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Research Note

Diagnostics of *Echinococcus granulosus* particles in hepatic cysts punctate of seropositive patients

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Summary

Diagnosis of liver hydatid cyst disease was established by ultrasound and CT examination in 76 patients and confirmed with positive IF and ELISA tests. We found them elective for percutaneous punction after albendazole treatment. Obtained specimens were examined for presence of echinococcal particles and 48 were positive (63.16 %) and it reflects the specifity of applied serological tests. Development of more specific serological tests combined with existent radiological methods will contribute to more accurate diagnostic approach but analysis of cyst specimens will remain the cornerstone of exact diagnosis.

Keywords: hydatid disease; immunodiagnostic tests; albendazole; PAIR method; motility of protoscolices

Introduction

Unilocular echinococcosis or hydatid disease is a zoonosis caused by a dog tapeworm *Echinoccocus granulosus*. In human medicine, infection with three species of tapeworm presents a significant health problem: *E. granulosus,* with dogs and some other canids as definitive hosts and humans, sheep, goats, cattle, pigs and horses as intermediate hosts and carriers of larval stage (Markell *et al*., 2000). Other form is alveolar echinococcosis caused by *E. multilocularis,* with foxes, dogs and catsas definive hosts, small rodents as intermediate hosts and humans as intermediate (accidental) hosts (Dražilová *et al*., 2012) These two forms are of special importance due to wide distribution and serious medical and economic impact. Third form, polycystic echinococcosis caused mainly by *E. vogeli,* with bush dogs and dogs as definitive hosts, rodents as intermediate hosts and man and other mammals as accidental hosts is less frequent and restricted to Central and South America (Neva *et al*., 1994.; Garcia, 2007).

E. granulosus is distributed worldwide in temperate and subtropic areas in extensive cattle and sheep breeding areas (Manfredi *et al.*, 2013; Singh *et al.*, 2013). Hydatid disease is usually asymptomatic, clinical manifestations vary due to the size and site of hydatid cysts, allergic skin rashes can occur regardless of the localization. Liver, lungs, kidney and spleen are the most frequent sites of hydatid cysts but no organ is spared, and its pathogenic effect is due not only to mechanical pressure but to antigenic and toxic effect of the parasite as well.

Humans are infected after ingestion of food or water contaminated with embryonated eggs distributed by feces of definitive host, so they become the intermediate or accidental hosts and carriers of hydatid cyst. After ingestion of eggs, oncospheres (hexcanth embryons) are released in human intestine, penetrate the intestinal wall and are carried by blood stream into the liver, eventually lungs and other organs. After nidation, the oncosphere develops into a cyst, which slowly grows and can reach the size of more than 20 cm in diameter. Rupture of a fertile cyst causes dissemination of scolices and new daughter cysts develop in affected organ system.

Diagnosis of hydatid disease is established by imaging methods: radiography, echosonography, computerized tomography (CT), magnetic resonance (MR), by serologic tests: indirect fluorescency of antibodies (IF), immunoenzymatic tests (ELISA), and microscopy of cysts punctate searching for protoscolices and echinococcal particles.

Material and methods

Examination of hydatid cyst punctate was performed at Parasitology Department of University Clinic for Infectious Diseases "Dr. Fran Mihaljević" in Zagreb, serologic diagnostics was done in Laboratory for Human Serologic Diagnostics of Department of Parasitology and Invasive Diseases with Clinic on Faculty of Veterinary Medicine, University of Zagreb.

During the period of nine years (from 2003 to 2011) 76

patients 17 – 69 years of age were examined, 24 male (31.58 %) and 52 female (68.42 %).

In all 76 patients diagnosis of echinococcosis was established after performing standard laboratory tests: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), red blood cell count (RBC), white blood cells count (WBC), platelate (PLT), bilirubine, aspartate-aminotransferase (AST), alanine aminotranspherase (ALT), gamma-glutamyltransferase (GGT), echosonography and serology (positive in all 76).

