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Identification and Characterization of Echinococcus granulosus Antigen B (EgAgB) and their use for Dot-ELISA Development

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Abstract
Cystic echinococcosis (CE) is a neglected tropical zoonotic disease mostly prevalent in tropical and subtropical region of the world. The diagnosis of the disease is difficult and expensive and could not differentiate in current and past infection with higher cross reactivity. Hydated cyst fluid is a mixture of various excretory and secretory antigens which can be used for the development of an effective, most specific and sensitive diagnostic assay like Dot-ELISA. Hydated Cyst (HC) from slaughter sheep were collected and Hydated Cyst Fluid (HCF) were rinsed through a sterile syringe. Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) were used for the separation of various antigenic peptides followed by identification and characterization of most suitable antigenic peptide for the development of Dot-ELISA assay. a total of five different peptides of *EgAgB* having different bands sizes including 10-12 kDa, 22-24 kDa, 38-40 kDa, 70-75 kDa and 170-173 kDa were isolated from hydated cyst fluid (HCF) of sheep through SDS-PAGE analysis. The evaluation of entire separated bands for Dot-ELISA development showed that the EgAgB peptide of 22-24 kDa could work as a promising candidate for dot-ELISA development. The developed dot-ELISA procedure had a diagnostic sensitivity of 96.4% and sensitivity of 98.6% for diagnosis of the condition in human hydated cyst patients. In comparison to the commercially available ELISA kit which shows a sensitivity of 85.7% and specificity of 97.2% the current developed dot-ELISA Kit will be a better alternative with higher efficacy in human diagnosis. The current study concluded that HCF contain various antigenic peptides among which the antigenic peptide of 22-24kda band size could be used for the diagnosis of CE infections in under-developed and developing countries

Keywords: Cystic Echinococcosis, Hydated Cyst Fluid, SDS-PAGE, Dot-ELISA

Introduction

Hydatidosis/Cystic echinococcosis (CE) of Cystic hydatid disease (CHD) is an endemic, zoonotic infection caused by the larval stage of various species of an important helminth parasite *Echinococcus granulosus* sensu lato (*E. granulosus s.l*) complex. CE possess a serious threat to livestock and human population, causing significant economic and public health issues (Khan et al., 2021; Basharat et al., 2022; Thompson et al., 1986; Budke et al., 2006; Cardona et al., 2013; Nakao et al., 2013; Alvarez Rojas et al.,2014). The metacestodes (larvae) stage of *E. granulosus* is bladder-like, fluid-filled structure that establish and gradually grow in the parenchyma of host anatomical organs, most commonly liver and lungs. Generally, they are known as hydatid cysts (HC) however, strictly the term cyst includes a fibrous adventitial layer generated as a consequence of the host inflammatory reaction (Silva-Álvarez et al., 2016). The hydated cyst fluid (HCF) is the amount of fluid present within the cyst, containing a mixture of various excretory and secretory products, released by the wall of the cyst, germinal layer (GL) or cellular layer and by protoscoleces as well (Silva-Álvarez et al., 2016).

