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Development and evaluation of flow through technique for diagnosis of cystic echinococcosis in cattle

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ABSTRACT

A novel *in vitro* flow through technique was developed and evaluated for immunodiagnosis of cystic echinococcosis in cattle using hydatid specific non-cross reactive 8 kDa protein. The 8 kDa protein was prepared from hydatid cyst fluid by DEAE-Sepharose fast flow anion exchange chromatography. In this flow through technique, the 8 kDa antigen was coated on the nitrocellulose membrane of the flow through device. Protein A Colloidal gold was used as detector. The evaluation of the technique was performed by comparing 150 known positive hydatid serum and known negative serum collected from cattle. The test was shown to have high sensitivity and specificity that were closely correlated with those of Enzyme-linked immunoelctrotransfer blot. Furthermore, the immunofiltration based assay is rapid and easy to perform with no requirement of special skill, reagent or instrumentation. This suggests the flow through technique is an acceptable alternative for use in clinical laboratories lacking specialized equipment as well as large scale screening of cystic echinococcosis in the field with both animal and human populations.

Key words: cystic echinococcosis, flow through technique, 8 kDa antigen

Introduction

Cystic echinococcosis (CE) of animals and man causes severe economic loss and public health problems for 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 complete loss of an infected organ or at least the trimming and downgrading of that organ (IRSADULLAH, 1989). It has

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also been shown that only 6.5 percent of meat from infected cattle could be placed in the prime category for consumption compared to 22.4 percent from healthy animals. The body weight of infected animals will be 1 percent 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 intervention and the mortality may be up to 2 to 4 percent. 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 sheep, cattle, buffaloes and pigs, and wild animals are being increasingly reported from different parts of India, including Tamil Nadu (PARIJA and DEVI, 1999; RAMAN and JOHN, 2003).

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

The development of an inexpensive accurate serological assay could be of importance as a surveillance tool for diagnosis and sero epidemiology of hydatidosis 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 the control programme (DUEGER et al., 2003).

Antibody detection remains the method of choice for diagnosis. Indirect haemagglutination test, Counter immunoelectrophoresis (RAMAN and CHELLAPPA, 1998), Enzyme-linked imunosorbent assay (KITTELBERGER et al., 2002), Latex agglutination test (GOMEZ et al., 1980) and Enzyme linked immunoelectrotransfer blot (EITB) (DUEGER et al., 2003) are the most commonly used immuno diagnostic methods. Various immunodiagnostic tests for hydatidosis in man and animals have been attempted in India, including Tamil Nadu (DHAR et al., 1996; PARIJA, 1998; RAMAN and CHELLAPPA, 1998; JEYATHILAKAN, 2007) using hydatid cyst fluid antigens with various sensitivity and specificity. However, these assays, using crude hydatid antigens, have been non-specific due to cross reaction with C*ysticercus*, C*oenurus* and other helminthic infections (SHEPHERD and McMANUS, 1987). In order to overcome these difficulties, various novel tests using purified antigens are essential for confirmative diagnosis of hydatidosis 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). Recently, there has been a growing interest in the immunochromatographic strip for the rapid detection of analytes because of its easy of use and visual end point. It has been

widely used in medical fields for the detection of pregnancy, viral infection and drug abuse (NACHIMUTHU and RAJ, 2005). The flow through technique is a simple, novel, rapid and user-friendly *in vitro* diagnostic assay where the level of sensitivity and specificity is very high. These are otherwise called immuno- chromatographic assays. The current study envisages development of an easy to use hydatid specific flow through technique (colloidal dye immunofiltration assay) for the diagnosis of cystic echinococcosis in cattle using a hydatid specific non cross reactive 8 kDa antigen.

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. Examining a drop of hydatid fluid for the presence of protoscolices tested the fertility of the hydatid cysts. 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 dialysed against three changes of distilled water at 4 ^{o}C. The dialysed fluid was further taken into dialysis tubing (Sigma, USA) and concentrated using polyethylene glycol 6000 (SRL, India). The hydatid fluid was supplemented with 0.02 percent 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. A total of 150 known positive serum samples were collected from cattle showing the presence of hydatid cysts and 150 known negative serum samples collected from healthy parasite free cattle slaughtered at Perambur Slaughter House, Chennai.

