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Original papers

Molecular determination of suspected alveolar echinococcosis requiring surgical treatment in human cases from Poland

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ABSTRACT. In this study five cases of suspected alveolar echinococcosis from Poland in which surgical treatment was needed, previously diagnosed by means of imaging and serological techniques, were analyzed in terms to identify the causative agent. Samples of the parasite tissues taken perioperatively from the liver lesions were used for the histopathological and molecular examinations. The sequences of all isolates were identical to *Echinococcus multilocularis*; all nad1 sequences have been deposited in GenBank The histopathological examination revealed Pass-positive fragments of laminated layers typical for *E. multilocularis* metacestode. Obtained results confirm that the use of imaging techniques only may be insufficient to diagnose alveococcosis thus the recognition of the zoonosis should base on several procedures; especially valuable are highly sensitive and specific molecular methods.

Key words: liver lesions, human alveolar echinococcosis, mitochondrial DNA, PCR

Introduction

Echinococcus multilocularis, a tapeworm species belonging to the genus *Echinococcus,* family Taeniidae, involves wild carnivores – mainly red fox, *Vulpes vulpes* and arctic fox, *Alopex lagopis* as main definitive hosts in the sylvatic life-cycle [1–8]. It is possible that in synanthropic cycle adult tapeworms may develop in cats and domestic dogs. The adult strobilar form lives in the small intestine of definitive hosts, gravid proglottids and eggs are shed with their feces. Coprophagic flies may serve as mechanical vectors of the eggs of *Echinococcus* species. Eggs with oncospheres are the infective forms for various rodents that are the intermediate hosts of the cestode. Next larva – metacestode of *E. multilocularis* is a tumour-like, infiltrating structure; larval growth remains in the proliferative stage, resulting in invasion of the surrounding tissues. The metacestode may form a net of ramifications extending throughout affected organ, mainly liver.

The sylvatic cycle of *E. multilocularis* with foxes as definitive hosts is the predominant source of the infection for humans that are accidental intermediate hosts; however it is emphasized that dogs may also play a role in alveolar echinococcosis in man [4]; after oral infection with oncospheres, metacestodes, usually without protoscolices develop, primarily almost in human liver. A prolonged asymptomatic incubation period, subsequent chronic course, slow infiltrative growth of this larva result in difficulties in diagnostics of alveolar echinococcosis, this serious human zoonotic disease, with high fatal rate if untreated. A patient's history, clinical findings, imaging, immunological, immune-enzymatic techniques are assessed for diagnosis of liver lesions [4,9,10]. The alveolar echinococcosis may be mistakenly diagnosed as a liver cancer, thus, treated inadequately; a recognition of alveococcosis should

always base on several procedures. The examinations of samples of the infected liver taken during surgery with use of parasitology, histopathology and by means of transmission electron microscopy techniques may be helpful [4–12]. Molecular methods have been developed and, if available, are very useful especially in the diagnostically difficult zoonotic diseases [3,4,12–17].

The purpose of this study was to identify – using molecular techniques – the causative agent of suspected alveolar echinococcosis in human cases from Poland demanding surgical treatment.

Materials and Methods

Material deriving from five patients, four males and one female, with serious liver dysfunctions due to parasitic diseases, was analyzed in this study. The age of patients ranged from 19 to 54 years; four of them have been under medical supervision for several years due to infection with *Echinococcus* spp. and were conservatively treated with anti-parasitic chemotherapeutic, albendazole. However, as further morpho-physiological deteriorations occurred in course of the infections, it was decided that surgical treatment is necessary.

Before the hepatectomy, all patients underwent abdominal ultrasonography (US), computed tomography (CT) and immunological tests (ELISA, Western blot). Several persons were suspected of having the alveococcosis, although results of imaging techniques and immunological tests not confirm this diagnosis. Curative liver resections were carried out in clinics of the Medical University of Warsaw, Poland.

