

Comparative Diagnostic Evaluation of Crude and Isolated Fractions of *Echinococcus granulosus* in Dogs

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Abstract: The current study was designed to comparatively assess the diagnostic efficacy of crude somatic worm antigen (SWA), hydatid cyst fluid antigen (HCFA), protoscoleces antigen (S-A), specific and cross-reactive fractions in experimentally infected dogs with *Echinococcus granulosus* (*E. granulosus*) using ELISA. The best diagnostic performance was obtained with cross-reactive fraction (FIII) isolated by affinity column chromatography and its crude extract (SWA). Both antigens proved to exhibit good diagnostic parameters of natural echinococcosis in dogs although a variable range of potency was observed. The electrophoretic profiles of potent antigens revealed multiple components in SWA of molecular weight 103, 88.3, 81, 63.8, 54, 44, 38, 32, 18.7, 16.6 and 14.8KDa. While FIII was resolved into 5 bands of molecular weight 81, 64, 44, 18.7 and 14.7KDa. The immuno reactive bands recognized in SWA by dog naturally infected serum were 103, 88.3, 81, 64, 54, 44 and 16.6KDa using immunoblot. While only three bands of 81, 64 and 44KDa were immunologically responsible for diagnostic potentials in FIII. In conclusion, the current study added new diagnostic fraction of echinococcosis in dogs commonly exists in mature worm and cyst fluid and consists of only three bands (81, 64 and 44KDa).

Key words: Common antigens • Diagnosis • Echinococcosis • Affinity Column Chromatography • ELISA

INTRODUCTION

Echinococcosis caused by cestodes of the genus *Echinococcus* is one of the major zoonotic helminthiosis, causing considerable socio-economic consequences in endemic areas. Due to its world-wide distribution and its important impact in both human and animal health, *E. granulosus* is considered the most relevant species [1]. In Egypt, echinococcosis *granulosus*/hydatidosis is an endemic zoonotic disease [2-4].

The adult worm lives in the small intestine of dogs and other canids, in intimate contact with the intestinal epithelium. The intermediate larval stage (metacestode) can grow in a wide range of mammal species including humans that acquire infection through accidental ingestion of eggs. Currently, diagnosis of hydatidosis/echinococcosis is based on a combination of imaging techniques (ultrasonography, computerized axial tomography, X-rays) and immunodiagnostic methods such as ELISA and immunoblotting [5].

For immunodiagnosis of human hydatidosis and dog echinococcosis, hydatid cyst fluid (HCF), somatic antigens (S-Ag) and excretory-secretory products (ES-Ag) of *E. granulosus* protoscoleces and adult worms are

used as main antigenic sources. The choice of the most appropriate antigenic extract depends on the developmental stage of the worm and the host species. HCF is mainly used for the immunodiagnosis of human cystic echinococcosis [6-8]. The subunit of 8/12 kDa from antigen B is considered the most specific component of HCF in the genus *Echinococcus* [5]. Moreover, con A purified hydatid fluid fraction was successfully utilized in diagnosis of echinococcosis *granulosus* using ELISA [9]. S-Ag from protoscoleces has been used for the serodiagnosis of dog echinococcosis [10], as protoscoleces are the infective stage of the parasite in the definitive host. In the last few years, ES-Ag from protoscoleces has become the main antigenic source for the immunodiagnosis of dog echinococcosis, based on the detection of parasite antigens in fecal samples (coproantigens) by ELISA [11, 12]. While, little attempts were performed regarding diagnostic value of worm antigen.

Cross-reaction between *E. granulosus* and other helminthes [13, 14], among different *Echinococcus* species [15] and among different developmental stages of *E. granulosus* [16] was previously reviewed. Searching for the potent diagnostic antigen(s), the current study aimed

to focus on worm antigen which did not previously receive enough investigations of its diagnostic activities compared with hydatid cyst fluid and protoscoleces antigens. Also, to compare the diagnostic potentials of commonly existed antigens among developmental stages of *E. granulosus* and the exclusive one in experimentally infected dogs.

MATERIALS AND METHODS

Hydatid Cyst Fluid Antigen: HCFA was prepared from HCF obtained from cysts of camel origin as described by Varela-Díaz *et al.* [17]. Briefly, HCF was centrifuged at $2000 \times g$ for 45 min, then passed through a Millipore AP20 filter (Bedford, US) and dialyzed against distilled water, using dialysis tubing with a cut-off of 5 kDa (Medicell, London, UK). Finally, HCF was centrifuged at $6500 \times g$ for 30 min, lyophilized and stored at 4 °C.

