis japonica (10), and clonorchiasis sinensis (10). The third grouped

32 sera from infection with larvae of cestode: cysticercosis (10), sparganosis mansoni (12), and echinococcosis (10). Patients infected with *Schistosoma japonica*, *Paragonimus westermani*, *Clonorchis sinensis* and intestinal nematodes (*Ascaris lumbricoides*, *Trichuris trichiura*, and hook worm) were confirmed by parasitological examination of stool using the Kato Katz method. Cases of angiostrongyliasis cantonensis, sparganosis mansoni and cysticercosis cellulosae were diagnosed based on computerized tomography scan, exposure history and specific serological tests. Patients with echinococcosis were confirmed with surgical observation.

Preparation of crude antigen: L3 soluble extract

The *G. spinigerum* crude extract of L3 was prepared as described previously [14] with minor modifications. The liver of freshwater eels were collected from farmers' markets, gently sheared with forceps at the laboratory, and subsequently digested with 1% pepsin solution (pH2.0) at 37°C for 4 hr. About 500 live larvae were collected from the digested liver tissue under a dissecting microscope. The larvae were rinsed 3 times with sterile normal saline, then re-suspended in 3 ml pre-cooled phosphate-buffered saline (PBS, 0.01 mol/L, pH7.2) [containing proteinase inhibitors in Complete ULTRA Tablets, EDTA-free which inhibits serine proteases, cysteine proteases, and aspartic proteases (Roche, Penzberg, Germany), and 5 mM EDTA], and homogenized using a glass tissue grinder. The homogenate was sonicated at 20 kHz for 5 min on an ice bath and centrifuged at 20,000 ×g for 15 min at 4°C. The supernatants were pooled as the L3 crude extract. The protein concentration was measured by a Bradford Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, Illinois, USA)

Table 1. Rapid detection of specific IgG4 in human sera by DIGFA using *G. spinigerum* crude extract of L3 as antigen

Group	Subject	No. of sera	Positive rate (%)
A	Gathostomiasis	13	100
B	Negative healthy control	50	0
C	Other helminthiases	112	6.3
	Angiostrongyliasis	20	10
	Intestinal nematodiases (ascariasis, Trichuriasis ancylostomiasis,)	15	6.7
	Trichinosis	15	0
	Cysticercosis	10	0
	Echinococcosis	10	10
	Sparganosis	12	25
	Clonorchiasis	10	0
	Paragonimiasis	10	0
	Schistosomiasis	10	0

and estimated at 2.7 mg/ml.

ELISA

Indirect ELISA was used to detect the levels of specific anti-*Gnathostoma*-IgG4 in human serum with L3 crude extract as antigen. Each well of a 96-well micro-plate was coated with 100 µl (2 µg/ml) of the L3 crude antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 4°C overnight and blocked with 3% BSA solution at 37°C for 1 hr. The plates were washed 3 times and 100 µl of serum samples at a 1:100 dilutions were added and incubated at 37°C for 1 hr. Following a second washing, 100 µl of a mouse anti-human IgG4 horseradish per-oxidase conjugated antibody at 1:1,000 dilutions (Thermo Fisher, Carlsbad, California, USA) was added and incubated at 37°C for 1 hr. The plates were finally washed 3 times then 100 µl of tetramethylbenzidine substrate solution was added and incubated at room temperature for 15 min to determine the binding enzymatic activity. The enzyme reaction was stopped with 50 µl of 2 M H_2SO_4, and the optical density (OD) was measured at a wavelength of 450 nm using a microtitre plate reader (Bio-Rad, Hercules, California, USA). The mean OD plus $3 \times SD$ of 50 healthy control serum samples was set as a positive cut-off value.

Development of DIGFA for IgG4 detection from human sera

The DIGFA comprised of test cassettes, colloidal gold conju-gated with mouse anti-human IgG4 monoclonal antibody (McAb) and washing buffer. The test cassettes were assembled as previously described [16]. Two dots consisting of 0.5 µl of human IgG4 (0.1 mg/ml) used as positive control and 0.5 µl of L3 crude antigen (1 mg/ml) were put separately at the sides of "C" (positive control dot) and "T" (test dot) on the centre of nitrocellulose membrane (Fig. 1). For the preparation of anti-human IgG4-conjugated colloidal gold solution, 100 ml of mono-disperse gold solution (pH 8.5) was obtained according to the method described previously [16]. Then, it mixed with 0.25 mg of mouse anti-human IgG4 Fc antibody (Thermo fisher, Carlsbad, California, USA) by stirring. Tris-HCl (0.02 mol/L, pH 8.5, containing 3% bovine serum albumin and 0.05% tween-20) was used as washing buffer.

