Usefulness of PCR Analysis for Diagnosis of Alveolar Echinococcosis with Unusual Localizations: Two Case Studies

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The report presents two cases where diagnosis of alveolar echinococcosis was confirmed by *Echinococcus multilocularis* and *Echinococcus granulosus* PCR. The extrahepatic osseous involvement and the absence of initial hepatic involvement are unusual in both cases. Due to limitations of serological interpretation, PCR was useful to diagnose atypical echinococcosis.

**CASE REPORTS**

**Case 1.** A 48-year-old man from a rural area in Lorraine, France, initially presented with abdominal pain. Abdominal tomodensitometry detected collection involving paravertebral and retroperitoneal areas up to the kidneys. Surgical excision and drainage were required. Three years later, skin fistulization had developed. Disease progression led to destruction of bone in the 12th dorsal vertebra, which was stable for many years. The diagnosis of Pott’s disease was suggested, although all tests for mycobacteria were negative. After 17 years of evolution, the abdominal pain increased significantly. Imaging techniques showed paravertebral collection with multiple spinal thoracolumbar involvement, epidural infiltration with intradural abscess, and hepatic calcifications. Diagnosis of alveolar echinococcosis (AE) was made from the serum and fistula specimens. Despite surgical treatment and parasitostatic medication, the patient was admitted in a medical reanimation unit and died rapidly from an encephalopathy of unknown origin associated with a respiratory failure.

**Case 2.** A 59-year-old woman, also from a rural area in Lorraine, France, presented with dorsal pain. Radiological investigations (tomodensitometry and magnetic resonance imaging) diagnosed a spondylitis localized at vertebrae D10 and D11. The disease developed rapidly to osseous destructions within a few months. Two surgical operations were needed for medullar decompression, and osteosynthesis was performed. Biopsy specimens from the 11th dorsal vertebra were processed for histological examination. Eosinophilic and hyaline membranes heavily stained by periodic acid-Schiff suggested a pellucid membrane of an AE. No scolices or hooklets were observed (data not shown). Once osseous AE was confirmed by PCR of the biopsy sample, a careful screening of the liver was performed. No abnormalities were found in imaging studies of the liver. The disease has progressed slowly, with aggravation of the vertebral lysis despite appropriate albendazole treatment. Table 1 compares clinical features and diagnostic analysis of these two cases of osseous AE.

**Detection of Echinococcus-specific antibodies.** Detection of *Echinococcus*-specific antibodies is routinely based on the use of crude antigens and antigenic fractions (22). An in-house enzyme-linked immunosorbent assay (ELISA) with crude *Echinococcus granulosus* hydatid cyst fluid antigen (HCF) obtained from naturally infected sheep was performed as described previously (1). In addition, an Em2+ ELISA (Bordier Affinity Products, Crissier, Switzerland), using a combination of the recombinant antigen II/3–10 and the purified Em2 antigenic fraction of *Echinococcus multilocularis* (7), was performed in accordance with the manufacturer’s instructions. *Echinococcus* immunoglobulin G (IgG) Western blotting (LDBIO Diagnostics, Lyon, France) was performed as a confirmation technique for the diagnosis of echinococcosis and was carried out as reported previously (12). In case 1, both HCF ELISA and Em2+ ELISA detected a high titer of antibodies in serum. In case 2, HCF ELISA was positive whereas Em2+ ELISA was negative. The Western blotting detected *Echinococcus*-specific IgG in sera of both patients. A distinct antigen recognition pattern for each patient’s serum is used to distinguish both species, as shown in Fig. 1.

**Detection of E. multilocularis and E. granulosus DNA by PCR.** DNA was extracted from drainage material and biopsy specimens by binding to silica gel membranes (QIAamp DNA minikit; QIAGEN SA, Courtaboeuf, France). *E. multilocularis* DNA was detected by a modified PCR described by Dinkel et al. (6) and Stieger et al. (21) with the primer pair EM-H15 (5′-CCATATTACAACAATATTTCATC-3′) and EM-H17 (5′-GTTGAGTGGATTTGTAGGGGAAG-3′). A 200-bp product from the *E. multilocularis* 12S rRNA gene was amplified. PCR amplification was performed within a 50-μl reaction volume by using a hot-start *Taq* DNA polymerase (HotStar Taq; QIAGEN SA). The reaction mixture consisted of 10 μl of DNA template; 5 μl of 10× HotStarTaq PCR buffer (including 1.5 mM [final concentration] MgCl2); 5 μl of dATP, dTTP, dGTP, and dCTP (each at 200 μM [final concentration]); 1 μl
of each primer (1 μM [final concentration]); and 0.25 μl of HotStarTaq DNA polymerase (1.25 U [final amount]). The thermal cycling conditions were as follows: 95°C for 15 min; 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. Amplicons were detected after electrophoresis on a 2% agarose gel by staining the gel with ethidium bromide. Each sample was tested in duplicate. In one assay, an internal control plasmid containing a size-modified *E. multilocularis* target sequence was added to the reaction mixture to detect the presence of PCR inhibitors in samples. Failure to detect the corresponding 371-bp DNA band on gel electrophoresis would indicate the presence of an amplification inhibitor in the DNA template, and consequently the results in the corresponding sample would be considered inconclusive.

