

## Epidemiological Significance of Sero-positive Inhabitants against Sparganum in Kangwon-do, Korea

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Sparganum is a plerocercoid of pseudophyllidean tapeworms, *Diphyllobothrium* or *Spirometra* spp. Human sparganosis is endemic mainly in East and Southeast Asian countries where the custom of eating raw snake or frog meat, or poulticing with snake's skin exists. From January 1995 to November 1999, an epidemiological survey was undertaken to evaluate the serum levels of anti-sparganum specific IgG antibodies in Whachon-gun residents, Korea. An enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis of the sera from 316 subjects were used. In addition, a stool examination from 416 inhabitants and questionnaires regarding the consumption of raw meat were given. Out of 416 inhabitants examined coprologically, one was infected with *Clonorchis sinensis* and two were infected with *Metagonimus* spp. The sera from 36 inhabitants (11.4 %) showed a positive reaction to the sparganum antigen. Out of these 36 inhabitants, the sera from 25 people were examined 7, 19, and 50 months later. The sera were found to still show positive reactions without any remarkable changes of anti-sparganum specific antibody titers except for one. An analysis of the questionnaires suggested that a history of eating of raw snakes or frogs was important risk factor for clinical or covert sparganosis (odds ratio=15.6 and 3.1, respectively).

**Key Words:** *Spirometra erinacei*, sparganosis, enzyme-linked immunosorbent assay, IgG antibody, immunoblotting, epidemiology

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## INTRODUCTION

Sparganum is a plerocercoid of pseudophyllidean tapeworms, *Diphyllobothrium* or *Spirometra* spp. While the adult worm is an intestinal cestode in cats and dogs worldwide, the larva can infect a wide variety of vertebrate hosts including humans. When humans are infected, the larva migrates through several tissues forming a migration track, and can also invade the central nervous system where it may elicit several neurological manifestations. Human sparganosis is endemic mainly in East and Southeast Asian countries where the custom of eating raw snakes or frogs, or of poulticing with snake's skin exists.<sup>1</sup> In Korea, the standard positive rate for anti-sparganum antibodies in 850 normal adults was 1.7%.<sup>2</sup> In Kangwon-do and Chollanam-do, the positive rate was as high as 8%. In these areas, positive rates in males was as much as 10 times higher than in females, which suggests that the sparganum infection is possibly related to the consumption of intermediate hosts such as snakes and/or frogs. In the present study, the sero-positive rate in Kangwon-do residents was investigated. In addition, this study aimed to determine the risk for the sparganum seropositive inhabitants.

## MATERIALS AND METHODS

Stools were collected from 416 inhabitants in Whachon-gun, Kangwon-do, Korea, in January

1995. The formalin-ethyl acetate method was carried out once for intestinal parasites. The questionnaire regarding the consumption of raw snake and/or frogs was concomitantly surveyed from the examinee with individual contact. The topics on the questionnaire consisted of the age, sex, and the history of raw eating of snake or frog. Data on the age and sex were provided by the officers of the Health Center. Other past histories were provided by the examinees. The serum from each subject was collected from 316 out of 416 inhabitants. After the first screening of the serum levels for anti-sparganum specific IgG antibodies by an enzyme linked immunosorbent assay (ELISA), the serum from 25 out of 36 positive reactions against the sparganum antigen were followed up in August 1995, August 1996 and November 1999. The sera for the proven sparganosis patients and normal persons who denied any possible exposure to helminthic or protozoan infections were used as controls. The sera were stored at  $-70^{\circ}\text{C}$  until required.