Serum sample test was performed as described by Ma et al. [16] (Supplementary Fig. S1). First, 2 drops (~60 µl) of wash-ing buffer were added to the test well to block non-specific binding on the nitrocellulose membrane; second, 25 µl of se-
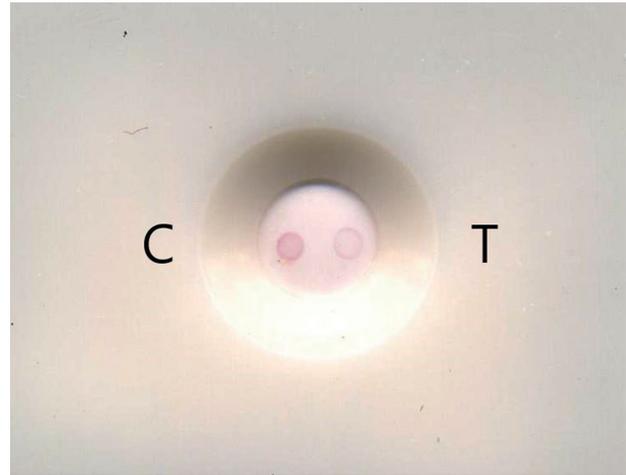

Fig. 1. Cassette for human IgG4 test by DIGFA displaying the re-sult of a positive case. C, positive control dot; T, test dot.

rum sample was then added to flow through the membrane before one drop of washing buffer was added; third, one drop of the colloidal gold conjugate was added to visualize the spe-cific antigen-antibody reaction before washing with another 2 drops of washing buffer; and finally observing the results with naked eyes. The appearance of a well-defined red dot at both sides of 'T' and 'C' in the test-well was considered to be posi-tive; only one red dot appeared at the "C" side was considered negative; If a red dot did not appear at the side of "C", the test was invalid.

Statistical Analysis

The ELISA data were reported as the means of duplicated tests of each sample. Student's t-test was used to determine the significance of differences between data groups. A *P*-value less than 0.05 indicated statistical significance.

RESULTS

Level of specific IgG4 in serum samples of patients with gnathostomiasis

The mean OD value of patients with gnathostomiasis was significantly higher than that of patients with healthy controls ($t = 6.791$, $P < 0.01$) and patients with other helminthiases ($t = 6.779$, $P < 0.01$). There was no significant difference be-tween mean OD values of patients with other helminthiases and healthy controls ($t = 2.846$, $P > 0.05$) (Fig. 2).

Sensitivity and specificity of rapid DIGFA IgG4 test

A total of 175 serum samples were tested for evaluation of rapid detection of specific IgG4 test. Results are summarized in Table 1. Rapid DIGFA IgG4 test using native L3 crude extract as antigen showed 100% (13/13) sensitivity and 100% (50/50) specificity for diagnosis of gnathostomiasis. Cross-reactivity with sera from patients with other helminthiases ranged from 0 (trichinosis, paragonimiasis, clonorchiasis, schistosomiasis, and cysticercosis) to 25% (sparganosis) with an average of 6.3% (7/112). Out of 7 serum samples with cross-activity, 4 samples showed weak reactions with light colour dots.

Specific IgG4 antibody level in follow-up cases

We have followed up 8 patients after treatment with single or multiple courses of albendazole and/or ivermectin. Post-treatment reduction in specific IgG4 levels for each patient was calculated by percentage, as follows: (1-post-treatment specific-IgG4 level/pre-treatment specific-IgG4 level) × 100%. Clinical
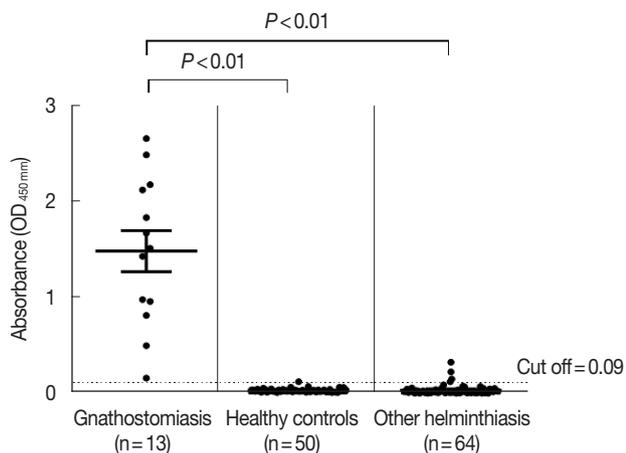

Fig. 2. Qualitative detection of specific IgG4 in human sera by rapid DIGFA.

characteristics of the cases that have been followed up are summarised in Table 2. Two individuals (case 1, 2) who have never relapsed after treatment showed significant reduction in specific IgG4 level but still over the cut-off threshold at 6 months post-treatment (Fig. 3A). This downtrend was well presented in DIGFA tests (Fig. 3C). In contrast, another 2 individuals (case 3, 4) who suffered from failure treatments with recurrences, kept the same high levels of specific IgG4 as at pre-treatment when they were followed up at 6 months post-treatment (Fig. 3A, B).

