Development of a Dipstick Dye Immunoassay for Diagnosing Hydatidosis

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We have developed a kit to diagnose hydatidosis, based on the detection of specific antibodies. This disease, caused by larvae of the cestode *Echinococcus granulosus*, is a zoonosis commonly found throughout the world. The diagnostic kit discriminates between positive and negative cases in a way that is easily interpreted, i.e., positive cases form a colored band on an inert substrate. The results

show test sensitivity to be some 94.87% and specificity 85.71%, which is comparable to those of the ELISA technique. The high sensitivity and specificity of this test affords the great advantage of speed in diagnosing this parasitosis. No other equipment or procedure is required. J. Clin. Lab. Anal. 17:219–222, 2003. © 2003 Wiley-Liss, Inc.

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INTRODUCTION

Hydatidosis, a zoonosis distributed throughout the world and particularly widespread in agricultural areas, is caused by *Echinococcus granulosus*. This is a small cestode of which the adult phase develops in the duodenum and jejunum of the canine species. In its biological cycle, the eggs of the parasite are expelled with the feces of the definitive host. When this is ingested by omnivorous animals, it gives rise to hexacanth larvae that perforate the intestinal wall and invade the different organs, developing into metacystodes or hydatids.

Hydatidosis causes considerable damage both to livestock and to humans. The highest levels of parasitism appear in areas that are devoted to raising livestock, such as Australia, Argentina, Chile, Brazil, and Uruguay, or where socioeconomic conditions are low, as in the Middle East or the Mediterranean basin.

Depending upon where it resides in the body, hydatidosis manifests itself in humans in diverse ways both in terms of pathology and symptoms (1). In 60% of cases, it is responsible for a cyst in the liver, a cyst in the lung (10%), and a cyst in the rest of the organs (30%).

Serological tests are useful for diagnosing hydatid disease caused by *E. granulosus* in humans since no parasitological diagnosis is possible without using invasive procedures, and the clinical signs of the disease

are nonspecific. The presence of specific antibody titers in patients with cystic hydatid disease has been assayed by various techniques, such as indirect hemagglutination or latex agglutination, immunoelectrophoresis, complement fixation, indirect fluorescence antibody tests, and immunoenzymatic ELISA (2–6).

We describe here a diagnostic method known as the dipstick dye immunoassay (7) and compare it with the immunoenzymatic ELISA system. The simplicity and speed of the dipstick dye immunoassay can be appreciated. Its simplicity also renders it particularly useful for application in the field or in any situation where a quick diagnosis of parasitism by the *E. granulosus* larva is required.

MATERIALS AND METHODS

Parasitic Material

Hydatid fluid (HF) was taken from sheep-liver cysts in the province of Zaragoza in Spain. The supernatant

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was extracted after centrifugation at 4,000 g for 30 min and 0.1 mg/L of NaN_3 was added together with 5 mM of ethylenediaminetetraacetic acid (EDTA) to prevent protease activity. This was divided into aliquots of 20 mL and frozen at -20°C until use.

Antigen Preparation

The HF preparation enriched in B antigens was obtained as described by Sbihi et al. (2), based on the procedure described by Williams et al. (8), providing a fraction enriched with the subunits of the main 5/B antigens (2,3,9). After concentration by dialysis against polyethylene glycol (PEG 20,000), the HF was dialyzed once more with an acetate buffer (5 mM) at pH 5.0 for 12 hr and then centrifuged at 48,000 g for 30 min. The precipitate was dissolved in 0.2 M phosphate buffer at pH 8.0, boiled for 15 min and centrifuged at 48,000 g for 60 min. The pellet was discarded and the supernatant passed through a G-Sepharose protein column (Pharmacia LKB, Uppsala, Sweden) to remove any immunoglobulin G (IgG) contaminant from the host. Finally, dialysis was performed once more with a phosphate saline buffer (0.15 M PBS at pH 7.4).

The concentration of protein in the antigen preparation was determined by a micro-Lowry assay (10).

Sera

Fifty-six positive serum samples were obtained from the Microbiology Department of the University of Valladolid in Spain and came from patients with surgically confirmed hydatidosis with different cyst locations: liver-lung, liver, lung, transient liver-lung, mediastinic, and others. Twenty of the patients with hydatidosis were from Chile; nine with schistosomiasis were from Egypt; four with cysticercosis were from Panama; four with toxoplasmosis were from Spain; four with leishmaniasis were from Spain; and 15 were used as controls and came from the blood bank belonging to the Hospital Virgen de las Nieves, Granada, Spain. The controls showed no abnormality in the medical examination and registered negative to hydatidosis according to ELISA (4).

All the serum samples were divided into aliquots and kept at -70°C until studied.

Dipstick Preparation

The dipstick is a nitrocellulose-membrane strip 4–5 cm long by 0.7 cm wide (Schleicher & Schuell, Dassell, Germany): AE cellulose nitrate membrane 99 (8 μm) with a binding capacity of more than 20 $\mu\text{g}/\text{cm}^2$ and porosity of 8 μm , allowing the uniform movement of particles by a simple lateral flow of 1.5–2 cm/min. A

trap made of 1.5 mg/mL purified antigen solution is applied in a horizontal band across the center of the membrane. Once the antigen is dry, the membrane is submerged for 12 hr at 4°C in blocking solution containing 0.3% polyoxyethylensorbitan monolaurate (Tween 20), 1% powdered milk, and 0.4% gelatine. After the support membrane is blocked, it is dried at room temperature. A fiberglass membrane is attached to one end of the nitrocellulose strip (Borosilicate Glass Fibre Filter; Schleicher & Schuell) and 20 μL of a suspension of colloidal-gold particles between 10 and 20 nm in diameter and conjugated to protein A are applied to the other end as described below. This suspension is allowed to dry on the fiberglass filter until used. On the opposite end of the strip, "Absorbent Pad" is attached—a highly absorbent paper which intensifies the lateral flow by dragging the particles over the analytical membrane (Cotton Linter Papers-900; Schleicher & Schuell).