Protoscoleces Antigen: S-Ag was prepared from protoscoleces as described by Carmena *et al.* [16]. Protoscoleces were obtained by aseptic puncture from fertile hydatid cysts of camel origin, washed with phosphate-buffered saline (PBS), freeze-dried and thawed several times and sonicated (10 cycles of 12 s at 60 Hz frequency) in PBS containing 2mM PMSF. Protoscoleces were freeze-thawed once more and centrifuged for 30 min at 20000 rpm. Supernatant was aliquotted and stored at -20°C.

Somatic Mature Worm Antigen: SWA was prepared by following the procedure of Elayoubi and Craigs [18] with slight modifications. The worms were collected from the small intestine of dogs and washed several times and homogenized in 0.15 M PBS pH 7.2 containing 2mM PMSF. The homogenate was repeatedly frozen and thawed and centrifuged at 20000 rpm for 30 min in cooling centrifuge. The supernatant was collected and used as antigen.

Protein Content Determination: The protein contents of the prepared antigens were determined by the method of Lowry *et al.* [19].

Hyperimmune Rabbit Serum: Antiserum against *E. granulosus* hydatid cyst fluid antigen was raised in rabbits immunized subcutaneously with 40µg/kg in Freund's complete adjuvant. A booster dose of antigen in Freund's incomplete adjuvant was injected on day 14. Second and third booster doses were given on day 21 and 28, respectively and blood samples were collected 4 days

post last injection [20]. Rabbit antiserum was aliquotted and stored at -20°C until use.

Experimental Infection of Dogs with *E. granulosus*: Five puppies were treated with piperazine citrate and praziquantel to prove their freedom from any parasites. The dogs were fed with dried food, milk and supplied with water *ad libitum*. Each animal was orally inoculated with 10.000 viable protoscoleces of hydatid cysts of camel origin for 2 successive days [21]. Three puppies were used as a control group. The infected animals were bled 60 days post infection for serum samples collection.

Antibody-Sepharose 4B Affinity Chromatography: The prepared hyperimmune serum against hydatid cyst fluid antigen was dialyzed against 100mM NaHCO₃ buffer pH8.3 containing 500mM NaCl and 0.02% NaN₃ and then coupled to CNBr-activated sepharose-4B by strictly following the manufacture instructions. HCFA, S-A and SWA were separately applied to the column and bound fractions were eluted using 50mM glycine containing 500mM NaCl.

ELISA: The diagnostic value of the crude antigens and isolated fractions was monitored by ELISA in experimental echinococcosis in dogs. The most potent antigens were adopted to detect the infection using ELISA in randomly collected dog serum samples. ELISA was performed according to Santiago and Hillyer [22] with little modifications. Antigens concentrations, sera and conjugate dilutions were determined by checkerboard titration. The cut off of optical density values was determined by the method of Allan *et al.* [23].

SDS-PAGE: SDS-PAGE was performed in polyacrylamide gels according to Laemmli [24]. SWA and its cross-reactive fraction with HCFA were separately mixed with sample buffer containing 2-mercaptoethanol before loading to the gel. After separation, gel was fixed in 50% methanol and stained with silver stain according to Wray *et al.* [25]. Relative molecular weights of bands were calculated using molecular weight marker.

Immunoblot: Proteins of SWA and its cross-reactive fraction with HCFA were fractionated by SDS-PAGE under reducing conditions according to Laemmli [24] and transferred to nitrocellulose membrane according to Towbin *et al.* [26]. After washing and blocking, membranes were incubated with naturally infected dog antiserum, examined by ELISA, diluted 1:500. Horse radish peroxidase-conjugated anti-dog IgG was used at 1:1000 dilution. Membranes were revealed by adding 4-chloro-1-naphthol solution.

RESULTS

The Purification process resulted in isolation of a single specific hydatid cyst fluid fraction (FI) and two cross-reactive fractions of S-A (FII) and SWA (FIII) with HCFA of *E. granulosus*.