In all cases, samples of the parasite tissue were removed from the liver lesions.

Cyst fragments and fluids collected during surgery were examined using molecular and histopathological methods. The samples were stored frozen at −20°C or fixed in 70% ethanol prior to molecular analysis; for histopathological examination, samples taken from liver lesions were fixed in formaldehyde solution.

DNA extraction and PCR. The tissue samples were rinsed with phosphate-buffered saline (PBS) several times to remove any ethanol and centrifuged at 3000× g for 10 min. Each pellet was dissolved in 100 µl PBS and genomic DNA was extracted using a NucleoSpin kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Part of the NADH dehydrogenase 1 (nad1) gene was amplified using primers JB11 (5'-AGATTCGTAAGGGGCC TAATA-3') and JB12 (5'-ACCACTAACTAATTC ACTTTC-3') [9]. The 50 µl reactions were comprised of 1 µl of DNA template, 50 pM of each primer, 0.2 mM of each dNTP, 1×PCR buffer containing 2.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Qiagen, Hilden, Germany). The following PCR was performed in a PTC-200 thermal cycler (MJ Research, Waltham, USA) in conditions: 3 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 50°C (nad1) or 55°C (cox1), and 1 min at 72°C. The PCR products were separated by electrophoresis on a 2% agarose gel (MetaPhor, FMC BioProducts, Philadelphia, USA), then stained with ethidium bromide and observed on a UV transilluminator. The nad1 gene fragments were purified and then directly sequenced in both directions using a BigDye Ready Reaction Cycle Sequencing kit and an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, USA). Chromatograms were manually checked and edited using Chromas 2.0. The obtained sequences were aligned with others retrieved from NCBI GenBank using ClustalW2 (http://www.ebi.ac.uk/Tools/ clustalw2).

Histopathological preparations. Samples of the parasite and liver tissues fixed in formaldehyde solution were embedded in paraffin, next cut into thin shavings and then stained with Schiff for microscopic assessed to reveal parasite structure.

Results

The *Echinococcus* cysts size ranged from 7 mm to 8 cm in diameter, mostly present as an unilocular cyst, but one was multilocular and consisted of numerous irregular cavities not sharply defined from the surrounding tissues. separated by collagen fibers, with necrosis and calcifications.

DNAs extracted from all five samples were used as the template in separate PCRs to amplify the region of the mitochondrial NADH dehydrogenase 1 (nad1, ~500 bp) gene. Each PCR produced a single band upon agarose gel electrophoresis. All samples were diagnosed positive by amplification of nad1. The nad1 fragments were sequenced and compared with sequences of Echinococcus genotypes available in NCBI GenBank. The sequences of all isolates were identical to *Echinococcus multilocularis.* All nad1 sequences were deposited in GenBank with accession numbers JX266825, JX266826, MH986749, MH986750,

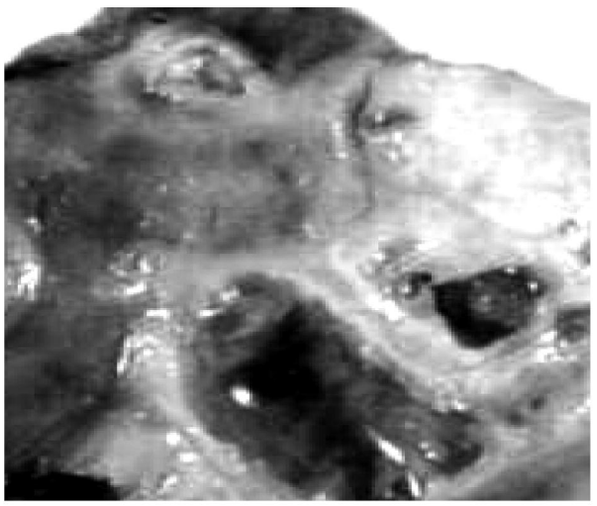

Fig.1. Liver sample with poorly delineated parasitic cysts in suspected alveococcosis

