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Review

Basic Problems in Serological Diagnosis of Cystic Echinococcosis

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ABSTRACT
Cystic echinococcosis (CE), which occurs in rural areas during most seasons, is an important public health problem in Turkey. Many challenges regarding both diagnosis and treatment of this disease have not yet been overcome, and despite significantly increasing patient care costs, surgical treatment remains the main option. Confirmation of diagnosis is usually performed by serological tests based on the detection of serum antibodies against crude parasitic extracts (hydatid fluid, HF); however, HF contains cross-reactive antigens that lead to false-positive results, indicating other parasitic and nonparasitic diseases. Moreover, certain patients are serologically negative for HF, despite suffering from CE, likely due to cyst stage, number, and size. The existing insensitive and nonspecific tests have been replaced with indirect hemagglutination test (IHAT), enzyme-linked immunosorbent assay (ELISA), and immunoblotting (IB) in recent years. As a result of the evident diagnostic problems, the World Health Organization/World Animal Health Organization recommendations were based on a sequential screening and confirmatory test model. The use of ELISA, IHAT, latex agglutination tests (LAT), immunofluorescence antibody test, and immunoelectrophoresis is recommended for primary screening. The accepted serological screening tests are IHAT, IFA, and ELISA in Turkey, with the Turkish Ministry of Health, Public Health Agency recommending that at least two serological screening tests are used to diagnose patients with CE, followed by confirmation using IB.In the present review, the laboratory tests used in the diagnosis of CE and their limitations and diagnostic algorithms are explained with reference to the current literature.
Keywords: Cystic echinococcosis, microbiology, serological diagnosis

INTRODUCTION

Cystic echinococcosis (CE), which occurs in rural areas during most seasons, is an important public health problem in Turkey. Although the disease does not discriminate among age and gender, its occurrence is greater in women aged 30-50 years who reside in rural areas and are in frequent contact with animals (1, 2).

Many challenges regarding both diagnosis and treatment of this disease have not yet been overcome, and despite significantly increasing patient care costs, surgical treatment remains the main option. Radiological imaging methods are generally used for the identification, evaluation, and screening of liver lesions (3, 4), with confirmation of the diagnosis, typically performed using serological tests (5).

A multidisciplinary team consisting of clinicians, radiologists, and microbiologists must work together for proper CE diagnosis. Clinician and laboratory cooperation is required for the differentiation of CE cysts from benign cysts, cavitary tuberculosis, mycoses, and benign and malignant neoplasms.

In the present review, the laboratory tests used in the diagnosis of CE and their limitations and diagnostic algorithms are explained with reference to the current literature, with a view to guiding clinicians with cases of CE.

CLINICAL AND RESEARCH CONSEQUENCES

Diagnosis

Detection of a cyst-like mass in an individual who works with livestock supports the diagnosis of CE in regions where *Echinococcus granulosus* is endemic. However, differential diagnosis from benign cysts, mycoses, cavitary tuberculosis, and benign or malign neoplasms must be made. Generally, a noninvasive confirmation of the diagnosis can be performed by the combined use of radiological imaging and immunological diagnostic techniques (5).

Radiological imaging methods, such as ultrasonography, computerized tomography, and magnetic resonance imaging, are frequently used for CE diagnosis. Radiological imaging is also used as a screening tool for the diagnosis of liver lesions, guided by a classification system for CE diagnosis defined by the World Health Organization (WHO) informal working group. Based on this classification, CE liver cysts are categorized as stages 1-5 (CE1-CE5), where stages CE1 and CE2 are considered as

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an active disease (3). Unilocular, oval, echo-free, double-walled cysts are classified as stage CE1. Generally, when the position of the patient is changed, a "snowflake effect" is observed due to free-floating protoscoleces. When daughter cysts are visible within mother cysts as a "wheel spoke" or "honeycomb" pattern, the cyst is classified as stage CE2. The laminate membrane on the inner surface of the cyst is dissolved as part of the degenerative process, described as the "water lily" sign, during stage CE3, which is the inactive staging transition phase. The cysts become inactive with a "ball of wool" appearance with heterogeneous internal structures during stage CE4. Finally, the cyst has a thick, arch-like, calcified wall that forms a conical ultrasonic shadow, and protoscoleces are no longer present during stage CE5, which is the inactive phase (3). These classification criteria enable the simple assessment and evaluation of cystic liver lesions in routine clinical settings (6).

