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RESEARCH ARTICLE

Comparative Evaluation of 8 Kda Antigen Based Serological Diagnostic Tests for Cystic Echinococcosis in cattle

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ABSTRACT

The dog tapeworm *Echinococcus granulosus* is the causative agent of cystic hydatid disease in domestic / wild herbivores animals and man. Accurate immunodiagnosis of the infection requires highly specific and sensitive antigens. The aim of this study was to develop and evaluate various immunoassays with principles of precipitation, agglutination and enzyme immunoassays for the identification of cattle infected with hydatid cyst. It would allow the monitoring of animals from endemic areas and identifying infected animals prior to slaughter. The immunoassays were developed and validated using hydatid specific, non cross reactive low molecular weight 8 kDa hydatid cyst fluid protein. Sera used for the assay validations were obtained from 150 cattle infected naturally with hydatid cyst and 150 non-infected cattle. The highest diagnostic sensitivity was obtained in Enzyme linked immuno electro transfer blot (EITB) at 87.5% followed by Latex agglutination test (85.5%) and Counter immunoelectrophoresis(75%) . The study demonstrated that EITB was most sensitive immunological test for detection of cystic echinococcosis in cattle. This test proved to be adequate for surveillance systems and for evaluating control programmes.

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INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic parasitic infection of many mammalian species caused by the larvae of *Echinococcus granulosus* which is a small tapeworm. The life cycle, dogs and other canids are typical definitive hosts and ungulates especially cattle, buffalo, sheep, goat, pig and horse are intermediate hosts in which the hydatid cysts occur (Daryani *et al*., 2007).It causes severe economic loss and public health problem to both human beings and livestock in many temperate and tropical areas of the world including India. The most tangible economic effects of this are the loss of offal from food animals. This may result in the entire loss of an infected organ or at least the trimming and down grading of that organ (Irsadullah, 1989). It has also been evidenced that only 6.5 per cent of meat from infected cattle could be placed in the prime category for consumption compared to 22.4 per cent from healthy animals. The body weight of infected animal will be 1 per cent less than uninfected animals (Torgerson, 2003). The global annual livestock production loss due to CE is estimated to be US$ 141, 605, 195 (Budke, 2006).

In addition, this disease is of utmost zoonotic importance in human beings and requires expensive and prolonged medical treatment, often surgical interventions and the mortality may go up to 2 to 4 per cent . The global annual monetary loss due to CE in man has accounted for US$ 193, 529, 740 (Budke, 2006).

The cases of CE in humans and domesticated animals such as cattle, buffaloes, sheep, pigs and wild animals are being increasingly reported from different parts of India including Tamil Nadu (Parija and Sheela Devi, 1999 and Raman and Lalitha John, 2003).

In livestock, infection with hydatid cyst is asymptomatic and diagnosis is made usually at necropsy. Lahmar et al., (2007) reported ultrasonography in animals, but a precise diagnosis of CE was not possible.

Development of an inexpensive accurate serological assay could be of importance as a surveillance tool for diagnosis and sero-epidemiology of CE in animals. In addition, such an assay could serve as a screening instrument for live animals prior to export and in the identification and elimination of isolated focal reservoirs of infection during the consolidation phase of control programme (Dueger *et al*., 2003).

Antibody detection remains the method of choice for diagnosis. Indirect haemagglutination test (Golassa *et al*., 2011), Counter immunoelectrophoresis(Raman and Chellappa, 1998), ELISA (Kittelberger et al., 2002), Latex agglutination test (Gomez *et al*., 1980)) and EITB (Dueger *et al* 2003) are the most commonly used immuno diagnostic methods. Various immunodiagnostic tests for CE in man and animals have been attempted in India, including Tamil Nadu (Dhar *et al*, 1996; Parija, 1998; Raman and Chellappa, 1998) using hydatid cyst fluid antigens with varied sensitivity and specificity. However these assays using crude hydatid antigens have been non-specific due to cross reaction with C*ysticercus* and other helminthic infections (Shepherd and McManus, 1987; Siracusano and Bruschi, 2006). In order to overcome these difficulties various novel tests using purified antigens are essential for confirmative diagnosis of CE in man and animals. Currently, the antigen B, 8 kDa is a highly immunogenic major component of hydatid cyst fluid and these properties have encouraged the preferential use of this antigen over other hydatid antigens, in the sero diagnosis of human CE (Mamuti *et al.,* 2006; Jiang *et al*., 2012).Hence the present study was envisaged to evaluate three immunological tests such as Counterimmunoelectrophorsis, Latex agglutination test and Enzyme linked immunoelectro transfer blot using hydatid specific non cross reactive 8 kDa antigen for diagnosis of CE in cattle.