Serology was performed by indirect fluorescency of antibodies (IF). According to principles described by Ambroise-Thomas (1969). As antigen of own production, cryostatic slices of echinococcal protoscolices were glued to a slide after removing fat with acetone. Diluted sera (1:100) or cerebrospinal fluid (1:10) with phosphate buffer pH 7.2 were incubated at 37 °C for 30 minutes. After rinsing (phosphate buffer pH 7.2) three times for 10 minutes a layer of conjugated sera (antihuman IgG antibodies labelled with fluorescein-isothiocyanate (FITC-Imunološki Zavod, Zagreb) in dilution 1:100 was incubated for 30 minutes at 37 °C. After rinsing, results were read under fluorescent microscope (Zarzosa *et al*., 1999).

Immunoenzymatic tests (ELISA), diagnostic kits were used: Nova Lisa, Echinococcus-IgG ELISA, Nova Tec, Immunodiagnostica Gmbh). Test was performed on microtitration plates or strips with bound antigen. Examined sera and cerebrospinal fluid were diluted 1:100 (Sample Diluent) and stirred. Positive, negative and cut off controls were placed in mictrotitration plate wells and after that the diluted sera. The plate was incubated for 60 minutes at 37 °C. After incubation wells were rinsed with 300 µl Washing solution. Protein A anti IgG conjugate 100 µl was applied to every well and incubated for 30 minutes at room temperature in dark. After rinsing with 100 µl with Washing Solution three times 100 µl of tetramethylbenzidine (TMB) Substrat Solution was applied and incubated for 15 minutes in dark at room temperature. A Stop Solution (100 µl) was then instilled in all wells and results read in 30 minutes on spectrophotometer with 450 nm filter (Zarzosa *et al*., 1999).

Patients were treated with albendazole 15 mg/kg/day for 28 days before percutaneous aspiration of the cyst. Contents of hydatid cysts were obtained after punction under echosonographic visualisation using the PAIR method (punction, aspiration, instillation, reaspiration) and searched for *E. granulosus* particles and vitality of protoscolices. Liquid samples after aspiration were divided in two portions. First part was examined under microscope as native specimen and as sediment after centrifugation. Native specimens were first evaluated for the presence of protoscolices or their fragments to confirm the diagnosis of echinococcosis. Further analysis aimed to estimate the level of protoscolex vitality in positive specimen based on motility, morphological characteristics and percentage of evaginated/invaginated protoscolices. Analysis of protoscolex-rich sediment obtained by centrifugation was deemed non-discriminative since virtually no motility was observed in any of the samples. To re-create conditions resembling those in the canine gastrointestinal system, in second part, 0.05 ml of canine bile or 0.2 % solution of sodium taurocholate was added to 1 ml of cyst fluid for assessing the vitality of protoscolices. Punctates were incubated in thermostate for 48 hours at 37 °C and examined under microscope for presence of echinococcal particles and motility of protoscolices after 24 and 48 hours (Sadjjadi *et al.*, 2009).

Results

After the examination of 76 hydatid cyst contents obtained with punction, echinococcal particles or protoscolices were present in 48 (63.16 %) specimens, gender distribution was 27 (56.25 %) in female and 21 (43.75 %) in male patients. We found no trace of echinococcal particles in 28 (36.84 %) cyst specimens.

All 48 positive specimens were examined for vitality of protoscolices. Three outcomes were assessed for vitality of protoscolices: a) presence of any protoscolex showing motility b) proportion of observed protoscolices showing morphological signs of destruction (complete disintegration and free hooks, destruction of tegmentum, loss off rostellar hook morphology and loss of hooks) c) percent of evaginated and invaginated protoscolices.

In 18 (37.50 %) signs of vitality (motility, higher percentage of evaginated protoscolices) were present after 24 hours incubation with canine bile or 0.2 % solution of sodium taurocholate. In 30 specimens (62.50 %) no signs of vitality were present after 24 hours incubation with canine bile or 0.2 % solution of sodium taurocholate. In all specimens (100 %) protoscolices were immobile before incubation with canine bile or 0.2 % solution of sodium taurocholate.

