

Original Research Article

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Serodetection of Hydatidosis in Buffaloes using Recombinant Ag-B Subunit 8 of *Echinococcus granulosus*

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ABSTRACT

Hydatidosis caused by metacestode stage of *Echinococcus granulosus* is one of the most important cyclozoonotic disease affecting both humans and livestock resulting in considerable economic losses to livestock industry which amounts to US\$ 3 billion annually (WHO factsheet on Echinococcosis, 2018). No reliable immunodiagnostic method is available for its sero-detection in animals. Native antigen-B represents one of the most immuno-dominant antigens in hydatidosis, therefore assessment of recombinant antigen-B subunit 8 was taken in the current study. The immuno-reactivity of recombinant 29-kDa EgAgB-8 protein was confirmed in the Western blot with rabbit anti-histidine antibodies and buffalo sera positive for hydatid infection. The sero-diagnostic potential of this recombinant protein was evaluated by indirect ELISA and DOT-ELISA platforms by screening two hundred and fifty buffalo sera where 44.8% (n=112) samples were revealed positive by indirect-ELISA by detecting antibodies against this antigen in sera. Immuno-reactivity with hydatid positive buffaloes (n=12) was also confirmed in DOT-ELISA. Cross-reactivity of the recombinant antigen with field sera positive for *Gigantocotyle explanatum*, *Paramphistomum epiclitum* and *Gastrothylax crumenifer* was also studied in DOT-ELISA that showed some of the buffaloes infected with parasites other than hydatid metacestode were cross-reacting with the recombinant antigen.

Keywords

Cystic
Echinococcosis,
DOT-ELISA,
Immunoreactivity,
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Introduction

Hydatidosis/Cystic echinococcosis (CE) is one of the most important zoonosis of global dimension caused by the larval stages of the taeniid cestode *Echinococcus granulosus* affecting both humans and animals worldwide (Thompson 2008). It is characterized by a slow growing cyst in the visceral organs like liver, spleen, lung etc. *E. granulosus* has a

cosmopolitan distribution and the disease is well known in Asia, Africa, South and Central America, the Mediterranean and Eastern Europe, with some foci in the United Kingdom (Craig *et al.*, 2003; Eckert and Deplazes, 2004; Samanta, 2008). About 4 million people are estimated to be infected and other 40 million at risk of this disease (Aziz *et al.*, 2011). WHO had put hydatidosis as an important neglected zoonosis sub-group for its

2008-2015 strategic plans devoted to the control of neglected tropical diseases (Siracusano *et al.*, 2012) and is working towards effective cystic echinococcosis control by 2020. *E. granulosus* is the most widespread species and 10 strains (G1-G10) of the genus *Echinococcus* have been identified including two sheep strains (G1 and G2), two bovine (G3 and G5), one horse strain (G4), one camel (G6), two pig (G7 and G9) and two deer strains (G8 and G10) (Mandal and Mandal, 2012). The most useful tools to monitor the incidence of the disease in asymptomatic high-risk human populations are imaging techniques and serology. Imaging methods such as sonography, computerized axial tomography (CAT), magnetic resonance imaging (MRI) are highly sensitive but serology is a supplement to these imaging tools (Macpherson *et al.*, 2003; Samanta *et al.*, 2009). However, there are no routine, reliable methods for diagnosis of the hydatid infection in living animals. The most reliable diagnostic method is through detection of cysts during meat inspection or at post-mortem examination.

One of the major antigens of hydatid cyst fluid is antigen B (*EgAg B*) (Lightowlers *et al.*, 1989; Barbeiri *et al.*, 1998; Valeria Silva-Alvarez *et al.*, 2015). There have been and are still going on important advances in the understanding of the molecular organization of *EgAg B* (Frosch *et al.*, 1994; Gonzalez *et al.*, 1996). It (*EgAgB*) is the most abundant and immunogenic antigen produced by the larval stage of *E. granulosus* and is postulated as a carrier of essential lipids of *E. granulosus* (Oriol *et al.*, 1971; Valeria Silva-Alvarez *et al.*, 2015) since cestodes have lost both degradative and biosynthetic pathways for sterols and cholesterol (Zheng *et al.*, 2013). It is a complex 120 kDa thermostable lipoprotein (Oriol *et al.*, 1971), showing 50% of alpha-helical conformation (Oriol and Oriol, 1975). SDS-PAGE analysis of hydatid cyst fluid

antigen using non-reducing conditions shows that *AgB* produces a ladder-like pattern consisting of sub-units of 8, 16 and 24 kDa (Shepherd and McManus 1987; Lightowlers and Gottstein, 1995).