Four patients (case 5-8) were followed up until 10 years after the initial treatment. Cases 5 and 6 complained suffering from intermittent recurrences after received multiple courses of albendazole. Both were specific IgG4 positive despite reductions of IgG4 levels by around 50% (Fig. 3A, B). For both cases 7 and 8, a *G. spinigerum* larva has been removed from their skin abscess (data not shown) during the first course of albendazole, and symptoms had never returned since then. Levels of specific IgG4 in their sera had fallen below the positive cut-off threshold and rapid DIGFA tests presented negative too (Fig. 3A, B).

DISCUSSION

IgG4 is known as an intriguing antibody with unique biological properties. Interaction between IgG4 and FcγRs is known to play an important role in host immune regulation of helminth infection [17,18]. In healthy population, IgG4 is the least represented IgG subclass, at less than 5% of total IgG. However, this odd immunoglobulin can rise to 50-95% of IgG and become the predominant antibody after human exposure to helminth antigens [17-19]. High concentration of IgG4 has been observed in the sera of patients with filariasis and the in-

Table 2. Clinical information and IgG4 test of eight gnathostomiasis follow-up cases

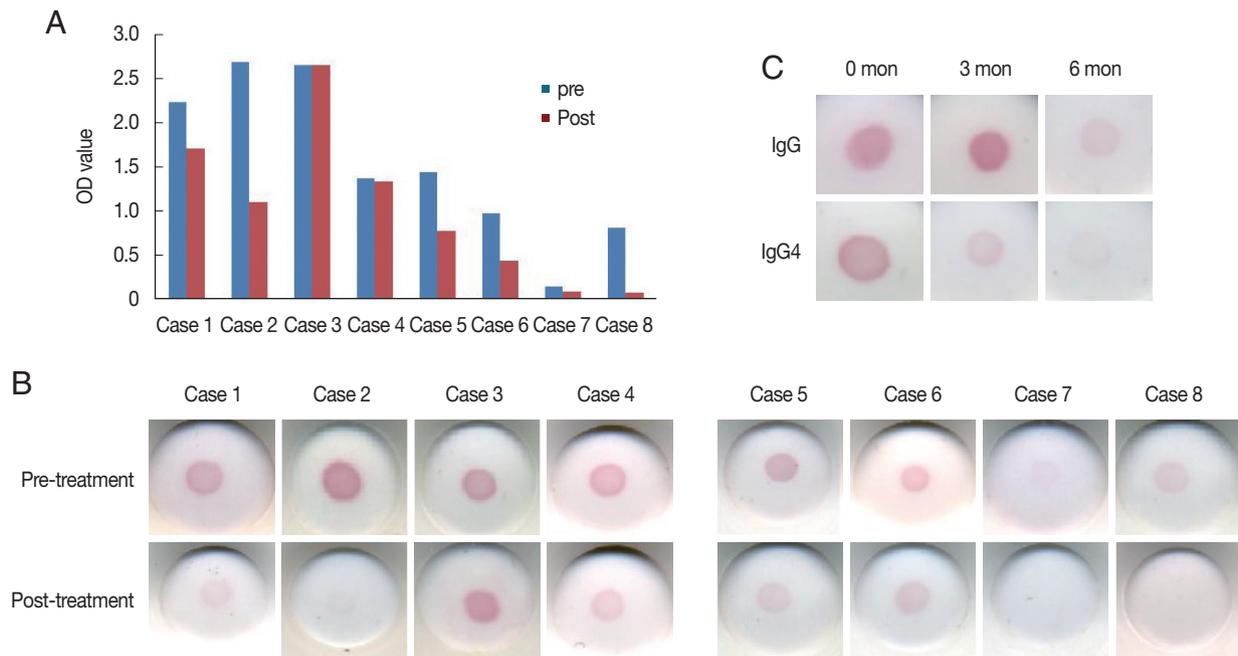| Case	Clinical manifestation	Treatment	Relapse	IgG4 reduction rate (%)	DIGFA IgG4 test	
					Pre-treatment	Post-treatment
1	Visceral	Alb+Ive	No	23.1	+++	+
2	Cutaneous	Alb	No	58.7	+++	+
3	Cutaneous	Alb	Yes	0	+++	+++
4	Cutaneous	Alb	Yes	2.90	++	++
5	Cutaneous	Alb	Yes	46.1	+++	++
6	Cutaneous	Ive	Yes	54.7	+++	++
7	Cutaneous	Alb	No	41.8	++	-
8	Cutaneous	Alb	No	89.6	+++	-

Alb, Albendazole; Ive, Ivermectin.