The diagnostic potency of crude antigens and isolated fractions was evaluated by ELISA. SWA (Fig. 1) and FIII (Fig. 2) showed higher potency in the diagnosis of experimental echinococcosis in dogs than other prepared antigens. FIII showed more potency in the diagnosis of natural echinococcosis in dogs (86%) (Fig. 4) than SWA (67%) (Fig. 3). Based on ELISA results proving the diagnostic advantage of SWA and FIII, both

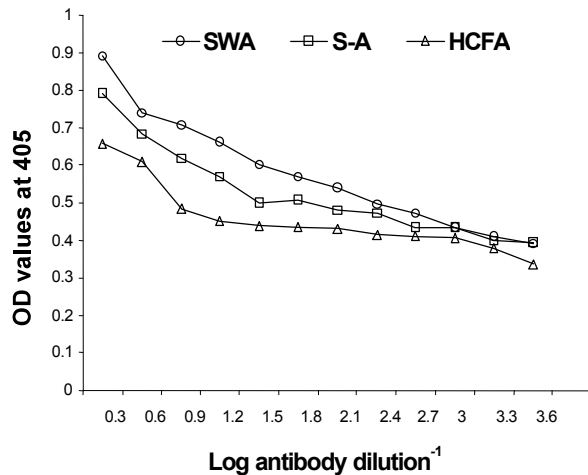


Fig. 1: Comparative diagnostic potentials of SWA, S-A and HCFA in dogs experimentally infected with *E. granulosus*

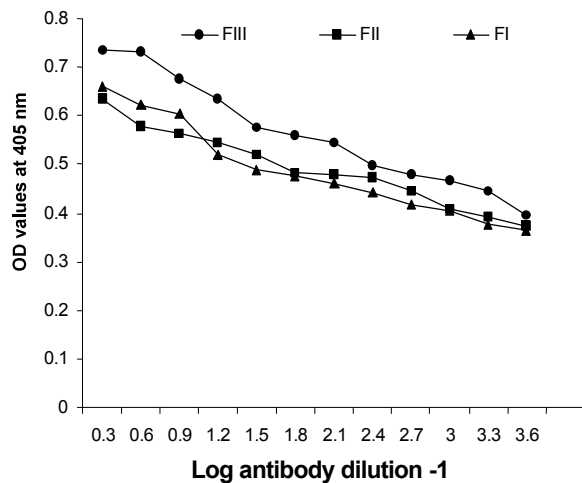


Fig. 2: Comparative diagnostic potentials of FI, FII and FIII in dogs experimentally infected with *E. granulosus*

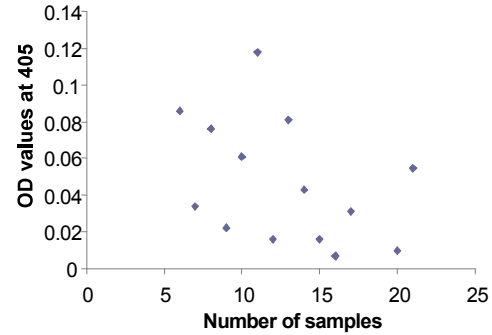


Fig. 3: Diagnostic potency of SWA in randomly collected dog sera

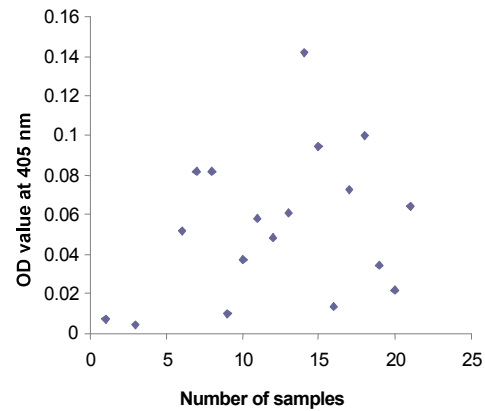


Fig. 4: Diagnostic potency of FIII in randomly collected dog sera

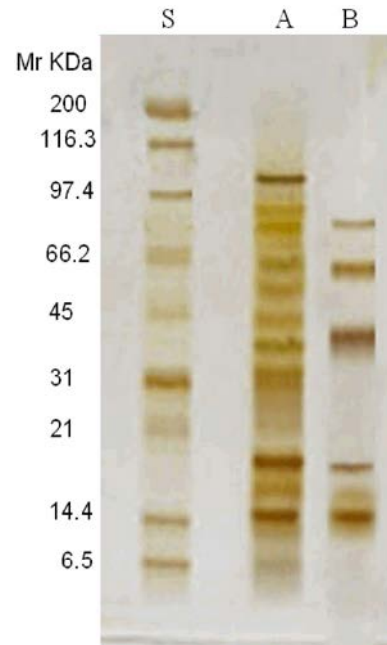


Fig. 5: Electrophoretic profile of *E. granulosus* SWA; Lane A, FIII; Lane B and molecular weight standards; Lane S