**E. granulosus.** DNA was detected by PCR amplification of a 255-bp product of the mitochondrial 12S rRNA gene, with the primers Eg1f (5′-CATTAATGTATTTTGTAAAGTTG-3′) and Eg1r (5′-CACATCATCTTACAATACACC-3′). The conditions of amplification reactions and the corresponding internal control target used were as previously described (20).

For patient 1, a test for *E. multilocularis* performed on material of fistula origin was positive (Fig. 2), whereas *E. granulosus* PCR was negative. For patient 2, a test for *E. multilocularis* carried out on biopsy specimens of vertebrae was positive (Fig. 2) whereas *E. granulosus* PCR was negative.

**Discussion.** Although primary extrapulmonary infection is well recognized in cystic echinococcosis (CE), it is an extremely rare event in AE. Indeed, only a few cases of AE without liver involvement have been reported in the literature (18). Bone manifestation of AE or CE is rare. Osseous localization occurs in only 0.9 to 2.5% of infected CE patients (2), and, to date, only 18 cases of osseous AE have been reported (3, 8, 14).

**FIG. 1.** *Echinococcus* Western blot IgG assays for patients 1 and 2. Most of the significant bands are indicated by arrows. Molecular sizes (in kilodaltons) are indicated. Lane N, negative-control serum; lane P, positive control (bands at 7, 10, 16, 18, and 26 to 28 kDa); lane 1, case 1 (*E. multilocularis* pattern with bands at 7, 12, and 26 to 28 kDa and a narrow band at 18 kDa); lane 2, case 2 (*E. granulosus* pattern with bands at 7 and 26 to 28 kDa and a diffuse band between 16 and 18 kDa). Echinococcosis serum IgG specifically recognizes antigens with molecular sizes below 30 kDa. The presence of one band at 7 kDa and/or one band at 26 to 28 kDa is indicative of the presence of *Echinococcus*-specific IgG in serum. *E. granulosus* serum IgG specifically binds to a component of 16 to 18 kDa as a diffuse band. By contrast, *E. multilocularis* serum IgG specifically binds to antigens of 16, 17, 18, and 20 kDa as sharp bands.

**FIG. 2.** *E. multilocularis* PCR on biological samples: material from the fistula of patient 1 (lanes 2 and 6), and biopsy samples of vertebrae from patient 2 (lanes 3 and 7). Lanes 1 and 9, molecular size marker; lane 4, negative control; lanes 5 and 8, positive control. Lanes 2, 3, and 5, *E. multilocularis* PCR positive (200 bp); lanes 6 to 8, internal control for inhibitor detection (371 bp).
both cases reported here, the presence of extrahepatic osseous involvement and the absence of initial hepatic involvement are unusual. Differential diagnosis includes tumors and infectious lesions, such as those due to tuberculosis and bacterial abscesses (16). In case 1, the patient had several treatments for tuberculosis over many years, without microbiological evidence. The lack of improvement with antibiotic therapy finally led to new etiological research. However, during the course of these years, AE had evolved and finally, the patient died. This case illustrates the pejorative prognosis of late diagnosis of AE. A fatal outcome may occur in more than 95% of untreated patients within a 10-year period following diagnosis (4).

Serological interpretation can be difficult in cases of extrahepatic manifestation and sometimes remains insufficient to differentiate AE from CE. HCF ELISA has relatively high sensitivity for echinococcosis: 91% for CE and 95% for AE (17). Nevertheless a lack of specificity and problems with the standardization of their use render interpretation difficult. Indeed, serological tests based on the use of total somatic antigens show a high degree of cross-reactivity with other parasites (17). By contrast, Em2 ELISA using purified species-specific antigens has been used for immunodiagnosis of AE with encouraging results (7). The Em2 ELISA has a high sensitivity of 97% in hepatic localization and a specificity of 74% with respect to cross-reactions due to infection with the closely related E. granulosus (7). In case 1, the positive Em2 ELISA suggested an AE with cross-reaction, as indicated by a positive HCF ELISA. In contrast, Em2 ELISA was negative in case 2, suggesting a CE. In both cases, Em2 ELISA’s results were confirmed by the Western blot pattern. Western blotting allowed the detection of serum IgG in 97% of Echinococcus-infected patients (12). It has a higher sensitivity than ELISA for the detection of echinococcosis and a specificity of 93%. However, it could differentiate E. multilocularis from E. granulosus in only 76% of cases (12).

PCR techniques have been developed to detect E. multilocularis and E. granulosus nucleic acids in biological samples (6, 9, 13, 19, 20). PCR is most frequently applied to drainage material and biopsy samples and is increasingly being accepted as a complementary diagnostic tool for echinococcosis (5, 10, 11, 14, 15). For patient 1, PCR confirmed the serological diagnosis, whereas for patient 2, PCR invalidated it. For patient 2, PCR and histological examination arrived at an accurate diagnosis of AE, whereas serological species identification failed. Serological misdiagnosis may be due to low E. multilocularis-specific antibody titers and to unusual AE localization. Precise identification of the parasite species is crucial, especially in countries where both E. granulosus and E. multilocularis infections occur, as their management and prognosis are different. Our cases highlight the efficiency of PCR by contrast to the limitations of serological interpretation in the diagnosis of extrahepatic AE.

We thank the Hôpitaux Universitaires de Strasbourg and the Université Louis Pasteur de Strasbourg for their financial support.

REFERENCES