Table 1. Review of analysed hydatid cyst specimens – period from 2003-2011

Year	Total	Positive	Negative
2003	5	3 (60%)	2 (40%)
2004	10	4 (40%)	6 (60%)
2005	5	5 (100%)	0 (0%)
2006	3	1 (33,33%)	2 (66,66%)
2007	11	9 (81,81%)	2 (18,18%)
2008	16	10 (62,5%)	6 (37,5%)
2009	11	5 (45,45%)	6 (54,54%)
2010	9	7 (77,77%)	2 (22,22%)
2011	6	4 (66,66%)	2 (33,33%)
total	**76**	**48 (63,16%)**	**28 (36,84%)**

Discussion

Throughout the period of nine years 76 patients (52 female and 24 male) with echosonographic and CT signs indicating hepatic hydatid cysts were evaluated at University Clinic for Infectious Diseases "Dr. Fran Mihaljević" in Zagreb. All patients were seropositive to *E. granulosus* after IF and ELISA testing and treated with albendazole 15 mg/kg/day divided in three doses during 28 days. At the end of the albendazole treatment they underwent diagnostic and therapeutic puncture of the cyst using the PAIR method under echosonographic surveillance. Specimens of cyst contents were analysed for presence of protoscolices or *E. granulosus* particles.

The presence of echinococcal particles was established in 46 of 76 cyst specimens (63.16 %) and 28 specimens were negative (36.84 %). All patients (100 %) were seropositive so we concluded that in 28 patients (36.84 %), cysts although considered as hydatid were either sterile hydatid cysts without protoscolices or cysts of other origin. This responds to results in other similar publications (Somily *et al.*, 2005).

All positive cyst specimens were tested for vitality natively, after 24 and 48 hours incubation with canine bile or 0.2 % solution of sodium taurocholate. In 30 specimens (62.50 %) protoscolices were determined as not vital, while in 18 cases (37.50 %) protoscolices showed some signs of viability after 24 hours incubation with canine bile or 0.2 % solution of sodium taurocholate. In all specimens protoscolices were immobile before incubation with canine bile. So, we can make conclusion that the technic of examination of echinoccocal cyst fluid specimen is very important, because native specimens can show immobility, but if we re-create conditions similar to those in the canine gastrointestinal system, some of protoscolices can show signs of vitality, mostly motility and evagination.

As the most clinical cases of echinoccocosis are asymptomatic (over 90 % in our material) and a diagnosis is established applying various imaging techniques such as ultrasonography, CT scanning or pure X-ray examination, the confirmation of primary diagnosis by serological tests remains of paramount importance. Although serological tests such as immunoelectrophoresis, double agar diffusion and indirect hemagglutination are replaced by immunoblotting, enzyme linked immunosorbent assay and indirect immunofluorescent antibody tests the problems remain the same: unsatisfactory performance of the available tests with occurrence of cross-reactions with false positive/negative findings, resulting from difficulties associated with the different sensitivity and specifity of various techniques (Clavel *et al.*, 1999).

PAIR method presents as excellent diagnostic and therapeutic approach to treatment of hydatid disease. It is relatively safe, rapid and is a powerful tool in resolving hydatid cysts in abdominal cavity (Neumayr *et al.*, 2011). However, it has its limits: superficially situated cysts are not suitable for punction because of the spill risk (Yagci *et al.*, 2005). Echinococcal cysts qualified by WHO (World helth organization) International classification of ultrasound images in cystic echinococcosis (CE) as CE4 and CE5 are not suitable for percutaneous puncture because CE types 4 and 5 are inactive cysts which have normally lost their fertility and are degenerated, have thick walls, septation and very dense content (International classification of ultrasound images in cystic echinococcosis for application in clinical and field epidemiological settings, 2003).

Conclusion

Examination of hepatic hydatid cysts contents evacuated after percutaneous punction demonstrate rapid and valid diagnostic tool that enables clinician to establish correct ethiological diagnosis and evaluate results of the various therapeutic algorhytms.

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