MATERIALS AND METHODS

Parasites

The hydatid cysts for this study were collected from cattle slaughtered at Corporation Slaughter House in Perambur and Department of Meat Science and Technology, Madras Veterinary College, Chennai, India. The collected hydatid cysts were thoroughly washed in distilled water to remove the adhering dirt and clotted blood. The fertility of hydatid cysts was tested by examining a drop of hydatid fluid for presence of protoscolices. The fluid was aspirated slowly using a 20 ml syringe. The aspirated fluid was pooled together and kept in a glass beaker for settling of brood capsules, protoscolices and dead tissues. The supernatant was collected and clarified by centrifugation at 10,000 rpm for 30 minutes to remove the sediments. The hydatid fluid was then poured into a 1000 Da cut-off membrane (Sigma, USA) and dialyzed against three changes of distilled water at 4^{o} C. The dialyzed fluid was further taken into dialysis tubing (Sigma, USA) and concentrated using poly ethylene glycol 6000 (SRL, India). The hydatid fluid was supplemented with 0.02 per cent sodium azide, 5 mM EDTA and 0.5 M PMSF. Aliquots of hydatid fluid were frozen at -20^{o} C for further use (Verastegui *et al.,* 1992).

Serum Samples

The serum samples were collected from cattle prior to slaughter and categorized positive and negative samples after thorough examination of internal organs for the presence/ absence of cysts. A total of 150 known positive serum samples were collected from cattle showing the presence of hydatid cysts in visceral organs and150 known negative serum samples collected from hydatid cyst free cattle slaughtered at Perambur Slaughter House,Chennai. India.

8 kDa antigen

The immunodominant 8 kDa antigen was prepared from hydatid cyst fluid by Anion exchange chromatography using DEAE- sepharose fast flow as per the method described by Gonzalez *et al.,* (1996) with minor modifications.

The hydatid cysts were collected and processed as mentioned above. The conductivity of hydatid fluid was adjusted with conductivity buffer pH 7.4 (20 mM sodium phosphate 1.2gm, 2M sodium chloride 58 gm and 500 ml distilled water) as equal to that of application buffer pH7.4(20mM sodium phosphate1.2gm, 200 mM sodium chloride 5.84gm and distilled water 500 ml). DEAE sepharose fast flow (Sigma, USA) was slowly packed to a 2.5 x 5 cm size column (Bio-rad, USA). The column was equilibrated with application buffer. Typically 1.5 liters of the hydatid cyst fluid supernatant were loaded in the column. The flow rate was adjusted to 3 ml / minute and the chromatography was undertaken at 4^{o} C. The column was washed with 5 column volumes of application buffer. The bound antigen fractions were eluted with elution buffer pH7.4 (20 mM sodium phosphate, 500 mM sodium chloride and 500 ml distilled water). The fractions were extensively dialysed against phosphate buffered saline (pH 7.2) and concentrated with polyethylene glycol 6000. The concentrated protein was Antigen B. The protein content of concentrated antigen B was estimated as per Smith *et al.,* (1985) using bicinchoninic acid protein estimation kit (Genei, India) at the absorbance of 562 nm.

The antigen B was resolved in 12.5 per cent SDS-PAGE to identify the 8 kDa protein band. The 8 kDa protein band strips were excised from gels. They were immersed in 2 per cent glutaraldehyde for 60 minutes. The strips were destained completely at 4^{o} C and pulverized with PBS (pH 7.2.). The material was centrifuged at 15,000 rpm at 4^{o} C for 30 minutes. The supernatant was collected. The procedure was repeated many times to collect 8 kDa antigen. The pools of supernatant were concentrated by polyethylene glycol with dialysis tubing (Mr cut of 1000 Da, Sigma, USA). The 8 kDa protein content was estimated as per Smith *et al.,* (1985) using bicinchoninic acid protein estimation kit (Genei, Bangalore) at the absorbance of 562 nm.

