# Western blot assay of anti-*Echinococcus granulosus* antibody positive serum samples by indirect haemagglutination method

## İndirekt hemaglütinasyon yöntemiyle anti-*Echinococcus granulosus* antikorları pozitif saptanan serum örneklerinin western blot testi ile değerlendirilmesi

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### ABSTRACT

**Objective:** Cystic echinococcosis (CE) is a zoonotic disease mainly caused by the larvae of *Echinococcus granulosus* which is common in rural areas in Turkey. A multidisciplinary approach consisting of clinicians, radiologists and microbiologists is required for the proper diagnosis of the disease. Enzyme-linked immunosorbent assay (ELISA) and indirect hemagglutination (IHA) tests are preferred in the primer diagnosis of cystic echinococcosis (CE), while western blot (WB) is used to confirm the disease. However the use of serologic tests alone in diagnosis and follow-up of the disease is not recommended due to variable sensitivity and specificity rates and multiple serologic tests are required for appropriate diagnosis. In this study, it was aimed to compare the test results of patients sera sent to Gazi University Medical Faculty Microbiology Laboratory, between December 2015 and December 2016, with the preliminary diagnosis of CE, by WB test after those titrated with IHA. It is also aimed to determine the consistency between the two tests.

**Methods:** CE suspicious specimens were first tested

### ÖZET

**Amaç:** *Echinococcus granulosus* larvasının insanlarda sebep olduğu kistik ekinokokkoz (KE), ülkemizde hayvancılığın yoğun olarak yapıldığı bölgelerde yaygın görülen ve tanı için klinisyen, radyolog ve mikrobiyologların multidisipliner yaklaşımını gerektiren bir zoonozdur. Enzim-linked immünassay (ELISA) ve indirekt hemaglütinasyon (IHA), hastaların tanısında ilk sırada tercih edilirken Western Blot (WB) testi daha çok doğrulama amacıyla kullanılmaktadır. Fakat serolojik testlerin hastalığın tanı ve takibinde tek başına kullanımı, değişken duyarlılık ve özgüllük oranları nedeniyle önerilmemekte, uygun tanı için birden fazla serolojik testin kullanımı gerekmektedir. Bu çalışmada, Aralık 2015-2016 tarihleri arasında Gazi Üniversitesi Tıp Fakültesi Mikrobiyoloji Laboratuvarı'na KE şüphesiyle gönderilen hasta serumlarında IHA yöntemiyle titrasyon veren örneklerin doğrulama testi olarak kabul edilen WB yöntemi ile değerlendirilmesi ve iki test arasındaki tutarlılığın saptanması amaçlanmıştır.

**Yöntem:** KE şüpheli örnekler, *E. granulosus* antijenleri ile hazırlanmış IHA (Fumouze Laboratoires,

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by the IHA method (Fumouze Laboratoires, France) prepared with *E. granulosus* antigens. Afterwards 54 samples were tested again with the WB method (Anti-Echinococcus EUROLINE-WBIgG, Germany). The presence and intensity of antigen bands on the WB strips was assessed using commercial EUROLINE Scansoftware.

**Results:** Of the 54 cases, we found that 44 (81.48%) were positive with IHA test while 46 (85.19%) of them were positive with WB method. Six patients (11.12%) were positive with WB while they were negative by the IHA (< 1/320 titer). Two of them were IHA-negative in the titer 1/80, four in the titer 1/160. Cohen's Kappa analysis showed fair (slight) consistency (κ = 0.26) between the two tests.

**Conclusion:** As a result, using only IHA test can miss out CE patients therefore, the combined use of immunoassay tests increases the sensitivity in diagnosis. In the case of screening with IHA and confirmation with WB, for the more accurate results, analysis of all sera titrating with IHA from 1/80 is recommended with WB, even if it is negative according to kit procedures.

**Key Words:** Cystic echinococcosis, serology, diagnosis, indirect haemagglutination test, western blot

Fransa) yöntemi ile analiz edilmiş, titre veren 54 örnek doğrulama testi kabul edilen WB (Anti-Echinococcus EUROLINE-WBIgG, Almanya) yöntemi ile tekrar çalışılmıştır. WB stripleri üzerindeki antijen bantlarının varlığı ve yoğunluğu ticari EURO-LineScan yazılımı kullanılarak değerlendirilmiştir.

