

*Echinococcus granulosus* ANTIGEN B FOR SERODIAGNOSIS OF CYSTIC ECHINOCOCCOSIS IN BUFFALO (*Bubalus bubalis*)

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### ABSTRACT

The present investigation was carried out to characterize buffalo cyst fluid and germinal membrane antigen by a double immuno diffusion test and immunoelectrophoresis. Attempts were also made to find out utility of antigen B for detection of cystic echinococcosis in buffalo by indirect enzyme linked immunosorbent assay (indirect ELISA). Hyperimmune sera raised against germinal membrane antigen and cyst fluid antigen has shown seroreactivity during the double immuno diffusion test and immunoelectrophoresis against germinal membrane antigen, cyst fluid antigen respectively as apparent by formation of precipitin lines and precipitation arc. A total of twenty sera samples (ten positive and negative samples each) of buffalo were used for indirect ELISA and the average optical density (O.D.) values of positive samples ranged from 0.498 to 0.783 with a mean of 0.655. The negative samples exhibited O.D. values ranged from 0.247 to 0.263 with a mean of 0.255. The cut-off value of the test system was 0.279.

**Keywords:** cystic echinococcosis (CE), buffalo, antigen B, *Echinococcus granulosus*, serodiagnosis

### INTRODUCTION

Cystic echinococcosis (CE) is a well-established cyclozoonotic disease which occurs throughout the world in animals and man. The parasite perpetuates through two different hosts. Canine serve as the definitive host of the parasite whereas ruminants and other ungulates serve as the intermediate host of the parasite. In the intermediate host, space-occupying lesion is produced by the cysts which develop mainly in the liver and lung (90%) (Gottstein, 1994) and as well as in other organs, namely, kidney, spleen, bone marrow, brain and heart. The protoscoleces can be seen in the fertile cyst fluid.

The public health and economic importance of the disease is assessed in terms of human and animal involvement. The loss due to human involvement is appreciated by the number of infected persons, their reduced functional capacity, cost of hospitalization before and after surgery. In animals, the economic loss is mainly due to condemnation of infected carcasses besides retarded quality of milk, meat and wool.

So far as the occurrence of the disease is concerned, CE has been well documented in Indian literature and the disease has been reported from the eastern and north eastern regions of India as

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well (Maity *et al.*, 2007).

Diagnosis of the infection with this parasite is a topic that covers a broad range of issues, since it deals with infection in human and animal intermediate hosts. The lack of non-invasive procedures for parasitological diagnosis of CE and the location of the cysts caused by *Echinococcus granulosus* has long prompted characterization of hydatid cyst fluid and the search for laboratory methods for diagnosis. If a sensitive, specific serologic assay is available, unnecessary surgery could be avoided in some cases of non-hydatid disease (Maddison *et al.*, 1989).

Hence, the present investigation was carried out to characterize buffalo cyst fluid and germinal membrane antigen by double immuno diffusion and immunoelectrophoresis. Attempts were also made to find the utility of antigen B for detection of CE in buffalo by indirect enzyme linked immunosorbent assay (indirect ELISA).

## MATERIALS AND METHODS

During the period of study all the glassware and plastic ware were sterilized following standard techniques. Chemicals and reagents used for this purpose were of analytical grade.

### Collection of sample

The samples were collected from slaughterhouses in Kolkata. Hydatid cysts were collected from the lungs of buffalo. The collected cysts were transported to the laboratory maintaining cold-chain. After collection of cyst fluid, the cyst was dissected carefully to collect germinal membrane.

Immediately after collection, cyst fluid, was centrifuged at 2,000 g for ten minutes. The

supernatant was collected and was stored at -20°C after addition of required concentration of phenyl methyl sulphonyl fluoride (PMSF; 2 µl/ml sample).

Collected germinal membrane was washed thoroughly three times at ten minute intervals with phosphate buffered saline (PBS, pH 7.2) and the material was further used for antigen preparation.

Sera samples were collected from buffaloes after examination of visceral organs for the presence or absence of visible cysts. During this study period, a total of ten positive and ten negative samples were collected from buffaloes. After separation of serum, the samples were preserved in aliquots at -20°C till further use.

