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Detection of specific IgG, IgM, IgE and IgG subclass antibodies for serological diagnosis of human cystic echinococcosis

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Summary

An enzyme linked immunosorbent assay (ELISA), based on sheep hydatid cyst fluid antigen was used for the detection of specific antibodies of IgG, IgM, IgE and IgG subclass in the serum samples of 62 clinically and radiologically diagnosed cystic echinococcosis (CE) patients, 8 clinically suspected cases of CE, 25 other parasitic disease controls and 25 healthy controls. The diagnostic sensitivity in the clinically and radiologically suggestive cases (n = 62) for IgG antibody detection was highest (93 %), followed by IgE, IgG4, IgG1, IgG2, IgM and IgG3 with 89 %, 87 %, 85 %, 76 %, 70 % and 55 % respectively. The detection of specific IgE, IgG1 and IgG4 antibody showed the higher diagnostic sensitivity and specificity to the extent that they can be safely used as better substitute to IgG. Even though, the diagnostic sensitivity of IgG was highest (93%) but was less specific (88 %) due to the frequent non-specific reactions in the sera of patients with other parasitic infections and healthy controls. None of the sera samples from healthy controls gave non-specific reaction with IgE, IgG1 and IgG4 and there was a considerably reduced cross-reaction with these antibodies. The most discriminatory and specific antibodies found in this study belonged to IgE, IgG1 and IgG4; therefore, these antibodies may serve as useful diagnostic markers for CE.

Keywords: cystic echinococcosis; immunological test; ELISA; cross-reactivity

Introduction

Hydatid disease generally known as echinococcosis or hydatidosis is caused by infection with larva (metacestode) of the tapeworms of genus *Echinococcus*. The genus *Echinococcus* contains four well-recognized species namely, *Echinococcus granulosus* causing cystic echinococcosis (CE), *E. multilocularis* causing alveolar echinococcosis (AE) and *E. oligarthrus* and *E. vogeli*, both causing polycystic echinococcosis (PE) (Eckert and Thompson, 1997). Cystic echinococcosis and alveolar echinococcosis are neglected parasitic diseases of both medical and public health importance. Cystic echinococcosis is a near-cosmopolitan zoonosis and responsible for most of the burden of echinococcosis globally (Budke *et al.*, 2006), although alveolar echinococcosis is endemic in Europe and is problematic in China (Eckert *et al.*, 2000; Craig, 2006; Deplazes, 2006). In India, *E. granulosus* is the most widespread species responsible for most of the causes of human and livestock hydatidosis. So far only few reports of *E. multilocularis* causing alveolar echinococcosis and *E. oligarthrus* causing poly-

cystic echinococcosis had been reported from India, while *E. vogeli* is yet to be documented (Parija & Swarna, 2005). The disease in man and other intermediate hosts is caused by ingestion of food or water contaminated with eggs of the dog tapeworm, *Echinococcus granulosus* (Devi & Parija, 2003).

The diagnosis of cystic echinococcosis mainly depends on radiological and immunological techniques. Radiological techniques are not cost effective and their non-availability at peripheral and rural hospitals makes it of limited use. Moreover, radiological picture of hydatid cyst often mimics other pathologies. Immunodiagnosis can play an important complementary role. It is useful not only for primary diagnosis but also for follow-up of patients after surgical or pharmacological treatment. Detection of circulating antigen in serum is much less sensitive than antibody detection. Thus, routine laboratory diagnosis of cystic echinococcosis is heavily reliant on detection of specific antibody response (Lightowers & Gottstein, 1995; Garcia, 2001). Cyst fluid (CF) of *E. granulosus* cysts of sheep or cattle origin is one of the most widely used antigens, and the enzyme-linked immunosorbent assay (ELISA) is

one of the most commonly used technique which received most attention as an immunodiagnostic method for various parasitic infections (Schantz & Gottstein, 1986). Therefore, the present study was intended to detect the specific antibodies of IgG, IgM, IgE and IgG subclass for the identification of best diagnostic markers for human cystic echinococcosis.

Material and Methods

Subjects and Samples

Seventy (70) patients, who were either clinically suspected and/or radiologically diagnosed to be having cystic echinococcosis, were enrolled in the study after obtaining their due informed consent. Detailed clinical history and radiological findings were recorded from all the patients. Twenty five (25) patients with other parasitic diseases, 5 each of cysticercosis, ascariasis, amoebic liver abscess, toxoplasmosis, malignancy were included as controls after taking their due informed consent. Twenty five (25) normal healthy subjects who were found negative for intestinal helminthic infections by stool examination were included as controls after taking due informed consent. Blood samples of 4 – 5 ml were collected from all the subjects. Serum was separated from blood samples and preserved at -70 °C till further use.