Spargana were collected from the European glass snake, *Rhadophis tigrinus tigrinus* Boie, 1826. The larvae were washed with physiological saline 3 times and homogenized with a teflon-pestle tissue homogenizer. The supernatants were saved by centrifugation at 20,000g for 1 hr. All procedures were done at  $4^{\circ}\text{C}$ .<sup>3</sup> To conduct an ELISA, 200  $\mu\text{l}$  of the antigen solution, diluted to 2.5  $\mu\text{g}/\text{ml}$  in a carbonate buffer (pH 9.6) was coated with micro-titer plate (Costar, Corning, Cambridge, CA, U.S.A.) overnight at  $4^{\circ}\text{C}$ . Each serum sample was diluted at 1:100 in a phosphate buffered saline solution containing 5% Tween 20 (PBS/T, pH 7.4) and incubated for 2 hr. Peroxidase conjugated anti-human IgG (heavy- and light-chain specific, Cappel, West Chester, PA, U.S.A.) was diluted at 1:1,000 and incubated for 2 hr. The substrate for the color reaction was o-phenylenediamine. The reaction was stopped with 4 M  $\text{H}_2\text{SO}_4$ . The absorbance was recorded at 490 nm with a micro-titer plate reader (Bio-Rad M3550, Richmond, CA, U.S.A.). The cut-off absorbance of a positive reaction was 0.22 as described previously.<sup>3</sup> The sera that showed a positive reaction against the sparganum antigen by an ELISA were further examined by immunoblot analysis. The crude extracts of the sparganum were separated

by 10-15% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and transferblotted to a polyvinylidene difluoride (PVDF) microporous membrane (Millipore, Bedford, MA, U.S.A.). Each serum sample was diluted at 1:200 then probed overnight. The serum antibody ligated to the antigenic protein bands was further incubated with peroxidase-conjugated anti-human IgG (Cappel) diluted at 1:1,000. The color reaction was detected with 4-chloro-1-naphthol (Sigma, St. Louis, MO, U.S.A.).

## RESULTS

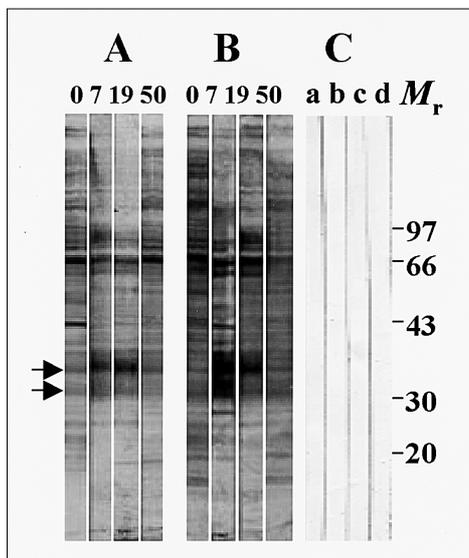
Out of 416 inhabitants examined, one was infected with *Clonorchis sinensis* and two were infected with *Metagonimus* spp. One person with metagonimiasis showed a positive reaction to the sparganum antigen by ELISA as well (data not shown). The patients were treated with praziquantel (Distocide<sup>®</sup>, Shinpoong, Korea). Thirty-six inhabitants (11.4%) exhibited a positive reaction against the sparganum antigen by ELISA. Of the 161 male inhabitants tested, 31 tested positive (19.3%) while only 5 out of 155 (3.3%) females tested positive. There was a significant difference between male and females ( $p=0.000$ ). The positive rates in the fifth, sixth, seventh and eighth decades were 5.9, 12.3, 13.9, and 22.0%, respectively (Table 1). There was no positive reaction in people whose age was less than 20. Among the positive reactions, the sera from 25 inhabitants were available 7, 19 and 50 months later. When the specific antibody levels in the follow-up were tested, all showed similar levels of antibody titers as before except for one case. The anti-sparganum antibody levels ranged from an absorbance of 0.22-0.79 in the follow up examination (Table 2). The sera that exhibited positive reactions by ELISA were further analysed by immunoblot analysis against crude sparganum extracts. Several protein bands appeared to be reactive including 36 and 31 kDa, which were shown to be specific to clinical sparganosis with strong intensities<sup>4</sup> (Data not shown). Other positive bands such as 72 kDa was also found in the positive sera. The positive sera during the follow-up periods showed the same patterns (Fig.