Laboratory Diagnosis

Preanalytical considerations

There is a risk, although small, of this parasite infecting the laboratory personnel who handle tissue biopsies from positive patients or stool samples from experimental animals. Although most laboratory animals are commercially available, animal species used in certain investigations are not guaranteed to be pathogen free. The American College of Laboratory Animal Medicine defines animal species as primary and secondary. *Echinococcus* spp. has been reported to be at risk of transmission from dogs and sheep to humans; however, it is classified as low infectivity in biomedical research laboratories (7).

Biosafety level 2 (BSL2) laboratory applications are required to handle hydatid disease in a clinical laboratory. Great attention must be paid to the use of personal protective equipment and good hygiene practices, such as hand washing, to protect the laboratory personnel from infection with *Echinococcus* (7). A laboratory-based case of CE was reported in 2012 in a 37-year-old laboratory technician who had been working with positive CE specimens in a non-BSL2 laboratory for 20 years (8). Gloves should be used when coming into contact with stools or surfaces contaminated with fresh stool samples (9).

Microscopic approaches

The pathogen can be detected directly with microscopic examination of fluid obtained from fine-needle aspiration or liver biopsy samples. Hooks, protoscoleces, and laminate membrane fragments can be detected in smears prepared from sediments of centrifuged cyst fluid with microscopy. Ziehl-Neelsen staining can also be performed, giving a better contrast of hooklets (10). Cyst fluid microscopy may show infection and cystic vitality (11); however, certain cysts are sterile (acephalocysts) without the presence of germination capsules. Therefore, a negative microscopy result does not exclude CE infection, and the diagnosis should be confirmed by serological tests (5).

Immunological approaches

Serological methods for CE are based on the detection of serum antibodies against crude parasitic extracts (hydatid fluid, HF); however, HF contains cross-reactive antigens that lead to false-positive results, indicating other parasitic and nonparasitic diseases. In addition, HF produces nonspecific reactions in certain samples from healthy donors, and conversely, certain patients are serologically negative for HF, despite suffering from CE, likely due to cyst stage, number, and size. As a result of the pitfalls in detecting antibodies, alternative laboratory methods have been developed, such as the detection of circulating antigens, peripheral cytokines, and parasitic DNA (12).

E. granulosus Antigens

Native antigens

Hydatid fluid is the main source of antigens most commonly used in serological tests for the detection of antibodies in patients affected with CE. The most pertinent disadvantage of HF as an antigen source is that it cannot be produced in the laboratory but has to be collected from naturally infected animal and human cysts (12). Hence, HF composition varies greatly depending largely on the host, the stage of cystic development, and the parasitic genotype (13).

Hydatid fluid is a complex mixture of glycoproteins, lipoproteins, carbohydrates, and salts formed during parasite metabolism, with certain components including serum albumin and immunoglobulins being internalized from the host. The most well-defined and abundant immunogenic antigens in HF are antigen B (AgB) and antigen 5 (Ag5) (12). AgB is a highly immunogenic 120-160 kDa protein that acts as a protease inhibitor, eliciting a Th2 cell response in patients with progressive CE, which inhibits neutrophil recruitment and activation of T helper cells (14, 15). AgB-like antigens are also present in parasites of the *Taenia* genus, including *Taenia solium* and *Taenia saginata* (16). Ag5 is a 400 kDa thermolabile protein that is highly abundant in HF (14) and is thought to have important functions in the cyst development (12). Since Ag5 shows high homology with antigens in the *Taenia* species, it can cause cross-reaction when used in diagnostic tests (17). Semi-purified fractions enriched in AgB and/or Ag5 can be obtained from HF in different ways; however, this has not yet been standardized. HF and its fractions are heterogeneous since they are usually collected from infected animals, leading to false-positive and -negative test results when used as an antigen for the detection of antibodies (12). Recombinant antigens have been developed as an alternative due to the cross-reactivity of native antigens.