Preparation of the Metal Conjugate

Colloidal gold, made up of AuCl_3 particles, is hydrophobic and kept stable by electrostatic repulsion. The addition of an electrolyte such as NaCl causes the particles to flocculate and form aggregates, which make the colloid turn from red to blue. This can be avoided by stabilizing the colloid by the absorption of hydrophobic substances. Prior to its sensitization, it is submitted to a process of reduction with 1% trisodium citrate in an aqueous solution. After adjusting the pH to 6.0, the quantity of bioselective reagent is calculated that is needed to stabilize the colloid, in this case protein A (Sigma) (11).

The particles are centrifuged at 60,000 g for 1 hr at 4°C , leading to the formation of a red precipitate at the bottom of the tube made up of a complex of protein and colloidal gold. At the same time, the nonstabilized particles adhere to the tube walls as a darker precipitate. The pellet is resuspended in a 25-times smaller volume with a solution composed of PBS 0.15M and 20% glycerol, with the addition of 0.2 mg/mL of polyethyleneglycol-20,000 and 20 mM of sodium azide. This suspension remains stable for several months at 4°C .

Development of the Method

The biological sample (serum in this case) is applied to the end with the colloidal-gold particles bound to protein A. The quantity of sample applied is not crucial, although at least 20 μL is necessary to soak the fiberglass strip and keep it wet for 2 or 3 min. If not enough sample is applied, 30 μL of 0.15M PBS solution at pH 7.4 can be added afterwards. When the blood samples have been treated with anticoagulant, hydrogen

peroxide (110 volumes) at 75% should always be added to this solution.

Reliability and Specificity of the System

To judge the reliability and specificity of the kit, we tested the serum from patients whose infection with *E. granulosus* had been surgically confirmed, from patients with other parasitoses, and from healthy donors. To do this, we used the ELISA immunoenzymatic test, fixing it to the microtiter plates with the same antigen as that absorbed by the nitrocellulose strip as described previously by Sbihi et al. (4)

ELISA

We used ELISA to follow the recognition of the hydatid antigen by human serum according to Sbihi et al. (2). The antigen was prepared as described above and 20 µg/mL coated onto polystyrene microtiter plates (Nunc, Roskilde, Denmark). The antibodies retained were developed by peroxidase-conjugated goat anti-human total antibodies and O-phenylenediamine (OPD) (Sigma Immunochemical, St. Louis, MO) and 10 µL of 30% H₂O₂ per 25 mL. The reaction was measured at 495 nm after 20 min in an ELISA reader (METERTech Σ960, Torrance, CA). Mean and standard deviations (SD) of the optical-density (OD) values for the control plasma were used to establish a cutoff value—cutoff = X mean of OD + 3x (SD). OD values higher than the cutoff value were considered to be positive.

RESULTS

The sensitivity of the kit was 94.87% and specificity was 85.71%. These results coincide with those found with ELISA. Of the four serum samples for cisticercosis, only one gave a positive reading, both with ELISA and the dipstick method. In this case the optical density with ELISA was 0.2 and the cutoff value with the dipstick was 0.19. In the ELISA method, the highest

value was 0.9 with a mean OD value of 0.49 for the positive hydatidosis sera. Of the nine samples with schistosomiasis, eight were negative according to both ELISA and the dipstick. The four *Toxoplasma* sera all showed negative, while one of the four *Leishmania* sera was positive to hydatidosis both with the kit and ELISA with a percentage similar to that found with the cisticercosis sera.

In Fig. 1, we showed the image of the positive and negative results with the diagnostic system. The comparative ELISA results for serum samples against an antigen B fraction and the dipstick results are shown in Fig. 2.

DISCUSSION

We describe here the high sensitivity of a quick diagnostic method (3–5 min), which is easy to use and requires no equipment other than the kit described here. In addition, this kit can use sera, blood, or other biological fluid in which IgG can be found as well as any secretion in which, together with IgA, IgG can be found perhaps as a consequence of mucous irritations. The specificity shown is also very high and the possibility of crossed reactions described between *E. granulosus* and the other infectious agents are well known and determine the specificity of the serological tests. Crossed reactions have been reported for infections by: *Echinococcus multilocularis*, *Echinococcus vogeli* (12); *Taenia saginata* (1,13–15); *Tenia solium* (16–18); *Hymenolepis nana* (18); *Ascaris lumbricoides*, *Trichinella*, *Fasciola* (16).

Although we used antigen, we found only a very low number of false positives: one out of four for cisticercosis; one out of four for leishmaniasis; and one out of nine for schistosomiasis. This system can be useful for the diagnosis of hydatidosis both in epidemiological campaigns as well as in situations with a scarcity of equipment or where a quick diagnosis of this parasitosis is needed.

Fig. 1. Dipstick analyses of the positive and negative sera. The positive serum shows a central colored band. In the negative serum, the band appears at the end of the development of the stick.

Fig. 2. Standard ELISA results for laboratory serum samples against a semipurified fraction of antigen B. Positive/negative cutoff value is shown as a horizontal line. Sera numbers 1-58 from Valladolid, Spain (A); 59-78 sera from Chile (B); 79-87 sera of schistosomiasis from Egypt (C); 88-91 sera of cysticercosis (D); 92-95 sera of toxoplasmosis from Spain (E); 96-99 leishmaniasis sera from Spain (F), and 100-114 negative control from clinically normal people residing in Spain (G). Filled square, positive in the dipstick assay.

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