**Table 1.** Positive Rate of Anti-sparganum Antibodies in 316 Peoples Analyzed by Age and Sex

Age (year)	No. positive/No. examined (%)		
	Male	Female	Total
0-9	0/0	0/0	0/0
10-19	0/3	0/1	0/4
20-29	0/2	0/5	0/7
30-39	1/23 (4.4)	0/14	1/37 (2.7)
40-49	1/15 (6.7)	1/19 (5.3)	2/34 (5.9)
50-59	10/43 (23.3)	2/55 (3.7)	12/98 (12.3)
60-69	8/35 (22.9)	2/37 (5.4)	10/72 (13.9)
70-79	11/32 (34.4)	0/18	11/50 (22.0)
> 80	1/8 (12.5)	0/6	1/14 (7.2)
Total	31/161 (19.3)	5/155 (3.3)	36/316 (11.4)

**Table 2.** Serum Level Distributions of Anti-sparganum Antibodies of Positive Inhabitants in Whachon-gun, Kangwon-do, Korea during Follow-up Period (1995-1999)

Date of survey	No. of examined	Distribution of absorbance		
		0.01-0.21	0.22-0.49	0.50-0.79
January, 1995	36	0	10	26
August, 1995	25	1	4	20
August, 1996	25	1	3	20
November, 1999	24	1	3	20

**Fig. 1.** Immunoblot results of the positive reactions to sparganum. The proteins were separated by 10-15% SDS-PAGE, transferred to a polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA, U.S.A.), and subjected immunoblot analysis. Each sample was diluted at 1:200. Peroxidase-conjugated anti-human IgG was used to detect the immuno-reaction. The color was developed with 4C1N chromogen.  $M_r$ , molecular mass in kDa. A & B: sparganum ELISA positive reactions. The numerals at the top of A & B indicate the follow-up month, C: negative controls of 4 persons a, b, c & d. Arrow indicates the 31 and 36 kDa protein bands.

1). Analysis of the questionnaire in 36 ELISA positive inhabitants revealed that eight had a history of eating raw snake meat, 14 had a history of eating frogs. Meanwhile, of 280 ELISA negative people, four had a history of eating of raw snake

and 35 had ate raw frog. The odd ratio of the anti-sparganum antibody positive reaction for the history of eating raw snake meat was 15.6 and that for eating raw frogs was 3.12.

## DISCUSSION

In this epidemiological survey, the sero-positive rate against sparganum and the risk factors for these diseases were investigated. Sparganum infections in Korea might be caused by eating raw snake and frog meat as is the case in other countries.<sup>1</sup> In this study, the risk from eating raw snake is as high as 5 times that of eating raw frogs (15.6/3.12). These results might have significance in terms of the seroepidemiological survey. One of the reasons is that the antibody test is reliable for detecting the disease with a sensitivity and specificity of as high as reached 85 and 97%. The other reason is that all the sera that showed a positive reaction in this study exhibited strong reactive bands at 36 and 31 kDa, which are specific for clinical sparganosis.<sup>3-6</sup> A 27 kDa cysteine protease is only expressed in the stages involving the active migration of the parasite in the host tissue. However, it is reactive to IgE and not to IgG.<sup>7</sup> The results in this study suggest that the people showing positive reactions during the follow-up periods may have had a dormant infection, because there were no changes in the antibody titers as measured both by ELISA and immunoblot analysis. The fact that the positive rate from inhabitants were higher in males than in females, and that it was higher in people over 50 years of age coincides with a history of eating raw snakes or frogs. Another source of sparganosis is drinking mountain pond water that is contaminated with infected Cyclops. Therefore, in the future the number of seropositive inhabitants in the surveyed area will decrease due to the decrease of custom of eating raw snakes or frogs and drinking mountain pond.

The seropositive rate, in the subjects (11.4%) is higher than average positive rate of 1.9% in other areas in Korea and coincide with previous data.<sup>2</sup> Kangwon-do has the highest incidence of sparganosis, which can be explained in ways. Firstly,

Kangwon-do is a mountainous area where there are lots of snakes compared with other provinces in Korea. Secondly, the habit of eating raw snake or frog still persists. The people who tested positive stated that there were no subjective symptoms for cutaneous mass or focal neurological manifestations, However, the possibility of a covert infection could not be ruled out since there have been some reports on asymptomatic sparganosis.<sup>1,8,9</sup> It is uncertain whether those positive cases may complain any symptoms or signs in the future. Therefore, regular follow-up examination was necessary.

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