Generally, ultrasonographic and radiological examination in combination with immunodiagnostic techniques are used for complete diagnosis of hydated cyst/hydatidosis (Parija et al., 1998; Sadjjadi et al., 2001). A large number of immunoblot assay have been developed for diagnosis of echinococcus antigens and anti-echinococcus antibodies in the serum of infected patients (Ortona et al., 2003). These diagnostic assays include enzyme-linked immunesorbent assay (ELISA), radioimmunoassay (RIA), counter-current immunoelectrophoresis (CIEP), immunoelectrophoresis, indirect immunofluorescence (IFA) and indirect hemagglutination (IHA) (Sarkari et al., 2007; Sadjjadi et al., 2007; Siavashi et al., 2005). Furthermore, immunoblot assay, time-resolved fluoroimmunoassay (TR-FLA), enzyme-linked immunoelectrodiffusion assay (ELIESA), and enzyme-linked immunoelectrotransfer blots (EITB) have been developed for anti-hydatid cyst antibodies diagnosis (Aceti et al., 1991; Babba et al., 1994; Moosa et al., 1994; Ortona et al., 2000). Each and every immunodiagnostic method has its own limitation and currently no reported diagnostic assay with 100% sensitivity and specificity is present.
The hydated cyst fluid contain AgB (EgAgB), the major lipoprotein which is constantly synthesized/expressed by the larvae stage of E. granulosis (Oriol et al., 1971). Molecular characterization showed that these genes are continuously expressed with different level and within distinct tissues of a given developmental stage (Zhang et al., 2010). The EgAgB of echinococcus-genus is one of the most specific and immunogenic antigens which can be used for serodiagnosis of human infection therefore, most of the studies are carried out on its composition and antigenic abilities (Ioppolo et al., 1996; Ortona et al., 1995; Virginio et al., 2003; Carmena et al., 2006). Various studies of HCF through western blot analysis reported five peptides antigenic bands of 16 to 17kDa, 55kDa, 16kDa and 24kDa (Rajabiyoun *et al*., 2006; Maddison *et al*., 1989; Burgu *et al*., 2004). Other studies reported three bands of 8kDa, 16kDa and 22-24kDa in HCF sample by (Al-Olayan *et al*., 2012), and 4 subunit of antigenic peptide 8kDa, 12kDa, 16kDa and 24kDa (Lightowlers *et al*.,1989) through SDS–PAGE electrophoresis.

There is a need for the development of new diagnostic method for echinococcosis (Latif at el., 2013). Dot-ELISA is One of the best, simple and rapid diagnostic technique intend high potential for the detection of serum hydatid antigen and needs minute volume of reagents. Majority of EgAgB based ELISA studies reported that this can be used as an authentic, valuable and cost-effective test for the diagnosis of human hydatidosis (Rokni, 2009). Moreover, ELISA has the potential to be served as a vehicle to detect the seroprevalence of some diseases (Ilbeigi et al., 2015), so it was utilized in this study to develop HCF-based Dot-ELISA assay which can be used for immunodiagnosis of cystic echinococcosis in human populations of developing countries including Pakistan, in order to investigate the actual human disease burden of cystic echinococcosis.

Methodology

Study Design and Ethical Statement

The current study was designed to evaluate and characterize the immunogenic peptides founds in HCF and their use for development of dot ELISA assay. Ethical approval for the study was taken from Ethical Approval Committee of COMSATS University, Islamabad under Reference No. CUI-Reg/Notif.2255/19/2661.

Collection of Sheep Hydated Cyst and Human Echinococcus Patients Serum

A total of 28 serum samples from confirmed cystic echinococcosis patients were collected from surgery ward of Dera Ghazi Khan Teaching hospital, Punjab, Pakistan. The hydated Cyst present in liver of sheep were obtained from Sihalla Slaughter House, Islamabad, Pakistan. The cyst external surface was disinfectant with heat and alcohol and the cyst fluid were aspirated through a sterile 10cc syringe (Figure 1). By using an optical microscope, the fluid was examined to determine the presence of protoscoleces (Figure 2) and assess the turbidity of the fluid (since bacteria turn the fluid turbid). The controlled fluid was stored in sterile dishes at -70°C until isolation (Sarkari et al., 2007; Rafiei et al., 2008).