### Preparation of antigen

#### a) Crude cyst fluid antigen

This antigen was prepared following the methodology of Maddison *et al.* (1989) and ten fold concentration of cyst fluid antigen was made by lyophilisation. The concentrated cyst fluid material was stored at -20°C after addition of PMSF.

#### b) Antigen B

The antigen was prepared following the methodology described by Ibrahim *et al.* (1996). The final antigen concentration was adjusted to 25 µg/ml, which was determined by Lowry's method (Lowry *et al.*, 1951). The antigen was analyzed by 15% SDS-PAGE to ascertain its purity.

#### c) Germinal membrane antigen

The germinal membrane was sliced into pieces and homogenized in ice cold PBS, pH 7.2 (10% w/v) in an all glass homogenizer. The homogenized material was centrifuged at 10,000 g for one hour at 4°C and the supernatant was collected in aliquots at -20°C after addition of required concentration of PMSF.

### **Preparation of antisera against cyst fluid and germinal membrane antigen**

For raising of hyperimmune serum, the protocol described by Hudson and Hay (1989) was followed. Healthy New Zealand white rabbits with an average weight of 1.5 kg were used for inoculation. The serum samples were stored in aliquots at -20°C till further use.

### **Double immuno diffusion (DID) test**

For the double diffusion test, the protocol described by Hudson and Hay (1989) was followed.

#### **Immuno electrophoresis**

For immuno electrophoresis, the protocol described by Hudson and Hay (1989) was followed. The electrophoresis was run at a constant current of about 8 mA / slide for one hour. Finally the slide was stained with Coomassie blue staining solution.

#### **Indirect ELISA**

Hudson and Hay's (1989) protocol was followed for this assay. The ELISA microplate was coated with antigen B (0.5 µgm / well) after diluting with coating buffer and kept overnight in an incubator at 37°C. The following day, it was washed with washing buffer, i.e. PBST (250 µl tween 20 in 500 ml PBS) and then blocked with blocking buffer (400 mg bovine serum albumin in 20 ml PBST) for one hour at 37°C. Then again washing was done. Serum, both positive and negative, for hydatidosis (collected from buffaloes from an abattoir in Kolkata, India) was then diluted 1:100 with blocking buffer and added to the microplate. After one-hour incubation at 37°C, it was washed and antibovine conjugate (1:2500 dilution with blocking buffer) was added. Substrate (5 mg OPD tablet in 10 ml distilled water with 4 µl H<sub>2</sub>O<sub>2</sub>) was added after one-hour incubation. The stopping solution (5.5 ml H<sub>2</sub>SO<sub>4</sub> in 94.5 ml distilled water)

was added after desirable colour development. The absorbance was taken at 492 nm.

## **RESULTS AND DISCUSSION**

So far antigens of *E. granulosus* has been prepared from cyst fluid, protoscoleces and germinal membrane (Lightowers and Gottstein, 1995). Amongst the three sources, the protein patterns of protoscolex are most complex, and hence, it could not be used for characterization of the isolate. On the contrary, cyst fluid (excretory/secretory product of parasite) and germinal membrane are useful sources for characterization of the larval stage of this worm (Siles-Lucas and Cuesta-Bandera, 1996).

### **Double Immuno Diffusion (DID) test of germinal membrane and cyst fluid antigen**

Hyperimmune sera raised against germinal membrane antigen produced two and one precipitin line(s) by DID against germinal membrane antigen, and normal buffalo serum, respectively. However it did not produce any precipitin line against cyst fluid antigen.

On the other hand, hyperimmune sera raised against cyst fluid antigen produced two precipitin lines by DID against cyst fluid antigen but no visible precipitin line was recorded against germinal membrane antigen or normal buffalo serum. The result of DID have been shown in Figure 1.

### **Immuno electrophoretic analysis of germinal membrane and cyst fluid antigen**

Hyperimmune sera raised against germinal membrane antigen produced four and one arc against germinal membrane antigen and cyst fluid



Figure 1. Double immuno diffusion of germinal membrane antigen (GM), cyst fluid antigen (CF) and normal buffalo serum (BS) against anti-germinal membrane (GM-HIS) and anti-cyst fluid (CF-HIS) hyperimmune sera.



Figure 2. Immunoelectrophoresis of germinal membrane (GM) and cyst fluid antigen (CF) against anti-germinal membrane hyperimmune sera (GM-HIS) (after staining with Coomassie blue).