Western blot analysis

The purity of the 8 kDa protein was tested by western blot. SDS-PAGE (12.5 per cent) of 8 kDa protein from hydatid cyst fluid was carried out on a mini protein-3 electrophoresis apparatus (Biorad, USA) using 1 mm thickness gel using a discontinuous system as described by Laemmli (1970). The 8 kDa protein bands were, then

transferred to PVDF membrane as described by Towbin et al. (1979) using Mini Trans – Blot Electrophoretic Transfer Cell (Biorad, USA). After the completion of transfer, the PVDF membrane was removed, washed briefly in distilled water and dried with paper towel. PVDF membranes with resolved 8 kDa protein were incubated with hyperimmune sera raised in rabbits against hydatid cyst fluid antigen and other metacestode fluid antigens such as Cysticercus *tenuicollis* separately for one hour with gentle shaking. The unbound antibody was washed in washing buffer 3 times for 5 minutes each. The PVDF membranes were probed with 1:1000 anti rabbit IgG HRP conjugate (Sigma, USA) for 1 hour at 37^o C. The membranes were again washed three times in washing buffer for 5 minutes each and treated with substrate Diaminobenzidine solution till the appearance of reaction.

Counter immuno electrophoresis (CIEP)

CIEP was carried out as per the method described by Ravinder and Parija (1997) with minor modifications. 1 per cent Agarose solution was prepared in 0.05M Veronal buffer pH 8.4(Sodium barbitone 10.31gm, Barbituric acid 1.84gm, sodium acetate 6.8gm and distilled water 1000ml). Five milliliter of 1% molten agar was coated on the clean glass slide and allowed to solidify under room temperature. Parallel rows of wells 4 mm in diameter and 3 mm apart were punched out on the slides with the aid of a template (Biorad, USA). Six pairs of wells were punched out on each slide. The cut wells were sealed with 5 µl of 1 per cent agar. The 8 kDa antigen was loaded at first, third and fifth row of wells at the rate of 10 µl respectively. The hyper immune HCFA antisera at the rate of 10 µl were loaded in the second row, normal rabbit serum in fourth row and test sheep serum in sixth row of wells. The antigens were placed on the cathode side well and sera at anode side well. The electrophoretic chambers were filled with veronal buffer. The slide was placed on the electrophoresis tank with contact wicks (Presoaked Whatman filter paper strips). Electrophoresis was carried out with constant current of 8 mA per slide for 1 hour. The CIEP slides were read unstained, immediately after completion of electrophoresis with the use of oblique light against black back ground. The precipitation lines between antigen and hyper immune serum were defined. The slides were incubated at room temperature in the moist chamber for 24 hours to obtain stronger reactions. The slide was washed with PBS, dried and then stained with 0.1 per cent coomassie blue. The slides were destained with destaining solution, dried and stored.

Latex agglutination test (LAT)

Latex agglutination test was carried out as per the method described by Eckert et al. (1981) with modifications using 8 kDa antigen. Polystrene Latex particles (Sigma, USA) of approximately 0.81 µm in diameter size were used. 8 kDa antigen @ 0.5 ml solution (100 µg / ml) was added to 0.5 ml of Latex working solution (5% in glycine buffered saline) for sensitization. The mixture was agitated for 30 minutes, and then incubated at 37^o C for 2 hours. The mixture was mixed with 1 per cent PSA and 1 per cent Tween-20 and stored at 4^o C. The test was standardized using hyper immune hydatid fluid serum and normal rabbit serum (negative control) and Latex control. The serum was diluted at 1:5 with glycine buffered saline and inactivated by heating in a water bath for 30 minutes at 56^o C. Twenty µl each of hyper immune serum ,negative rabbit serum and hyper immune serum were placed in first, second and third well of 3 well cavity slide (Blue star, India) respectively. Then 20 µl each of sensitized latex particles in first and second well and 20 µl of non sensitized latex particles in third well were added and mixed with a tooth pick. The slide was gently rotated for 8 minutes and the reaction was read against a dark background. Agglutination of latex particles after 5 minutes was considered as positive test and was compared with negative control and Latex control. The latex particles remained as suspension in negative control and Latex control.