Recombinant antigens

The AgB isoforms, such as AgB1, AgB2, AgB3, and AgB4, are produced as recombinant proteins by different laboratories for use as antigens; however, recombinant AgB5 is yet to be successfully produced. The methods used to obtain recombinant antigens have not reached consensus among different laboratories, leading to different diagnostic performances (12). Synthetic peptides have been identified as alternatives to recombinant proteins, and their standardization is thought to be better since they are produced chemically following the amino acid sequence. Nevertheless, a single peptide cannot provide sufficient diagnostic sensitivity; thus, several peptide antigens can be combined to

increase sensitivity (18). Although Ag5 is produced as a recombinant protein, its various available versions and different immune reactivities render it less than ideal. Recently, other recombinant antigens from protoscolices, oncospheres, and adult worms have been described (12).

Serological diagnosis

In recent years, the existing insensitive and nonspecific tests have been replaced with indirect hemagglutination test (IHAT), enzyme-linked immunosorbent assay (ELISA), and immunoblotting (IB) (19, 20). CE elicits a strong antibody response in many patients with different isotypes (IgG, IgM, IgA, and IgE). Antibodies against oncosphere antigens first appear several weeks after infection, followed by those against the laminar layer, cystic fluid, and protoscolices (12). The most commonly used methods for CE diagnosis are the detection of specific IgG antibodies using an HF antigen in diagnostic tests, such as ELISA and IHAT, and in the confirmatory IB test.

Studies have reported that the sensitivity of IgG-ELISA varies from 63% to 100%. False-negative results with ELISA are due to various factors, such as early and inactive cyst stages, cyst number and size, cyst placement outside the liver, and parasitic genotype (21-23). Another problem with ELISA is false positivity. When HF is used as an antigen, it causes various false-positive results in healthy donors from different geographical regions (24). Cross-reactivity to HF can be observed in other parasitic (alveolar echinococcosis, cysticercosis, ascariasis, and amebiasis) and nonparasitic (malignant) diseases (23, 25, 26). Moreover, the HF anti-echinococcal antibody level cannot be used as an indication of successful treatment, since it can remain high for many years despite cyst removal. Therefore, when antibodies other than IgG were investigated, they were found to provide better results with respect to patient follow-up, although this is still a question of debate (27).

Antibody responses to HF have been found to be highly variable both qualitatively and quantitatively at different times during infection, both in different patients and in the same patient. This variability is due to cyst number, size, stage, and location, in addition to parasitic genotype and the applied treatment (12). Recombinant antigens have also been used in studies with more sensitive and specific diagnostic purposes; however, most of the current studies have been conducted in a small number of patients, often with unknown clinical variables. Variable susceptibility and specificity rates are common for all tested recombinant antigens (12).

There are various commercial kits available that are based on ELISA, IHAT, and immunochromatography (IC), containing crude or semi-purified HF fractions; however, the antigenic source is rarely specified. IHAT tests have reported a sensitivity of 34.9%-88% and a specificity of 44%-70% (28-31). Several commercial ELISA kits were tested and compared with in-house ELISA, determining variable false-negative and -positive results. IB has been found to be more sensitive than either ELISA or IHAT techniques (12). As a result of the evident diagnostic problems, there exist the WHO/World Animal Health Organization recommendations based on a sequential screening and confirmatory test model. The use of ELISA, IHAT, latex agglutination test, immunofluorescence antibody test, and immunoelectrophoresis is recommended for primary screening (32). Over the past few years, sensitive and easy-to-use tests, such as IC and the dot immunogold filtration assay, have been commercially developed. Compared with other tests, IC is more advantageous with respect to its various features, such as a short test duration, no requirement for specialist staff, and easy interpretation of results (12). In addition to being more economical than other techniques, IC does not require to be transported or stored under refrigeration. Higher sensitivity ratios have been determined for IC compared with the antigens used in other tests (33, 34). A flowchart adapted from the "Republic of Turkey Ministry of Health, Public Health Agency, Cystic Echinococcosis, Field Guide for Laboratory Diagnosis of Infectious Diseases" hown in Figure 1 (35).