Figure 3. Immunoelectrophoresis of germinal membrane (GM) and cyst fluid antigen (CF) against anti-cyst fluid hyperimmune sera (CF-HIS) (after staining with Coomassie blue).



Figure 4. Showing peptide profile of antigen B of *E. granulosus* in 15% SDS-PAGE (monochromatic silver stain).

antigen, respectively (Figure 2).

Hyperimmune sera raised against cyst fluid antigen produced two and one arc against cyst fluid and germinal membrane antigen, respectively (Figure 3).

Immunoelectrophoretic study depicted the individual antigenic components separated under potential voltage difference, which was seen as precipitation arcs. Moreover, cross-reacting antigenic components were also evidenced for cyst fluid and germinal membrane antigen. Similar observation on cyst fluid and germinal membrane antigen has been evidenced by different groups of workers, which was appreciated with the presence of precipitation arcs both with homologous and heterologous hyper immune serum. From India, efforts have been made with buffalo cyst fluid antigen by Raina and Singh (1997) who found five and three bands with undiluted and diluted serum. The findings of Raina and Singh (1997) partially corroborate the present finding, since

during the present investigation two precipitation arcs were recorded with cyst fluid antigen when it was reacted against hyperimmune serum raised against the same. However, the result of germinal membrane could not be compared with the work carried out by Raina and Singh (1997) since they did not characterize germinal membrane antigen by immunoelectrophoresis. However, the presence of three precipitation arcs by immunoelectrophoresis recorded by Khan *et al.* (1999) with germinal membrane corroborate the present finding. Besides Indian workers, more than one precipitation arc has also been recorded by Oriol *et al.* (1971), Pozzuoli *et al.* (1972) and Gottstein *et al.* (1987), who described the presence of different antigenic components of different charges and also indicated the complex nature of the antigen (both the germinal membrane and cyst fluid antigen) which demands purification.

Table 1. Mean O.D. values of individual serum samples by indirect ELISA.

<b>Positive samples O.D. Value (mean ± SE)</b>	<b>Negative samples O.D. Value (mean ± SE)</b>
0.659 ± 0.011	0.257 ± 0.002
0.670 ± 0.011	0.248 ± 0.005
0.783 ± 0.017	0.259 ± 0.005
0.701 ± 0.012	0.247 ± 0.002
0.498 ± 0.003	0.251 ± 0.003
0.617 ± 0.009	0.257 ± 0.005
0.592 ± 0.011	0.263 ± 0.003
0.624 ± 0.009	0.259 ± 0.007
0.732 ± 0.005	0.261 ± 0.003
0.671 ± 0.009	0.252 ± 0.004
Mean O.D. value of positive samples = 0.655	Mean O.D. value of negative samples = 0.255

**Indirect ELISA using antigen B**

Monochromatic silver staining of antigen B (prepared following the methodology described by Ibrahim *et al.*, 1996) after 15% SDS-PAGE revealed presence of three peptides having  $M_r$  of 23, 17 and <14.3 kDa (Figure4). This corroborates the results of other workers who also detected three subunits of antigen B within similar molecular weight ranges (Oriol *et al.*, 1971).

A total of twenty sera samples of buffalo were used for indirect ELISA. Of these, ten animals had lesions of cystic echinococcosis (CE) and the other ten samples were from apparently healthy animals. The mean O.D. (optical density) value of individual serum sample has been depicted in Table1. The O.D. values of positive samples ranged from 0.498 to 0.783 with an average of 0.655. The negative samples exhibited O.D. values between 0.247 and 0.263 with an average of 0.255. The cut-off value of the test system was 0.279, which was calculated on the basis of mean O.D. value of the negative sample added with three times standard deviation (S.D.) of the same.

The result of indirect ELISA using antigen B showed clear-cut difference between O.D. (optical density) values of infected animals and apparently healthy control animals. Thorough study of utility of antigen B has been done by Ibrahim *et al.* (1996) who found 90% sensitivity and 99% specificity. Although during the present study, 100% sensitivity and specificity was observed, the result needs further validation with a greater number of samples. However, the results of indirect ELISA with antigen B in the present investigation has shown promising result.

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