Enzyme linked immuno electrotransfer blot (EITB)

This test was carried out as described by Verastegui et al. (1992) with modifications. SDS-PAGE (12.5 per cent) of 8 kDa protein from hydatid cyst fluid was carried out as mentioned earlier. The 8 kDa protein bands were, then transferred to PVDF membrane as described earlier. After the completion of transfer, the PVDF membrane was removed, washed briefly in distilled water and dried with paper towel. The PVDF membrane was then cut into 3 mm wide strips. The membrane strips were placed in blocking buffer overnight at 4^o C. The strips were then washed in washing buffer 3 times with gentle agitation for 5 minutes each. One PVDF strip was incubated with 1:100 dilutions of hyper immune anti-HCFA serum and other strip with 1:100 dilution of normal rabbit serum separately for one hour with gentle shaking. The unbound antibody was washed in washing buffer 3 times for 5 minutes each. The strips were probed with 1:1000 anti rabbit IgG HRP conjugate (Sigma, USA) for 1 hour at 37^o C. The strips were again washed three times in washing buffer for 5 minutes each. Then the strips were treated with substrate solution till the appearance of bands. Immediately after the appearance of bands, the strips were thoroughly rinsed with distilled water to stop the reaction. The PVDF strips were allowed to dry on a paper towel. The appearance of brown band (8 kDa) was considered as positive while the absence of band indicated a negative reaction.

Statistical analysis

The statistical method used for assessment of sensitivity, specificity, efficiency and predictive values of CIEP, LAT and EITB for the diagnosis of CE using 150 known positive and 150 known negative serum samples of cattle and keeping post mortem examination as a gold standard are as follows; Sensitivity % = true positive x 100 / (true positive + false negative); specificity % = true negative x 100 / (true negative + false positive); efficiency % = (true positive + true negative) x 100 / (true positive + false positive + true negative + false negative); positive predictive value = true positive x 100 /(true positive+ false positive) ; negative predictive value =true negative x 100 /(true negative + false negative).

The differences between the test in relation to sensitivity and specificity were assessed using χ^2 test.

RESULTS

Isolation of 8 kDa antigen

The hydatid cyst fluid antigen was purified by anion exchange chromatography using DEAE Sepharose fast flow. The antigen B was eluted in 7, 8, 9 and 10th fractions. These fractions were pooled together and concentrated using PEG 6000. The protein concentration of antigen B was estimated by BSA method. The protein content was 0.987 mg / ml. SDS-PAGE analysis of DEAE Sepharose fast flow anion exchange chromatography fractions revealed the antigen B protein bands at 8 kDa and 24 kDa. The 8 kDa protein band was isolated and the protein content was estimated by BSA method. The protein content was 0.320 mg / ml. SDS-PAGE analysis of isolated protein revealed a single band at 8 kDa in the gel (Fig. 1).

Western blot probing of 8kDa hydatid cyst fluid antigen with anti cyst fluid antibodies of hydatid and cysticercus cyst revealed the reaction between 8kDa antigen and hyperimmune sera raised against hydatid cyst fluid antigen only. Hyper immune sera of cysticercous fluid antigen did not react with 8kDa antigen.

Evaluation of CIEP, LAT and EITB for diagnosis of CE in cattle

The tests were evaluated with 8kDa antigen,150 hydatid positive cattle sera and 150 hydatid negative cattle sera. The sensitivity, specificity, positive, negative predictive value and efficiency of these three immunoassays in detecting serum antibodies in cattle was 75 per cent , 89.6 per cent , 92 per cent , 69.3 per cent and 80.6 per cent respectively for CIEP(Fig. 2 and Table 1), 85.5 per cent , 94 per cent , 94.6 per cent , 84 per cent and 89.3 per cent respectively for LAT (Fig. 3 and Table 2) and 87.8 per cent , 96.29 per cent , 96.6 per cent , 86.6 per cent and 91.6 per cent respectively for EITB (Fig. 4 and Table 3). The sensitivity and specificity of CIEP (χ^2 = 118.97** P<0.01), LAT (χ^2 = 187.79** P<0.01) and EITB (χ^2 = 210.44** P<0.01) were statistically significant. Comparative evaluation of CIEP, LAT and EITB for detecting serum antibodies of cystic echinococcosis in cattle showed that EITB had higher sensitivity and specificity than CIEP and LAT (Table 4 and Fig. 5). However statistically no significant difference between these tests regarding sensitivity and specificity was noticed (χ^2 = 1.18 NS P>0.05).