Serological studies have alternatively been performed using noninvasive urine specimens, with similar sensitivity and higher specificity rates being detected by ELISA (36). Currently, there is a need for the production of easy-to-use tests containing few recombinant antigens, which can be used as both primary screening and secondary confirmation tests. To date, there is no ideal test for use in patient follow-up; accordingly, it is necessary to validate the standardized antigens identified from patients with detailed clinical traits from a large number of serum samples (12).

Antigen detection

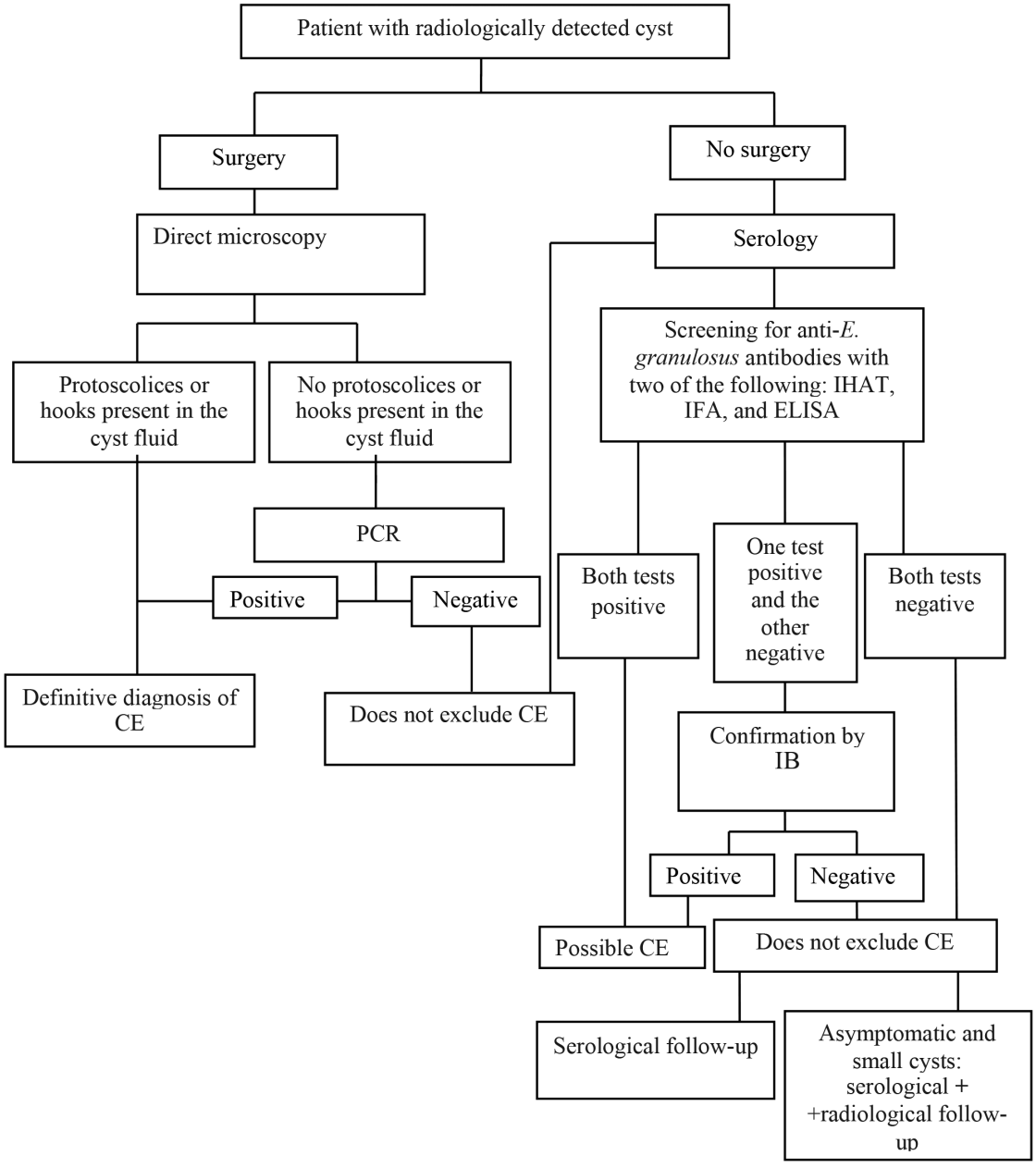
Antibody detection does not only show the presence of disease but also the exposure to an *Echinococcus* infection, and serum antibodies can often be detected for up to 10 years after removal of a hydatid cyst (24). An alternative for CE diagnosis is the detection of antigens in bodily fluids and serum, which may be more advantageous than the detection of antibodies in the early stages of infection and during patient follow-up, since circulating antigens initially decrease in successfully treated patients (12). Antigen detection may be useful in diagnosing antibody-negative patients; however, circulating antigens in patients with CE are often below the detection limits due to a low release or binding of released antigens to antibodies in the circulation (37). Diagnostic samples may need to be treated prior to testing to separate antigens from antigen-antibody complexes, and for this reason, antigen detection may be more time-consuming (12). The combined use of two tests that detect antigens and antibodies has been reported to increase sensitivity (38).