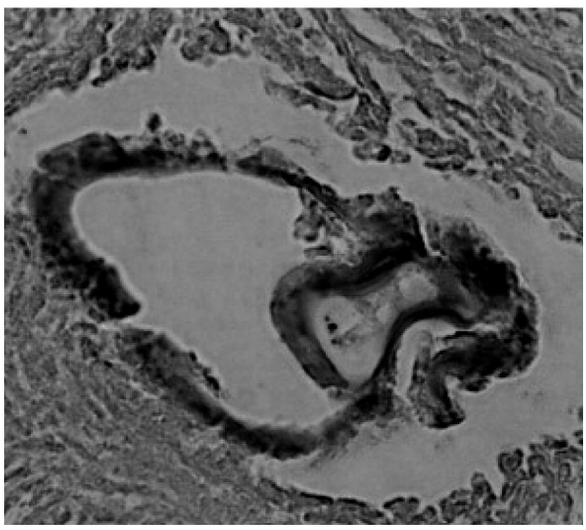

Fig. 2. Sections throughout infected liver samples - Schiff stained.
Note Pass-positive fragments of *E. multilocularis* laminated layer – the light microscope.

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Assessment of the liver sample taken post-operatively showed poorly delineated lesions with parasitic cysts various in size and shape in the suspected alveococcosis (Fig. 1).

The histopathological examination of the samples revealed the parasite structure (Fig. 2) in hepatic lesions – Pass-positive fragments of laminated layers typical for *E. multilocularis* metacestode. In some hepatic regions directly adjacent to these larval fragments occurred signs of echinococcosis with granulomatous inflammations**.**

Discussion

The alveococcosis is widespread in the northern regions of our globe, with a tendency to endemic occurrence. Recent epidemiological studies have shown that in the central Europe including Poland the distribution of *E. multilocularis* is wider than it was anticipated previously [4,5,10,13–17].

Humans are nonspecific hosts of the tapeworm.The alveolar echinococcosis is one of the most serious parasitic zoonosis with poor prognosis if untreated and a high fatality rate reaching 50–70% [4,14]. The increasing population of infected foxes affect most infections in man; it is regarded that hosts that penetrate nearby the village and urban buildings, infected dogs including, cause a certain risk to human health [4,12]. The ingestion of vegetables and fruits, including berries, mushrooms contaminated with *E. multilocularis* eggs is the most important transmission routes; coprophagic flies may serve as mechanical vectors of the cestode eggs. Moreover, it is considered that certain occupations: farm workers, animal herders are at an increased risk of the disease [4,9,11–14].

The early proper diagnosis is crucial for better prognosis in the dangerous zoonosis; particularly, the long-term chemotherapy with application of albendazole indicating parasitostatic efficacy against the metacestode of *E. multilocularis* may reduce the need for a surgery [4,9,12]. The liver lesions caused by *E. multilocularis* are diagnosed by means of ultrasound and computerized tomography, however metacestode growth may remain asymptomatic long time, the infection may be diagnosed as caused by *E. granulosus* or may mimic hepatic carcinoma, thus incorrect diagnosis may result in ineffective treatment.

For this reason, the use of imaging techniques only may be insufficient to diagnose alveolar echinococcosis. Immunological diagnostic approach combined with comparative morphology are helpful, but ELISA-based methods may be variable in sensitivities and there are cross-reactions.

In this study we analyzed several cases of suspected alveolar echinococcosis from Poland in which surgical treatment was needed, previously diagnosed by means of imaging and serological techniques. Results obtained with use of the histopathological and molecular examinations of the liver material taken perioperatively are the subsequent evidence that the recognition of alveococcosis should always base on several procedures.

Especially, highly sensitive and specific molecular techniques are indeed valuable for diagnostics in the zoonotic disease and for determination/verification of a causative agent in the suspected cases of human alveococcosis.

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