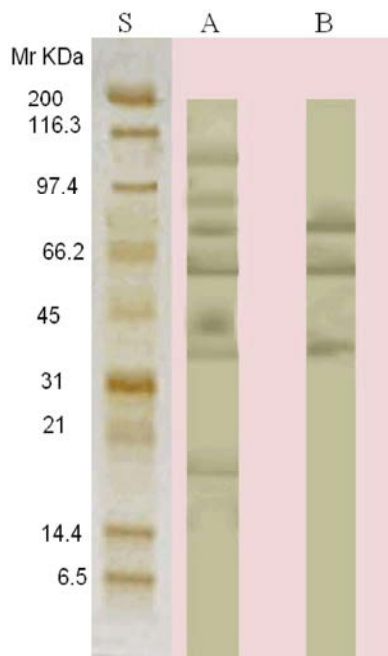


Fig. 6: Immunoreactive bands recognized by naturally infected dog sera in SWA; Lane A, FIII; Lane B and molecular weight standards; Lane S.

were characterized by SDS-PAGE and the electrophoretic profile of SWA revealed multiple components of molecular weight 103, 88.3, 81, 63.8, 54, 44, 38, 32, 18.7, 16.6 and 14.8KDa (Fig. 5 Lane A). While FIII was resolved into 5 bands of molecular weight 81, 64, 44 18.7 and 14.7KDa (Fig. 5 Lane B).

Searching for the components responsible for immunological reactions in both antigens, immunoblot was adopted. The immunoreactive bands recognized in SWA by dog naturally infected sera were 103, 88.3, 81, 63.8, 54, 44 and 16.6KDa (Fig. 6 Lane A). While the identified immunodiagnostic bands in FIII were 81, 64 and 44KDa (Fig. 6 Lane B).

DISCUSSION

For accurate serological diagnosis and for support of clinical diagnosis of echinococcosis, the selection of a particular immunodiagnostic antigen(s) is essential. In the current study, SWA proved higher diagnostic value than HCFA and S-A, although both were previously known to possess potent diagnostic potentials [8, 10]. So far, little information is available in regard to adoption of worm antigen in the diagnosis of dog echinococcosis. Consequently the main concern of the present study was to investigate its diagnostic activities compared with other antigens.

With special attention paid to the commonly existed components and to the potent diagnostic potentials of the three antigens, the current study proved that the commonly existed bands in both SWA and HCFA (FIII) showed higher potentials than specific fraction of HCFA. Previously, canine antibodies evidenced lesser avidity for their specific antigens than antibodies from human origin [16]. This observation explained the advantage of cross-reactive antigen, observed in the current study, than specific one in the diagnosis of dog echinococcosis. This phenomenon may be explained by the concept of antibody affinity maturation through the course of species evolution. It is known that less evolved species have antibodies with lower affinity for their specific epitopes than higher evolved species [27, 28]. FIII also showed higher diagnostic values of echinococcosis than cross-reactive fraction between HCFA and S-A which supported the diagnostic significance of SWA which was previously proved by Javare Gowda *et al.* [29] who successfully utilized the antigen in detection of *E.granulosus* specific antibodies in dogs using indirect ELISA. However, until now little studies have been performed to determine the homology degree among the antigenic components of HCFA, S-A and SWA and to find out their non-shared and shared proteins. In the current research, the shared antigens between SWA and HCFA which proved potent diagnostic value included three bands of 81KDa, 64KDa and 44KDa. Carmena *et al.* [16] identified the major antigenic components shared by HCF, S-Ag and excretory secretory antigen of protoscoleces with apparent molecular masses of 4-6, 20-24, 52, 80 and 100-104 kDa, including doublets of 41/45, 54/57 and 65/68 kDa. But, unfortunately, they did not examine its diagnostic potentials.

Collectively, the current study proved that SWA, which did not extensively studied previously, possesses higher diagnostic potentials than HCFA and S-A in experimentally infected dogs with echinococcosis. Moreover, three of its bands that also exist in HCFA proved the highest potentials than the crude antigen and the specific fraction of HCFA. So, the current study introduces new antigen for diagnosis of echinococcosis in dogs consists of three bands with molecular weight of 81, 64 and 44KDa. More attention must be paid to the shared antigens between different developmental stages of the parasite that probably possess diagnostic value of echinococcosis at any phase of infection.

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