8 kDa antigen. The immunodominant 8 kDa antigen was prepared from the 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 frozen hydatid fluid was thawed and conductivity adjusted with conductivity buffer as equal to that of the application buffer. DEAE sepharose fast flow (Sigma, USA) was slowly packed to a 2.5 5 cm size column (Bio-rad, USA). The column was equilibrated with application buffer. Typically 1.5 litres 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. The fractions were extensively dialysed against the phosphate buffered saline (pH

7.2) and concentrated with polyethylene glycol 6000. It was known as 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 percent SDS-PAGE to identify the 8 kDa protein band.The 8 kDa protein band strips were excised from the gels. They were immersed in 2 percent glutaraldehyde for 60 minutes. The strips were destained completely at 4 °C and pulverized with PBS (pH 7.2). The material was centrifuged at 15,000 rpm at 4 °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.

Flow through technique (FTT). The flow through technique was carried out as described by XIANG et al. (2003). The test was performed in an immuno-filtration kit in which the 8 kDa antigen coated Nitro cellulose membranes (NCMs) were pressed tightly to a water absorbing pad in a plastic housing. The easy pack immuno filtration kit (Mdi, India) comprises of the Flow through device and the Flow director funnel. The Flow through device is a small, rectangular, openable, plastic box (3.5 cm × 2.5 cm) with a test hole cut in the centre. The box was fitted with a water absorbing pad and the prepared NCM membrane was pressed tightly on top of the absorbing layer in the test well.

The nitrocellulose membrane was soaked in 80 percent alcohol for 10 minutes. The membrane was air dried and cut into 3.5 × 2.5 cm size pieces. The NCM pieces were pressed tightly on top of the water absorbing pad. The kit was stored at room temperature.

Three µL of 8 kDa antigen (10 µg/mL) was added to NCM as a small dot, using the flow director funnel in the device. The device was stored in a sealed plastic bag at room temperature.

Test procedure. Two 8 kDa antigen coated flow through devices were taken to carry out the test. The NCM of flow through device was wetted with 100 µL washing buffer pH 7.4 (25 mm PBS pH 7.4, 0.5% Bovine serum albumin, 0.05% Tween-20 and 0.2% Sodium azide) through the flow director funnel for one minute. A 50 µL volume of test serum was then pipetted into the well and allowed to absorb completely.

After that, 100 µL of protein-A colloidal gold conjugate solution (Sigma, USA) was added to the test hole and was allowed to soak for 2 minutes. Finally, 100 µL of washing buffer was added to remove the unbound conjugate. The whole procedure was completed within 2 minutes and the results were easily determined with unaided eyes. Likewise, a known negative serum test was done to compare the result. The appearance of red dots in

the NCM indicated a positive reaction and the absence of a red dot formation indicated a negative reaction.

Enzyme-linked immuno-electro transfer blot (EITB). EITB was used as a reference test for detection of seroreactivity between 8 kDa hydatid antigen band and serum samples. This test was carried out as described by VERASTEGUI et al. (1992) with modifications. SDS-PAGE (12.5 percent) of 8 kDa protein from hydatid cyst fluid was carried out on 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 a Mini Trans - Blot Electrophoretic Transfer Cell (Biorad, USA). The free binding sites were saturated with 5% skimmed milk powder. Anti bovine IgG HRP conjugate (Sigma, USA) was diluted at 1:1000 (v/v) with hydrogen peroxide / diamino benzidine (DAB) substrate system.

Evaluation of FTT and EITB. The 150 known positive and 150 known negative serum samples of cattle tested by EITB were subjected to the flow through test to assess the sensitivity and specificity of the assay. The following definitions are used to calculate the diagnostic parameters. Sensitivity % = true positive × 100 / (true positive + false negative); specificity % = true negative × 100 / (true negative + false positive); efficiency % = (true positive + true negative) × 100 / (true positive + false positive + true negative + false negative); positive predictive value = true positive × 100 / (true positive+ false positive); negative predictive value = true negative × 100 / (true negative + false negative).