Isolation of Antigen B (EgAgB) Peptides of HCF through SDS-PAGE Analysis

Echinococcus granulosus antigen B (EgAgB) was isolated according to (Rahimi et al., 2011) Briefly the collected HCF (50ml) was poured in polyethylene glycol (PEG 4000) containing bag for 2 h in order to concentrate of HCF proteins, followed by filteration through a 0.2 microfilter and centrifugation at 1500 g for 30 min. The preparation was dialyzed against 0.005 M acetate buffer (pH=8) overnight at 4 °C. The content of dialysis bags was centrifuged at 30,000 g at 4 °C by an ultracentrifuge for 30 min. The precipitates were then dissolved in 10 ml of 0.2 M phosphate buffer (pH=8). The preparation was saturated with ammonium sulfate 40% and centrifuged at 3000 g for 30 min and placed in incubator for 30minutes on 90°C. SDS-PAGE were run according to Laemmli in 1970 by using resolving gel 12.5% (30% Acylamide 8ml, Distilled water 8.4ml, 3M Tris-HCL (8.8pH) 3ml, 10% SDS 200µl, 10% APS 250µl, TEMED 14µl) and Stacking Gel 4.5% (30% Acylamide 940 µl, Distilled water 4.9 ml, 1M Tris-HCL (6.8pH) 350 µl, 10% SDS 55 µl, 10% APS 230µl, TEMED 5µl). The prepared HCF sample containing (SDS, beta mercaptoethanol and bromophenol blue) was heated at 70°C for ten minutes. BME is a reducing thiol and act as breaker of the disulfide bond in proteins and the heating breaks the hydrogen bonds disintegrate the secondary structures while the blue dye act as a visibility aid. The samples were poured into each well and a ladder of 170Kda was poured into a separate well for to measure the size of the protein bands.

Transfer of Peptides bands to Nitrocellulose Membrane and Dot-ELISA Development

The characterization of the most efficient antigenic part used for the development of Dot-ELISA were carried out in the following steps:

1. The SDS-Gel containing isolated protein bands were placed into transfer buffer (glycin 39mM, Trisbase 48mM, SDS 0.1%, Methanol 20%) at pH 8.3.

2. Both the blot papers and Nitrocellulose membrane (NCM) (Hybond ECL, Amersham bioscience, Germany) were rinsed with transfer buffer.
3. The soaked blot paper was placed on platinum anode of Transblot Turbo System (BioRad, USA) by removing the bubbles through ruller.
4. The pre-soaked nitrocellulose membrane was placed on the top of the wetted blot paper.
5. The equilibrated gel or SDS-Gel having separated peptide bands were placed on the top of the nitrocellulose membrane.
6. Two pieces of pre-wetted blot paper were placed on the top of the above-mentioned equilibrated SDS-Gel and the bubbles were removed.
7. The cathode plate of the cassette was placed above the mentioned gel system and closed off properly.
8. The transfer unit were run at 18V for 01 hour and the nitrocellulose membrane were rinsed with H_2O.
9. The Nitrocellulose membrane was stained with Poncea Red Solution cat # P3504 (Merck KGaA, Darmstadt, Germany) for 12 hours.
10. The stained membrane was washed extensively for 20 times with nuclease free, sterile water or TBST (sterile PBS 7.2 containing 0.1% Tween-200 water until the protein bands were well defined.
11. The nitrocellulose membrane was incubated for 01 hour in blocking buffer (5% non-fat milk in TBST) in rotatory incubator.
12. After 01 hour the blocking buffer were poured off and the nitrocellulose membrane were incubated with anti-echinococcus primary antibodies (sera from confirm echinococcosis patients) at 4^0 C for 12 hours in rotatory incubator.
13. The primary antibodies were poured off and the membrane were washed for 5 times (5 to 8 minutes) with TBST.
14. The membrane was incubated in rotator with secondary antibodies (peroxidase-labeled rabbit anti-human IgG; Sigma Aldrich, Spain) and blocking buffer at 25^0C for 2 hours.
15. The secondary antibodies solution was poured off and washed the membrane with TBST for 10 times (each time for 3 minutes).
16. The enhanced luminal solution (Cytiva RPN2232) (Merck KGaA, Darmstadt, Germany) was mixed (Solution A and Solution B) and poured into the membrane followed by incubation at 25^0C for 3 minutes.
17. The formation of brown colored dot/spots was seen with mobile and CCD camera.

Comparison of Dot-ELISA Results with Commercial ELISA

The entire surgically confirmed 28 human serum samples were evaluated through HYDATIDOSIS ELISA IgG Kit (Cat # G1006) (Vircell, S.L., Parque Tecnológico de la Salud Avicena 8 18016 Granada, Spain) according to the standard protocol provided. Comparison among our developed Dot-ELISA and commercial ELISA were carried out.