Though, several antigens of *E. granulosus* including antigen B have been expressed and evaluated for their potential in the detection of cystic echinococcosis in humans but in animals limited studies have been conducted on the evaluation of the panel of antigens available for humans. Furthermore, studies on the development of routine sero-diagnostic tests for domestic animals especially buffaloes are scanty which form the main backbone of dairy and meat industry in the country. Therefore, the present investigation was aimed at serodetection of cystic echinococcosis in buffaloes using a prominent diagnostic antigen of *E. granulosus* recombinant *EgAg-B* sub-unit 8 and preliminary cross reactivity with some important trematodes by DOT-ELISA as evaluated.

Materials and Methods

Collection of hydatid cysts from infected buffaloes and recombinant protein expression

Buffaloes were examined at post-mortem for infection with *E. granulosus* hydatid cysts. Cysts were categorized as fertile/sterile on teasing in the laboratory for the presence of live protoscolices. Hydatid fluid was retrieved from each fertile cyst and live protoscolices were collected in sterile phosphate buffered saline (PBS) pH 7.2. The protoscolices were washed several times in sterile PBS and examined under microscope for their viability and RNA was extracted using standard RNA extraction procedures. The *EgAgB-8* cDNA (accession no: U15001) synthesized from RNA was cloned in prokaryotic expression vector pET-32a(+) (Novagen, USA) and

recombinant protein was expressed in *E. coli* BL21(DE3) pLysS cells. The recombinant *EgAgB-8* was purified by Ni-NTA affinity and was subsequently evaluated in the sero-detection of cystic echinococcosis in buffaloes by IgG-ELISA.

Collection of sera from buffaloes

Sera were collected from 250 buffaloes slaughtered at the local abattoir. Blood was collected from each buffalo at the time of their slaughter and subsequently probed for the infection with hydatid cysts. Animals positive for hydatid infection *Paramphistomum epiclitum* (n=15), *Gastrothylax crumenifer* (n=9) and *Explanatum explanatum* (n=11) were separately labelled. Sera from buffaloes negative for these infections were used as negative control.

Sero-detection of *E. granulosus* antibodies by ELISA

Sera collected from the buffaloes were probed for anti-*E. granulosus* antibodies with the recombinant *EgAgB-8* using IgG-ELISA. Checker-board titration was initially performed to determine the optimum quantum of antigen, sera dilution and anti-globulin enzyme conjugate concentration. Polystyrene microtitre plates (Nunc, Denmark) were sensitized with 100 µl of 0.05M carbonate-bicarbonate buffer, pH 9.6, containing 1 - 2 µg/ml of recombinant antigen at 4°C overnight (ON). The wells were washed with PBS containing 0.05% Tween-20 (PBS-T) thrice for 5 min each. Subsequently, the plates were blocked with 3% skimmed milk in PBS at 37°C for one hour. After 3 washings with PBS-T, 100 µl of buffalo serum (1:100-1:200 dilution) in 1% skimmed milk-PBS was added to each well and incubated at 37°C for 1 h. Plates were washed five times with PBS-T and incubated for 1 h at 37°C after addition of 100 µl of 1:10,000-1:12,000 diluted rabbit anti-

bovine IgG-HRP-conjugate in 1% skimmed milk-PBS.

Finally, after 5 washes with PBS-T 100 µl substrate buffer (OPD-8 mg; 10 ml of citrate buffer, pH 5.0; 10 µl of H₂O₂) was added to each well and allowed to react in dark for 5-10 min. The absorbance was read at 490 nm on microplate ELISA reader. The results expressed as the mean of the optical density (OD₄₉₀) were obtained from duplicate samples.

Sera from *E. granulosus* positive buffaloes (n=12) and uninfected controls (n=14) were used as positive and negative controls, respectively. Cut-off values were determined by adding 2±SD to the mean OD₄₉₀ of *E. granulosus* negative sera.