DISCUSSION

Hydatid cyst fluid (HCF) is a complex mixture of glycol lipoproteins, carbohydrates and salts. Crude HCF has a high sensitivity, ranging typically from 75 per cent to 95 per cent (Zhang *et al.,* 2012). However, its specificity is often unsatisfactory and cross-reactivity with sera from patients infected with other cestode (89 per cent), nematode (39 per cent) and trematode (30 per cent) species is commonly observed (Eckert and Deplazes, 2004). Hence, the crude HCF is specifically recommended for mass serological screening and it has now become more frequent to purify components such as the lipoproteins, antigen B and antigen 5, the most relevant components of HCF for diagnostic purposes. The 8 kDa

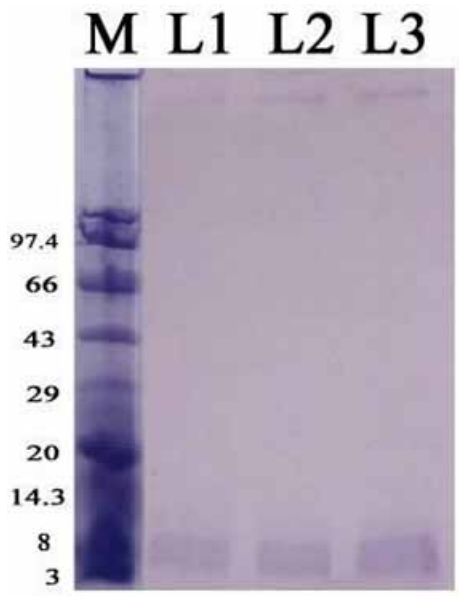

Fig. 1: 15% polyacralamide gel,commassie-stained, of 8 kDa antigen preparation used in this study; lane1-3 -Presence of 8 kDa protein band; lane M- Marker

Fig. 2: Counterimmunoelectrophoresis slide showing bands between 8kDa antigen(wells1,2,9 and 10) and positive control(3,4) and positive serum(11,12) and absence of bands between 8kDa antigen (5,6)and negative serum (7)and negative control(8).

Table 1: Evaluation of Counter immuno electrophoresis (CIEP) for detecting hydatid specific antibodies in serum samples of cattle (2x2 contingency table)

True Positive (TP) 138	False Positive (FP) 12
False Negative (FN) 46	True Negative (TN) 104

χ^2 = 118.97** P<0.01

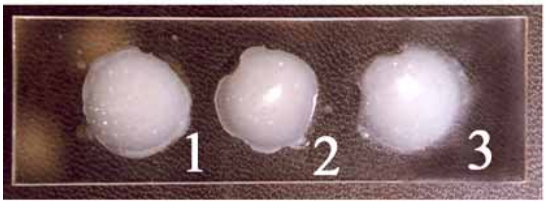

Fig 3: Latex agglutination test slide showing agglutination in positive serum (1) and suspension in negative serum (2) and latex control (3)

Table 2: Evaluation of Latex agglutination test (LAT) for detecting hydatid specific antibodies in serum samples of cattle

True Positive (TP) 142	False Positive (FP) 8
False Negative (FN) 24	True Negative (TN) 126

χ^2 = 187.79** P<0.01

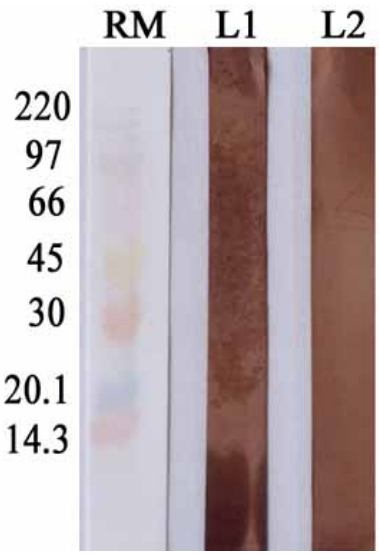

Fig 4: Enzyme linked immunoelectro transfer blot (EITB) test showing 8kDa protein reacted with positive serum (L1) and no reaction with negative serum (L2), RM- Prestained molecular weight markers.