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 the BSA method. The protein content was 0.987 mg/mL. SDS-PAGE analysis of the 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 the BSA method. The protein content was 0.320 mg/mL. SDS-PAGE analysis of the isolated protein revealed a single band at 8 kDa in the gel (Fig. 1).

Enzyme-linked immuno-electro transfer blot (EITB). The EITB test was standardized by using 8 kDa antigen, hyper immune rabbit serum, anti rabbit HRP conjugate and DAB substrate. Positive results were indicated by the appearance of the 8 kDa protein band, while no band was visible when the reaction involved negative sera. The sensitivity, specificity, positive, negative predictive value and efficiency of EITB in detecting serum

antibodies in cattle are 87.8 percent, 96.29 percent , 96.6 percent , 86.6 percent and 91.6 percent respectively (Table 1).

Table 1. Evaluation of enzyme- linked immunoelectrotransfer blot (EITB) for detecting cystic echinococcosis specific antibodies in cattle

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

Table 2. Evaluation of Flow through technique (FTT) for detecting cystic echinococcosis specific antibodies in cattle

True Positive (TP) 146	False Positive (FP) 4
False Negative (FN) 12	True Negative (TN) 138

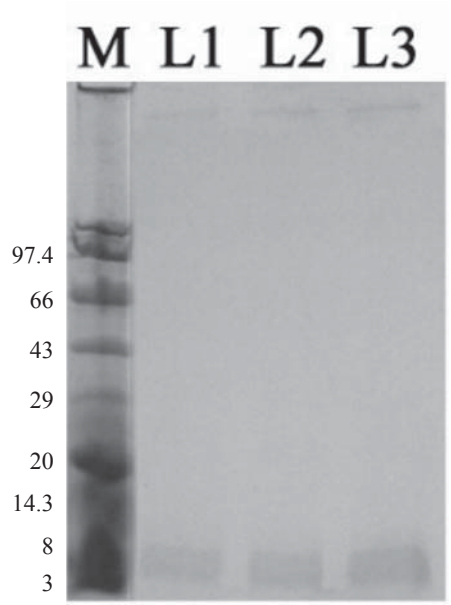

Fig. 1. 15% polyacralamide gel, commassie-stained, of 8 kDa antigen preparation used in this study; lane 1-3 presence of 8 kDa protein band, M - molecular weight marker.

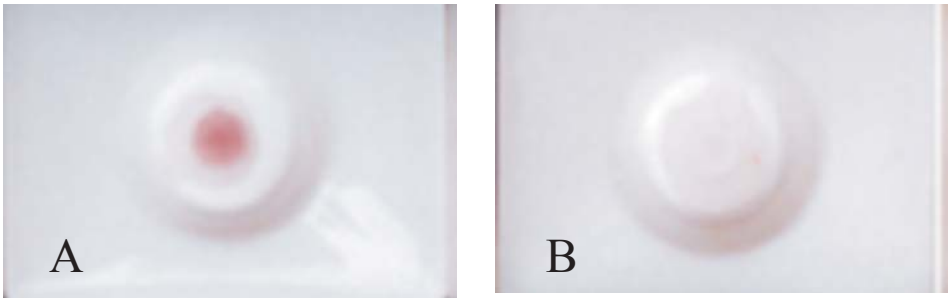

Fig. 2. Flow through technique. A - Flow through device showing red dot in cystic echinococcosis positive serum, B - Absence of dot in cystic echinococcosis negative cattle serum.

Flow through technique (FTT). The test was carried out by using 8 kDa antigen, 150 known positive and 150 known negative cattle sera. Formation of red dots was observed in positive serum while the absence of the dot formation indicated a negative reaction (Fig. 2). The sensitivity, specificity, positive, negative predictive value and efficiency of the flow through test in detecting serum antibodies in cattle was 92.40 percent, 97.18 percent, 97.33 percent, 92 percent and 94.66 percent respectively (Table 2).