Statistical analysis

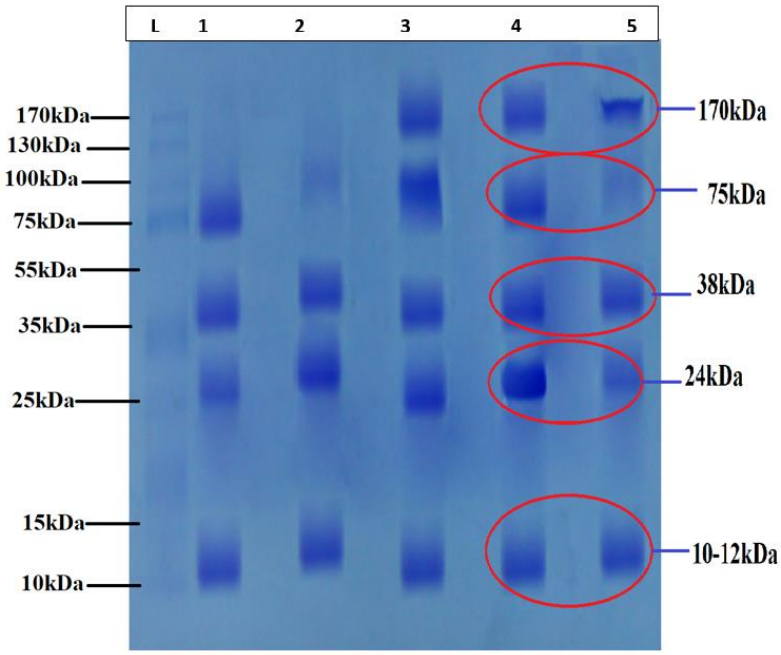
The entire Dot-ELISA results were evaluated for Diagnostic efficiency like positive predicted value (PPV), Negative predicted value (NPV), Specificity and Sensitivity according to Standard protocol (Park, 2005) by using Clinical Utility Calculator; http://www.clinicalutility.co.uk/.

Results

Separation of Hydatid cyst antigenic Proteins (SDS-PAGE)

Using the standard protocols for protein isolation and screening method we got various five (05) band of 10-12kDa, 22-24-kDa, 34-38kDa, 70-75kDa and 170-175kDa from the Hydated cyst fluid of sheep as shown in the figure 1.

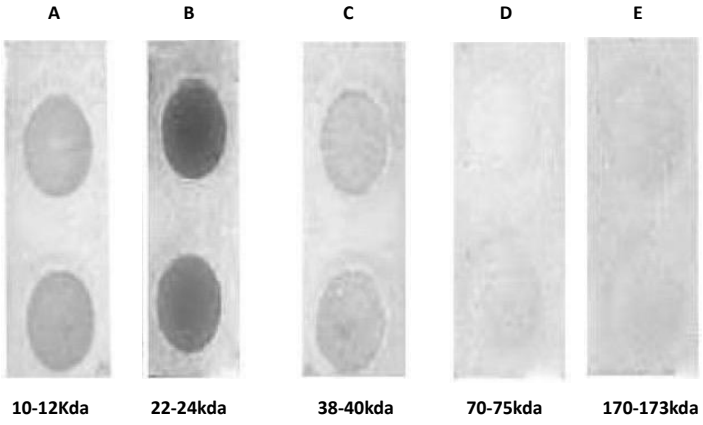
Figure 1. Characterization of HCF Antigens through SDS-PAGE Analysis. L represent the Ladder of 170kDa, Wells 1-5 represent the Peptides bands of Respective Size in sheep HCF



Development of Dot-ELISA

The separated antigenic peptide bands of 22-24kda showed a higher as compare to 10-12kda and 30-34kda band size as shown in the Figure 2. The results showed that the peptide of 22-24kDa size can be easily used for the development of Dot-ELISA diagnostic assay for human echinococcosis.