Levels of cytokines and peripheral blood mononuclear cells (PBMCs) in seronegative patients have also been investigated as diagnostic markers of the disease. Highly proliferative PBMC responses have been reported in certain patients with low antibody titers. Of note, the use of PBMC levels during the follow-up of treated patients may be impractical, since these levels remain high for a long period following treatment (39). Although a relationship between cytokine levels and CE has been established in certain clinics, further investigation is required (12).

Molecular diagnosis

DNA-based molecular tests for the presence of *Echinococcus* should measure true infection status with high sensitivity and

Figure 1. Flowchart for the laboratory diagnosis of patients with CE in Turkey. Adapted from the Republic of Turkey Ministry of Health, Public Health Agency, Cystic Echinococcosis, Field Guide for Laboratory Diagnosis of Infectious Diseases (35)



specificity, and be safe, and be cost-effective for the laboratory personnel. With the emergence of molecular and biochemical approaches for the detection of parasites, different methods have been developed to identify *Echinococcus* strains (12). Such studies, mainly based on polymerase chain reaction (PCR) approaches, have identified species, genotypes, and haplotypes of *E. granulosus*. PCR is the preferred method for parasite identification, molecular epidemiological studies, and confirmatory purposes (40, 41). Real-time PCR (qPCR) offers many advantages over conventional PCR in detecting parasitic infections due to the increased sensitivity and specificity, reduced reaction time, and quantitative detection of the amount of DNA in the sample (41, 42). However, DNA detection by PCR-based methods cannot assess parasitic viability or exclude the presence of a PCR-negative disease (43).

CONCLUSION

Despite the standardization and cross-reactivity challenges, a combination of radiological imaging and screening with confirmatory serological tests is the preferred choice for the diagnosis of CE. The specificities and sensitivities of hydatid serological tests differ depending on the antigen used. An ideal test should

be highly sensitive and highly specific; however, it is challenging to develop novel serological tests that are better than HF-based tests. PCR should be used for seronegative patients with radiologically suspected CE cases.

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