Table 3: Evaluation of Enzyme linked immuno electro transfer blot (EITB) for detecting hydatid specific antibodies serum samples of cattle

True Positive (TP) 145	False Positive (FP) 5
False Negative (FN) 20	True Negative (TN) 130

$\chi^2 = 210.44^{**}$ P<0.01

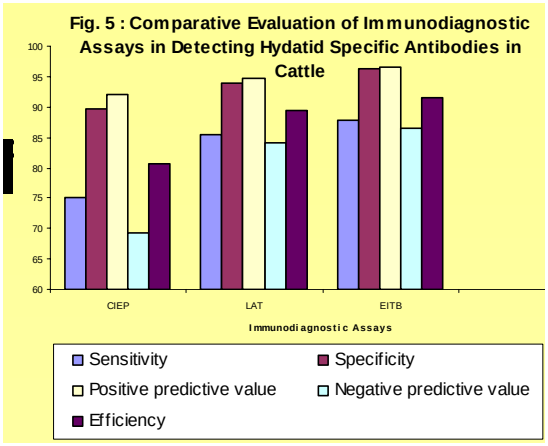

Table 4: Comparative evaluation of the serological assays for diagnosis of CE in cattle

Tests	Sensitivity %	Specificity %	Positive Predictive value %	Negative predictive value %	Efficiency %
CIEP	75	89.6	92	69.3	80.6
LAT	85.5	94	94.6	84	89.3
EITB	87.8	96.29	96.6	86.6	91.6

$\chi^2 = 1.18$ NS P>0.05

antigen has been shown to be hydatid specific. Antigen B which comprises of 8 and 24 kDa may have the opportunity to accumulate in the cyst fluid after being secreted by the parasite in such a way that the protein has the chance to aggregate into a form that is more immunogenic before the antigen gains contact with the host immune system (Mamuti *et al.,* 2006). Various authors have used different protocols to isolate 8 kDa antigens from hydatid cyst fluid, (Kanwar and Kanwar, 1994; Ioppolo *et al.,* 1996; Ibrahem *et al*., 1996; Ito *et al*., 1999 and Kittelberger *et al*., 2002), but the quantity of antigen available from the above methods was scanty. Therefore the method described by Gonzalez *et al*., (1996) using DEAE Sepharose fast flow was followed and it resulted in production of a large quantity of antigen. Gonzalez *et al.,* (1996) isolated 8 kDa proteins from 1-2 liters of hydatid cyst fluid.

Studies were conducted to detect CE in naturally infected and apparently healthy cattle from slaughterhouse by counter immuno electrophoresis. The sensitivity, specificity, positive and negative predictive value of CIEP in detecting serum antibodies in cattle was 75, 89.6, 92 and 69.3 per cent respectively. Similarly, Sangaran (1999) reported 71 per cent sensitivity and 88 per cent specificity in cattle.

The sensitivity, specificity, positive and negative predictive value of latex agglutination test in detecting CE in cattle was 85.5, 94, 94.6 and 84 per cent respectively. Gomez *et al*., (1980) reported 66.7 per cent sensitivity in cattle which differed from the present finding. The use of crude hydatid antigen by Gomez *et al*., (1980) should be the reason for the lower sensitivity when compared to 85.5 per cent sensitivity recorded in the current study using 8 kDa specific antigen.

Serodiagnosis of CE by EITB in cattle showed 87.8 per cent sensitivity, 96.29 per cent specificity, 96.6 per cent positive predictive value and 86.6 per cent negative predictive value. Perusal of available literature indicated that no study has so far been attempted on sero diagnosis of CE by EITB test in cattle and the present study is considered as an original attempt. Researchers reported that EITB assay was more sensitive than conventional serological diagnosis such as CIEP and LAT (Simsek and Koroglu, 2004)

The variation of sensitivity and specificity in diagnosing CE in cattle by these tests could be due to strain variation, nature of antigen, level of antibody in the serum etc. The false positive diagnosis were due to unspecific granulomas, pseudotuberculosis, emphysema and fatty degeneration and false negative diagnoses were due to small intra parenchymal cysts(Larrieu et al., 2001) The selective expression of EgAgB 8 kDa monomers in different hosts and / or different level of host immune response against AgB may be relevant factors in relation to the variability that is observed in the immunogenicity of AgB. It is possible that the differential expression of AgB 8 kDa monomers in different developmental stages of the parasite might be relevant to the different biological functions of each individual monomer in the host parasite interactions (Mamuti *et al.,* 2006). Further, the antigen B, 8 kDa is a highly immunogenic major component of hydatid cyst fluid and these properties have encouraged the preferential use of this antigen over other hydatid antigens, in the sero diagnosis of CE (Carmena *et al.,* 2006; Mohammadzadeh *et al.,* 2012). The main problems in the immuno diagnosis of echinococcal disease are the often unsatisfactory performances of the available tests and the difficulties associated with the standardization of

antigen preparations and techniques. However EITB proved adequate for the detection of the infected cattle on a flock basis for surveillance and evaluating control programmes.

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