Preparation of hydatid (Echinococcal) antigen

Hydatid antigen was prepared from hydatid cysts obtained from freshly slaughtered infected sheep at the local abattoirs. Hydatid fluid was aspirated aseptically from fertile hydatid cyst and centrifuged at 2000 x g for 45 min at 4 °C. The fluid was passed through a Whatman WCN type membrane filter (cellulose nitrate, 47 mm diameter, 0.45 µm pore size) and dialyzed against distilled water overnight at 4 °C using dialysis tubing (Sigma Aldrich) with molecular weight cut off 2000 Dalton (Wattal *et al.*, 1986). The protein concentration of antigen was determined by Lowry's method (Lowry *et al.*, 1951).

Antibody detection in serum

Antibody detection was performed by indirect enzyme linked immunosorbent assay (ELISA). The specific IgG, IgM, IgE in serum were detected according to Wattal *et al.*, (1986) with few modifications. Briefly, microtitration plates were coated with 2µg/100µl of crude hydatid sheep antigen (Optimum concentration of antigen was determined by the checker board titration method) diluted in 0.1M carbonate bicarbonate buffer (pH 9.6). Patients sera were diluted 1:400 for IgG, IgM, IgE and 1:200, 1:100, 1:50, 1:50 for IgG1, IgG2, IgG3 and IgG4 respectively. Diluted sera were incubated for 1 hr at 37 °C in the respective wells of microtitre plate and the plates were then washed three times with PBS-Tween. The different assays were then continued as follows.

Total IgG, IgM and IgE assays

Goat anti-human IgG, IgM and IgE conjugated to horseradish peroxidase (Sigma, USA), diluted 1:4000, 1:8000 and 1:4000 respectively were used as the secondary antibody and 100 µl was dispensed to each well and the plates were incubated for 1 hr at 37 °C. After three time washing of plates with PBS-Tween, the

substrate 3,3',5,5' tetramethylbenzidine (TMB) was used to visualize the antigen antibody reaction. Optical density (OD) was recorded at 450nm after the addition of 100 µl of stop solution (2M, H₂SO₄) to each well.

IgG subclass assays

The assays for IgG subclasses were performed as described by Lawn *et al.*, (2004) with some modifications. For the detection of hydatid specific IgG-subclass in serum monoclonal mouse anti-human IgG1, IgG2, IgG3 and IgG4 (Sigma Aldrich) were diluted 1:500, 1:2,500, 1:1,000 and 1:2,500 in PBS respectively and 100 µl of each dilution was added to their respective plates. Peroxidase conjugated goat anti-mouse Fc-specific IgG was used as secondary antibody diluted 1:24000, 1:10,000, 1:4000 and 1:24000 for IgG1, IgG2, IgG3 and IgG4 respectively. Plates were incubated for 1 hr at 37 °C and washed three times with PBS-Tween. Substrate solution was added and the plates were developed and read as described for total IgG, IgM and IgE assays.

Data analysis

The results of the test samples were recorded as optical densities (OD). The cut off OD was determined by adding 3 standard deviations (SD) to the mean of 5 negative samples. Samples having ODs higher than was cut-off values were considered as positive, where as samples having ODs lower than cut-off value were reported as negative. Surgically and/or radiologically confirmed cases were taken as true positive for sensitivity calculations whereas specificity was calculated from control group i.e. Group II and Group III subjects (n = 50).

Results

Out of 70 clinically suspected cystic echinococcosis (CE) patients, 21 were adult males, 47 were adult females and 2 were male children. The age group of the adult patients ranged from 18-85 years, while 2 children were 9 and 11 years old. Among the 70 patients, 62 were clinically and radiologically diagnosed as cystic echinococcosis, out of which 12 were also confirmed surgically. Forty three of the clinically and radiologically diagnosed cases had hepatic cysts while 19 had extra hepatic cysts (14 lung, 4 liver and lung and 1 thigh cyst). The remaining 8 cases were clinically suspected CE.

The anti-hydatid antibody responses in clinically and radiologically diagnosed patients were found positive in 58 of 62 (IgG), 44 of 62 (IgM), 55 of 62 (IgE), 53 of 62 (IgG1), 47 of 62 (IgG2), 34 of 62 (IgG3) and 54 of 62 (IgG4). Among the 12 surgically confirmed patients the number of cases with positive serology in IgG, IgM, IgE, IgG1, IgG2, IgG3 and IgG4 were 12, 9, 12, 12, 10, 8 and 12 respectively. Among 8 clinically suspected CE patients, 3 were positive in IgG and 1 each in IgE and IgG4 (Table 1). Considering the surgically confirmed cases as gold standard, the sensitivity for specific total IgG, IgE, IgG1, IgG4 antibody detection in serum was highest (12/12 cases) followed by IgG2 (10/12 cases), IgM (9/12 cases) and IgG3 (8/12 cases) respectively. However, in the clinically and radiologically suggestive cases (n = 62) the sensitivity for specific IgG antibody detection in serum was highest (93 %),