Western blotting of recombinant proteins

Immuno-reactivity of the recombinant *EgAgB-8* protein was checked by Western blotting. About 5 µg of the purified recombinant protein was resolved on 15% SDS-PAGE and subsequently transferred to a nitrocellulose membrane using a semi-dry blotting apparatus (ATTO, Japan). Successful transfer of the protein to the membrane was confirmed by staining with Ponceau's stain. The unbound surface of the membrane was blocked overnight with 5% skimmed milk at 4°C and washed 3 times with PBS- 0.05% Tween-20 (wash buffer). Rabbit anti-histidine antibody (Sigma Chemicals, USA), buffalo hydatid positive sera and healthy rabbit serum were used in the immuno-reactivity assay at 1:50 dilution at 37°C for 1 h. The membrane was washed 3 times with PBS-Tween-20 and incubated with bovine anti-rabbit/ rabbit anti-bovine IgG-HRP conjugate (1:2000 dilution) at 37°C for 1 h. The membrane was given a final wash with wash buffer and subsequently developed with diaminobenzidine (DAB) substrate solution for visualization of the

reactivity. Immuno-reactivity on the membrane was stopped by washing the membrane with distilled water.

DOT-ELISA

The immuno-reactivity of the buffalo sera with the recombinant antigen was also checked by DOT-ELISA. One μl (~200 ng) of the recombinant antigen was coated on the nitrocellulose membrane and reacted with buffalo sera at different dilutions for determining the reactivity of the sera. Standard protocols of DOT-ELISA using rabbit anti-bovine IgG-HRP conjugate were followed.

Results and Discussion

Western blotting of the recombinant protein

The immuno-reactivity of the recombinant *EgAgB-8* protein was confirmed in the Western blot using rabbit anti-histidine antibodies that showed the fusion protein reactive with rabbit anti-serum at 29 kDa (Fig. 1). The recombinant *EgAgB-8* antigen was also reacted with the buffalo sera positive for hydatid infection that showed reactivity at 29 kDa. No reactivity was detected with uninfected buffalo serum (Fig. 2).

Sero-detection of *E. granulosus* infection in buffaloes

Two hundred and fifty buffalo sera were screened for anti-*EgAgB-8* IgG antibodies with recombinant *EgAgB-8* in ELISA and 44.8 % (n=112) were positive for these antibodies. These positive animals showed OD₄₉₀ values ranging from 0.45-1.52, with negative control sera OD₄₉₀ of 0.23-0.38 (cut-off=0.32) (Fig. 3). The ELISA results indicated some of the hydatid infected buffaloes under field conditions had higher

antibody titre in IgG-ELISA while others were poor responders for antibody production with lower titre in the ELISA. Sensitivity of the recombinant antigen was confirmed with sera of 12 buffaloes positive for fertile hydatid cysts that showed all hydatid positive buffaloes were reacting with the recombinant antigen. Therefore, sensitivity of the ELISA in the detection of cystic echinococcosis in buffaloes was high indicating this antigen has a potential in the diagnosis of cystic echinococcosis in buffaloes. However, no cross-reactivity of the recombinant antigen with other helminths infecting buffalo was carried out in this ELISA format. The positive reactivity of these 12 animals was also confirmed in DOT-ELISA where all the positive sera reacted with recombinant antigen at 1:100-1:200 serum dilutions and 200 ng/dot antigen concentrations. Negative control sera used in this study did not react with this antigen in DOT-ELISA (Fig. 4). DOT-ELISA results also indicated that the recombinant antigen is highly sensitive in the detection of hydatid positive animals.

Cross-reaction study of the recombinant antigen was conducted with field sera positive for *Explanatum explanatum* (n=11), *Paramphistomum epiclitum* (n=15) and *Gastrothyla xcrumenifer* (n=9) in DOT-ELISA. Recombinant antigen did not react with majority of the sera from buffaloes infected with the above parasites, except for antigen reacting with three sera of *P. epiclitum* infected buffaloes. Also, the recombinant antigen reacted with 2 sera positive for *G. crumenifer* and 4 sera positive for *E. explanatum* infection. These results indicated that recombinant *EgAgB-8* antigen is either cross-reacting with these parasites or these animals were co-infected with minute hydatid cysts which may have been missed during manual investigation since hydatid cysts grow slowly over long course of time. Antigen B - one of the major lipoprotein antigens present

in hydatid cyst fluid has received wide attention in regard to immune-diagnosis of cystic echinococcosis (Lightowers *et al.*, 1989; Valeria *et al.*, 2016). It has now been well characterized by immune-blotting, immune-precipitation of radio-labelled antigen and SDS-PAGE (Shepherd and McManus 1987; Siracusano *et al.*, 1991) and is approximately 150 kDa in size comprising three subunits in the range 8–24 kDa (Shepherd and McManus 1987; Lightowers *et al.*, 1989; Siracusano *et al.*, 1991). The literature describing the immunological diagnosis of hydatid disease in humans is extensive (Brunetti *et al.*, 2010; McManus *et al.*, 2012; Zheng *et al.*, 2013).