Figure 2. Development of Dot-ELISA Through Various Antigenic Peptides. A represent the brown/black dot development in 10-12kDa Peptide, B represent the brown/black dot development in 22-24kDa Peptide, C for 38-40kDa, D for 70-75kDa and E for 170-173kDa.



Sensitivity and Specificity of Dot-ELISA

According to the obtained results of brownish-black spot/dot development among 27/28 confirmed CE serum the sensitivity of the test was found as 96.4% while the absence of dot development among the remaining 71/72 confirmed non-hydatidosis/normal group blood confirmed its specificity of 98.6% as shown in the Table 1.

Table 1. Efficacy Evaluation of Developed Diagnostic Assay

S. No	Evaluation of Diagnostic criteria	Hydated Cyst Fluid antigen 24Kda
1	Diagnostic Efficiency	98% (Excellent)
2	Specificity	98.6%
3	Sensitivity	96.4%
4	Negative predictive Value (NPV)	98.6%
5	Positive predictive value (PPV)	96.4%

Comparison of Dot-ELISA Results with Commercial ELISA

A total of 24/28 (85%) of the confirmed patients showed the presence of anti-echinococcus IgG in the serum of the sample however, false positive results among 02/72 (2.8%) were observed. The comparison of both commercial ELISA Kit and our developed Dot-ELISA Kit is given in the table 2.

Table 2. Comparison among Commercially Available ELISA and Our Developed Dot-ELISA Kit

Dot-ELISA of the Current study			**Commercial ELISA**	
Diagnostic Efficiency	98% (Excellent)		Diagnostic Efficiency	94%
Specificity	98.6%		Specificity	97.2%
Sensitivity	96.4%	**Vs**	Sensitivity	85.7%
Negative predictive Value (NPV)	98.6%		Negative predictive Value (NPV)	94.6%
Positive predictive value (PPV)	96.4%		Positive predictive value (PPV)	92.3%

Discussion

Cystic echinococcosis (CE) is a global neglected tropical disease infecting a huge amount of livestock and human population. CE is caused by the larval stage of helminth parasite *Echinococcus granulosus* sensu lato (s.l) species, responsible for huge public health and financial loses (Khan et al., 2021; Basharat et al., 2022; Thompson et al., 1986; Budke et al., 2006; Cardona et al., 2013; Nakao et al., 2013; Alvarez Rojas et al.,2014). The diagnosis of CE in patient is based on the cyst structure investigation by computer tomography, ultrasound technique, X-ray examination, immunodiagnostic test for detection of specific antibodies and slide agglutination test (Craig, 1997; Gottstein, 2000; Grimm *et al*., 1998; Kern *et al*., 2001; Pawlowski *et al*., 2001;Teggi *et al*., 2002).

The hydatid cyst fluid (HCF) of *Echinococcus granulosus* contains multiple recombinant antigens (Liang *et al*., 2020) which can be used for the development of diagnostic assay for echinococcosis. The Antigen B (AgB) is one of the most abundant and major immunogenic antigen which can be used for the diagnosis of human CE. AgB is highly used in serodiagnosis and it gives three bands on SDS-page the smaller subunits of 8kDa are mostly used in diagnosis (Arend *et al*., 2004; Folle *et al*., 2017). Antigen 5 is also used for the diagnosis of CE but little information is known (Pan *et al*., 2017). Sreo-diagnosis of Echinococcosis with the help of antigenic peptides were already performed by many researcher (Shepherd *et al*., 1987; Manzano-Román *et al*., 2015; Barbieri *et al*., 1998; Nova *et al*., 2008). The current study was designed to identify the various antigenic peptides of HCF through SDS-PAGE and Western Blotting technique and used for the development of immunoblot assay for the diagnosis of human echinococcosis infection.