Fig. 3. Detection of specific IgG4 in sera of patients after treatment. (A) Specific IgG4 in 8 paired sera of patients measured semi-quantitatively by ELISA. (B) Detection of specific IgG4 from 8 paired sera of patients using DIGFA. (C) IgG and IgG4 tests in sera from case 8 before treatment, and 3 and 6 months after treatment.

crease in specific IgG4 was positively correlated with microfilaremia [20,21]. Serological immunoassays to detect filarial-specific IgG4 have been successfully developed and applied for diagnosis of filariasis with high sensitivity and specificity [22, 23]. In human gnathostomiasis, IgG subclass antibodies have been qualitatively analysed by immunoblot and IgG4 has been identified as the predominant antibody in patient sera [24,25]. In this study, we first measured the levels of specific IgG4 against *Gnathostoma* L3 in serum samples by ELISA. Results showed that specific IgG4 levels in patients with gnathostomiasis were significantly higher than those of healthy controls and other helminthiases. This implied that specific IgG4 was a potential biomarker for diagnosis of human gnathostomiasis.

In this study, the rapid format DIGFA using L3 crude extract as antigen for detection of specific IgG4 against *Gnathostoma* presented 100% in both sensitivity and specificity for the diagnosis of gnathostomiasis, decreased cross-reactivity with other helminthiases, and increased specificity with respect to total IgG test by DIGFA [16]. The result of this study tallied with the data obtained previously by immunoblot analysis [15,24,25]. Specificity in sero-diagnosis of human helminthiasis could be enhanced by removing immune response to phosphocholine, carbohydrate, and polysaccharide antigens, which usually leads to false-positive reactions [26,27]. In our previous studies, the native L3 extract antigen-removed polysaccharide antigens by TCA/acetone precipitation had high specificity for gnathostomiasis diagnosis [16]. In this study, IgG4-based DIGFA using crude L3 extract attained similar diagnostic sensitivity and specificity as total IgG detection by DIGFA using partially purified L3 antigen.

Human gnathostomiasis has long been neglected and its immunological response characteristics still poorly understood. Albendazole and ivermectin are widely used to treat human gnathostomiasis with significant effectiveness, however the initial course is not always successful [28]. Recurrences are common from within a few weeks to years after chemotherapy, and 2 or more courses are needed in some cases [29-31]. Therefore, clinicians need a rapid test that detects biomarker of active infection or indication of cure to timely declare treatment success or failure. According to studies on serological diagnosis of filariasis, specific IgG4 response dramatically reduced then correlated with clearance of parasites, and high specific IgG4 level related with active infection [20]. As a same tissue dwelling nematode, *Gnathostoma* spp. worms might induce similar antibody response profile. According to the data of 8 patients' follow-up 6 months post treatment, significant reduction in specific IgG4 level coincided with successful treat-

ment, indicating that such reduction is a promising indicator of effective treatment. However, this data is based on limited numbers of gnathostomiasis patients in China, and investigation on large-scale population is necessary to further prove the potential of specific IgG4 as an indicator of treatment efficacy. Better understanding of the dynamics of specific IgG4 responses in post-treatment patients is required for interpretation of follow-up data and more precise assessment of anti-helminth chemotherapy.

Unfortunately, due to the rarity of human gnathostomiasis, we were not able to collect enough serum samples from gnathostomiasis patients and pre/post-treatment paired samples. In the future, there is need to develop a model of infection with *G. spinigerum* worms in experimental animals and monitor the dynamic changes of specific antibodies after anti-helminth treatment. Another limitation from the current study is that there is no evidence to show the kit can distinguish species of *Gnathostoma* [32,33].

This study shows that DIGFA has high sensitivity and specificity to detect specific IgG4 in human sera. The test was not only more specific than detection of total IgG for the diagnosis of human gnathostomiasis, but also useful for evaluating therapeutic effects. Thus, this novel immunoassay is a promising tool for the diagnosis of gnathostomiasis.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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