The hydatid cyst fluid has been used most frequently as a source of *E. granulosus* antigens and its components have been comprehensively investigated for their applicability in serological tests. But much less research has been conducted towards the development of immune-diagnostic techniques for *E. granulosus* infection in domesticated ruminants especially buffaloes and the results have been generally contradictory. Accurate serological diagnosis of CE infection in livestock has been problematic due to the serological cross-reactivity with several other species of taeniid cestodes including *Taenia hydatigena* and *Taenia ovis* (Lightowers and Gottstein 1995) in sheep. Furthermore, natural intermediate hosts of the parasite produce very poor antibody responses or none at all (non-responders) to infection compared with the relatively high levels of specific antibody evident in human infection (Lightowers and Gottstein 1995). Overall, there appear to be difficulties associated with sub-optimal sensitivity and specificity with different antigens used in several assays (McManus 2014). Similar to Ag5, cross-reactivity of antigen B with antigens from other parasites, notably other taeniid cestodes, in sheep has been a recurrent challenge (McManus 2014). The recombinant *EgAgB-8* antigen was evaluated for the detection of hydatid infected

buffaloes under field condition. The sensitivity of the AgB-8ELISA in detecting the animals infected with the hydatid cysts (true positive) was very high. However, study on the cross-reactivity of this recombinant antigen with other helminths commonly infecting buffalo was not carried out.

A preliminary field study was also carried out on a few sera from buffaloes confirmed positive for infections with *E. explanatum*, *P. epiclitum* and *G. crumenifer* in DOT-ELISA. In this study sera of all hydatid infected buffaloes (12) tested showed a positive reaction, while as 2 sera out of 9 *G. crumenifer*, 4 sera out of 11 *E. explanatum* and 3 out of 15 *P. epiclitum* also gave cross reactions with this antigen. McManus (2014) reviewed that different antigens including antigen B have shown problems of cross-reactivity in sheep.

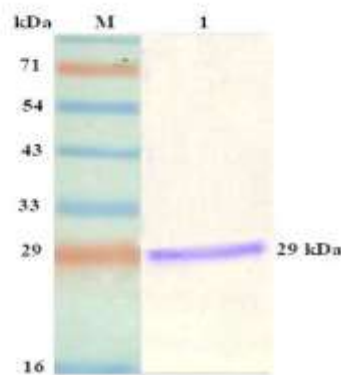
Hence, extensive studies on the cross-reactivity of this recombinant antigen with other parasites including protozoa need to be conducted not only in buffaloes but in sheep, goat and cattle where cystic echinococcosis is of common occurrence. Further, due to differential expression of the members of antigen B family (*EgAgB1-B5*) coded by different genes studies on the comparative sensitivity and specificity of these proteins need to be undertaken to identify the antigen with higher potential in the sensitive and specific diagnosis of cystic echinococcosis in domestic livestock.

Immunodiagnosis of CE has been investigated in a range of other natural intermediate hosts including pigs, cattle, goats, buffaloes and camels but in no case has specific or sensitive diagnosis been consistently achieved due to false-positive reactions and the weak serological response generated in infected animals (Lightowers and Gottstein, 1995; Ibrahim *et al.*, 2002; Golassaet *al.*, 2011).

However, Pan *et al.*, 2011 reported a very high level of sensitivity, specificity and accuracy of recombinant AgB-2 of 96.7, 94.7 and 95.9%, respectively in ELISA in cattle and buffaloes where the antigen did not show cross-reactivity with *Moniezia expansa*, *F. gigantica* in cattle and with *E. explanatum* in buffaloes.