The current study observed five (05) various antigenic peptides of 10-12kDa, 22-24-kDa, 34-38kDa, 70-75kDa and 170-175kDa band size on SDS-PAGE analysis justify that antigen B are the most abundant lipoprotein in hydatid cyst (Leggate and Mcmanus,1994). Various studies reports are in accordance as five bands of antigens having a size of 16 to 17kDa, 55kDa, 16kDa and 24kDa (Rajabiyoun *et al*., 2006; Maddison *et al*., 1989; Burgu *et al*., 2004). Other studies reported three bands of 8kDa, 16kDa and 22-24kDa in HCF sample by (Al-Olayan *et al*., 2012), and 4 subunit of antigenic peptide 8kDa, 12kDa, 16kDa and 24kDa (Lightowlers *et al*.,1989) through SDS–PAGE electrophoresis. Likewise Amelio *et al*., reported the 4 antigenic peptides of different molecular weight ranging from 13 to 32kDa (Amelio *et al*., 1985) and 4 types of the antigens in HCF i.e. 18kDa , 23kDa ,20kDa and 30.4kDa by (Hassanain *et al*.,2016). In contrast to the finding of the current study, numerous protein bands of greater size like 12kDa, 16kDa, 20kDa, 38kDa and 68kDa (Shepherd *et al* ., 1987) while antigen 5 showing two bands on SDS- PAGE having a size of 56kDa and 66kDa (Di Felice *et al*., 1986). Various 4 antigenic bands of greater size of 60kDa, 50kDa, 49.5kDa and 51.5kDa (Shaapan and Khalil, 2016) while 63kDa, 59kDa and 123kDa by (Latif *et al*., 2013) reported. The variation in the results might be due to the difference in handling and preparation of chemical solution, quantity, quality and preparation of antigenic solution (Burgu *et al*., 2000). In addition the quality and nature of antigens are different among the different host species, cyst status cyst location and parasite strain . This is one of the reason that different laboratories obtained different results of antigens (Poretti *et al*., 1999; Lightowlers *et al*., 1989).

There is a need for the development of new diagnostic method for echinococcosis (Latif at el., 2013). Dot-ELISA is One of the best, simple and rapid diagnostic technique intend high potential for the detection of serum hydatid antigen and needs minute volume of reagents. The current study, used 22-24kDa band of antigen B showing high efficacy of 98%, with sensitivity of 96.4% and specificity of 98.6% similarly the use of Antigen B as 100% specific and 80% sensitive for the diagnosis of echinococcosis is reported by (Immunodiagnosis, 2005). Other studies used various antigens with different sensitivity and specificity like urinary hydatid antigen through Dot-ELISA for the diagnosis of cystic Echiococcosis with a sensitivity of 53.33% reported by (Swarna & Parija 2012). In contrast to our study, several scientists used the small 8/12kDa subunits of AgB and observed a cross-reaction with serum antibodies of alveolar echinococcosis patients of *Echinococcus multilocularis* etiology and cysticercosis caused by *Taenia solium* (Leggatt et al., 1992; Poretti et al., 1999; Lightowlers et al., 1989). The current developed dot-ELISA can be justified as alveolar echinococcus caused by *E multilocularis* is highly prevalent in northern hemisphere while cysticercosis of *T. solium* etiology is not wide-spread in Islamic countries (Craig et al., 1995). Therefore, the current immunoblot assay based on 22-24kDa of AgB, will be significantly used for the confirmatory serodiagnosis of CHD in non-endemic area of *E. multilocularis* and *T. solium* (Immunodiagnosis, 2005). However the difference is due to the specificity and sensitivity of the different diagnostic test (Kagan et a!, 1966; Kagan, 1968).

Conclusion

The current study concluded that the hydated cyst of echinococcosis containing a lot of excretory and secretory antigenic peptides containing EgAgB which can be separated in different band size upon SDS-PAGE analysis. The peptide antigen of 22-24kDa can be significantly used for Dot-ELISA development which can be better and accurate option for human CE diagnosis. Th current developed dot-ELISA will play a significant role in the diagnosis of echinococcosis in developing countries to measure the exact burden of the disease and develop control strategies.

Conflicts of Interest:

"The authors declare no conflict of interest."

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