Table 1. Anti-hydatid antibodies detection by ELISA in serum samples of cystic echinococcosis patients

	Subjects tested	Number Positive						
		IgG	IgM	IgE	IgG1	IgG2	IgG3	IgG4
Group I: Cystic echinococcosis patients (n = 70)								
i. Clinically and radiologically diagnosed	50	46	35	43	41	37	26	42
ii. Surgically confirmed	12	12	09	12	12	10	08	12
Total (i + ii)*	62	58	44	55	53	47	34	54
iii. Clinically suspected	08	03	00	01	00	00	00	01
Group II: Parasitic Disease controls (n = 25)								
Cysticercosis	05	01	0	0	0	0	0	0
Ascariasis	05	01	0	1	0	1	0	0
Amoebic liver abscess	05	01	1	0	0	0	0	0
Toxoplasmosis	05	0	0	0	0	0	0	0
Malignancy	05	1	1	0	0	0	0	0
Cross reaction (%)	25	16	8	4	0	4	0	0
Group III: Healthy controls (n = 25)	25	2	1	0	0	0	0	0
Sensitivity (%)*		93	70	89	85	76	55	87
Specificity (%)#		88	94	98	100	98	100	100

*Surgically and/or radiologically confirmed cases were taken as true positive for sensitivity calculations.

Specificity was calculated from Group II and Group III subjects (n = 50)

followed by IgE, IgG4, IgG1, IgG2, IgM and IgG3 with 89 %, 87 %, 85 %, 76 %, 70 % and 55 % respectively. Although the diagnostic sensitivity of IgG was highest (92 %) but was less specific (85 %) due to the frequent non-specific reactions in the sera of patients with other parasitic infections and healthy controls with this antibody. The maximum percentage (16 %) of cross-reactions was observed with IgG followed by IgM (8 %) and IgE or IgG2 (4 % each). There were no cross-reactions observed when specific IgG1, IgG3 and IgG4 antibodies were analysed. None of the sera samples from healthy controls (Group III) gave non-specific reaction with IgE, IgG1 and IgG4 and there was considerably reduced cross-reaction with these antibodies (Table 1). Therefore these antibodies were the most specific antibodies for this parasite and can be considered best diagnostic markers for this disease.

Discussion

Immunodiagnosis is an important tool for diagnosis of CE infection. Thus, in addition to imaging techniques, a reliable serodiagnosis improves prognosis for patients, because medical treatment can then be specifically adapted to the CE problem (Ortona *et al.*, 2003). The results of our study demonstrated that specific antibodies of IgE, IgG1 and IgG4 are the most important antibodies for serological diagnosis of CE using sheep hydatid cyst fluid antigen. The important role of these antibodies has also been demonstrated in earlier reports (Felix *et al.*, 1998; Khabiri *et al.*, 2006). Our results are in corroboration with other studies, who also found predominance of IgE, IgG1 and IgG4 antibody response against cyst fluid antigen in hydatid patients (Short *et al.*, 1990; Shambesh *et al.*, 1997). In human hydatid patients, there is a frequent occur-

rence of elevated antibody levels, particularly of immunoglobulin G (IgG), IgM, IgE, IgG1 and IgG4 (Sterla *et al.*, 1999; Zhang *et al.*, 2008). Among IgG subclass responses in human cystic echinococcosis IgG1 and particularly IgG4 as the most predominant IgG isotypes observed (Wen & Craig, 1994; Virginio *et al.*, 2003). In the present study, false positive reactions were observed with the serum samples of ascariasis, amoebiasis, malignancy and cysticercosis infection. Such false positive reactions were observed highest with total IgG (16 %) and reduced with other antibodies. The probable cause of such high false positives could be the use of crude antigen in the study. Our results are in confirmation with the observations of earlier reports (Felix *et al.*, 1998; Khabiri *et al.*, 2006). They also recorded highest number of cross-reactions in total IgG and reduced in IgG subclass. Poretti *et al.*, (1999) reported frequent cross-reactions in the sera of patients with ascariasis, cysticercosis, amoebiasis and malignancy. The false positive reactions in hydatid cyst fluid antigen of *E. granulosus* with human antibodies of other cestodes and helminths are also reported in previous investigation (Sunita *et al.*, 2007). In the present study IgE, IgG1 and IgG4 antibodies proved to be highly specific as less cross-reaction with the sera of other parasitic disease patients was observed in them and there was no non-specific reaction with the sera of healthy controls for these antibodies. Therefore these antibodies were the most specific antibodies for this parasite and may serve as best diagnostic markers for this disease.

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