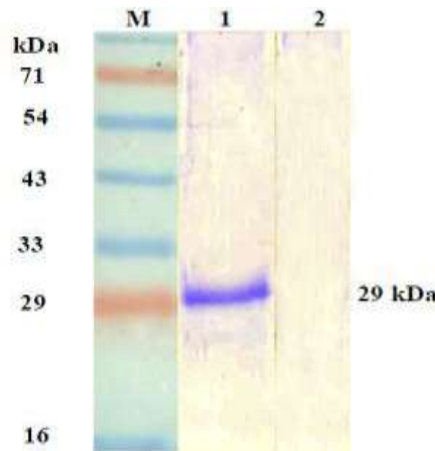
The smallest sub-unit of *EgAgB* has also been shown as the most useful target in terms of diagnostic values and AgB-2 has shown the best features in terms of diagnostic efficacy and significantly higher than native *EgAgB* (Rott *et al.*, 2000; Virgino *et al.*, 2003).

Fig.1 WB showing reactivity of recombinant *EgAgB*-8 with Anti-Histidine HRP Conjugate



Lane 1 : Purified recombinant antigen showing immuno-reactivity at 29 kDa size
Lane M : Pre-stained Protein Molecular Weight Marker

Fig.2 WB showing reactivity of recombinant *EgAgB*-8 with buffalo sera positive for CE



Lane 1 : Purified recombinant antigen showing immuno-reactivity at 29 kDa size
Lane 2 : Lane showing no reactivity with uninfected serum
Lane M : Pre-stained Protein Molecular Weight Marker

Fig.3 Field screening of buffaloes for IgG-antibodies with recombinant EgAgB-8 (n=48)

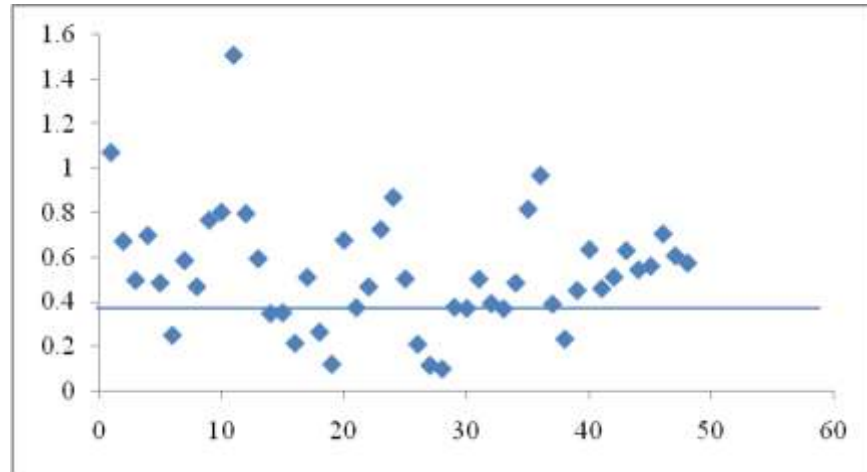
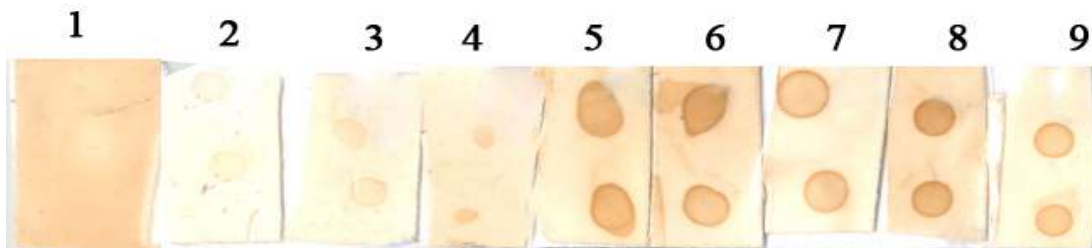


Fig.4 DOT-ELISA showing reactivity of EgAgB-8 with buffalo sera



No. 1: Hydatid negative sera

No. 2-4: Recombinant *EgAgB-8* blot with hydatid un-infected buffalo sera showing cross-reactivity with *E. explanatum*

No. 5-9: Recombinant *EgAgB-8* blot with hydatid infected buffalo sera

Therefore, *EgAgB-8* belonging to this protein family may also show the above attributes of the antigen B2 when tested for its sensitivity and specificity in buffaloes and other hosts on a larger scale.

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