Discussion

Hydatid cyst fluid (HCF) is a complex mixture of glycolipoproteins, carbohydrates and salts. Crude HCF has a high sensitivity, ranging typically from 75 percent to 95 percent (ZHANG et al., 2003). However, its specificity is often unsatisfactory and cross-reactivity with sera from patients infected with other cestode (89 percent), nematode (39 percent) and trematode (30 percent) species has been 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 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 antigen from hydatid cyst fluid, (KANWAR and KANWAR, 1994; IOPPOLO et al., 1996; IBRAHEM et al., 1996; ITO et al., 1999; 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 the production of a large quantity of antigen. GONZALEZ et al. (1996) isolated

8 kDa proteins from 1-2 litres of hydatid cyst fluid. The specificity and sensitivity of the immunofiltration test is largely dependent on the quality of the antigen used in the test (XIANG et al., 2003).

Enzyme-linked immuno-electrotransfer blot (EITB) was carried out to compare the results with FTT. The test showed 87.8 percent sensitivity, 96.29 percent specificity, 96.6 percent positive predictive value and 86.6 percent negative predictive value. Perusal of available literature indicated that no study has so far been attempted of sero diagnosis of CE by the EITB test in cattle and the present study is considered to be an original attempt.

The sensitivity, specificity, positive predictive value and negative predictive value of FTT for detecting hydatid specific antibodies in cattle sera were 92.45, 97.18, 97.33 and 92 percent respectively. FTT had higher sensitivity and specificity than EITB in detecting serum antibodies of CE in cattle. Perusal of available literature did not reveal any reference to the use of this test in cattle and other animals. But a similar kind of test was carried out for human CE using crude antigen and the results were variable, ranging from 67 to 100 percent sensitivity and specificity (MISTRELLO et al., 1995; ELIADES et al., 1998; PAUL and STEFANIAK, 2001; OLUT et al., 2005).

In this study, the flow through technique is considered as a simple, fast and user friendly *in vitro* diagnostic assay. The level of sensitivity and specificity is very high. The cost of the test is also very low ($1/test) Colloidal gold can be replaced by textile dyes, which would bring down the cost of this test considerably (XIANG et al., 2003). 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). For the reasons mentioned above, the test may prove to be a very useful new tool for the diagnosis of cystic echinococcosis in individual animal and human patients, especially for large scale screening both in the field with animal and human populations. Further studies are also required to evaluate its value as a screening test for cystic echinococcosis on the field level.

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JEYATHILAKAN, N., S. A. BASITH, L. JOHN, N. D. J. CHANDRAN, G. D. RAJ: Razvoj i prosudba protočne metode za dijagnosticiranje ehinokokoze u goveda. Vet. arhiv 80, 549-559, 2010.

SAŽETAK

U radu je opisana nova *in vitro* protočna tehnika prikladna za imunodijagnostiku ehinokokoze u goveda upotrebom visoko specifične bjelančevine od 8 kDa. Ta je bjelančevina bila pripravljena od hidatidne tekućine metodom DEAE-Sefaroze brzo protočne kromatografije s izmjenom aniona, a zatim naslojena na nitroceluloznu opnu u protočnom sustavu. Kao detektor rabljena je bjelančevina A obilježena koloidalnim zlatom. Metoda je vrednovana na temelju rezultata dobivenih pretraživanjem 150 pozitivnih i negativnih uzoraka seruma goveda. Istraživanje je pokazalo da je riječ o visoko osjetljivom i specifičnom testu vrlo sličnom metodi Western Bloting. Kako je opisani test brz i jednostavan i ne iziskuje posebne vještine, reagencije i opremu, smatra se da je osobito prikladan za opsežne probirne testove za dijagnosticiranje ehinokokoze u ljudi i životinja.

Ključne riječi: cistična ehinokokoza, protočna tehnika, antigen 8 kDa