**Bulgular:** 54 hastanın 44 (%81,48)'ü IHA ile pozitif saptanırken, WB yöntemi ile 46(%85,19)'sı pozitif olarak saptanmıştır. Altı (%11,12) hasta IHA ile negatif (< 1/320 titre) olarak saptanırken WB testi ile pozitif olarak saptanmıştır. Bunlardan iki tanesi 1/80, dört tanesi de 1/160 titrede IHA negatif olarak saptanmıştır. Cohen's Kappa analizi ile iki test arasında düşük (fair, slight) tutarlılık olduğu saptanmıştır.

**Sonuç:** Sonuç olarak KE tanısında sadece IHA testi ile pozitif olan hastalar atlanabilmekte; bu nedenle immüntanısal testlerin birlikte kullanımı tanıda duyarlılığı arttırmaktadır. IHA ile tarama, WB ile doğrulama yapılması durumunda kit kullanım kılavuzuna göre negatif olarak değerlendirilse dahi daha doğru sonuç verme açısından 1/80'den itibaren titrasyon veren tüm serumların WB ile analizi önerilir.

**Anahtar Kelimeler:** Kistik ekinokokoz, seroloji, tanı, indirekt hemaglütinasyon testi, western blot

## INTRODUCTION

Cystic echinococcosis, (CE) caused by larval form of the *Echinococcus granulosus* is endemic in many regions of the world, such as western and central Asia, south and south-eastern Europe and Middle East as well as in Turkey (1-11). The disease is a zoonosis and occurs after eggs of the parasite are being ingested by humans. The oncospheres hatch and penetrate the intestinal mucosa, enter the blood or lymphatic vessels and spread in the internal organs to form the fluid-filled cyst structure (12). Patients with CE are usually asymptomatic and it is possible to discover cysts by imaging methods incidentally (13). In general, only large cysts can cause clinical symptoms in humans, symptoms of the disease are nonspecific and vary according to the location of cysts. Clinical findings may be confused with the findings of the benign cysts, cavitary tuberculosis, mycoses, benign and malign tumors (7).

Radiological imaging methods, including ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI), are important for the diagnosis of CE but serological tests have only a complementary role in the diagnosis because of the low sensitivity and low specificity due to cross-reactions with a plenty of diseases (14-18). Sensitivity of serological tests was reported as 88-96% in liver cysts, 50-56% in lung cysts and 25-26% in other organ cysts (15). However, serology may provide valuable information when imaging studies are insufficient.

The most commonly used serological tests are indirect haemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody (IFA) and immunoblotting tests that detect specific IgG antibodies (7, 16, 19-23). The western blot (WB) test, one of the immunoblotting methods, is used for confirmation, while ELISA and IHA are the first-line tests for CE diagnosis (14, 19, 20, 22). Although the IHA test was considered to have variable (60-100%) sensitivity and poor specificity, it is one of the most frequently used test to screen CE (24, 25). Positive test results should be confirmed by immunoblotting although this technique is not widely available (26).

As a result, there is no standard serological test with high sensitivity and specificity that can be used in CE diagnosis today. This is due to the fact that the sensitivity and specificity of serological tests are influenced by a number of factors, such as the quality of the used antigen, location, number and size of the cysts and the individual differences in immunological response of the patients (27). For this reason, many laboratories use, at least two different serological tests, together to increase sensitivity in the diagnosis of CE (12).

In this study, we used IHA test as a screening test and WB assay as a confirming test in patients, who were prediagnosed CE. The aim was to determine whether there is consistency between the two tests.

## MATERIAL and METHOD

The present study included serum samples from 54 patients of both genders and different ages, with CE that presented between December 2015 and 2016 at the Gazi University Faculty of Medicine in Ankara, Turkey. The diagnosis of CE is confirmed by clinical findings, characteristic abnormalities in diagnostic imaging and demonstration of specific antibodies against *Echinococcus* spp. The presence of specific antibodies against *Echinococcus* species was demonstrated by two of the following tests: indirect haemagglutination (IHA, Fumouze, France) and WB (Anti-Echinococcus EUROLINE-WB IgG, Germany).

Samples were first studied with IHA (Fumouze Laboratories, France) prepared with *E. granulosus* antigens to detect antibodies. Serum was diluted to 1/80, 1/160, and 1/320 for IHA and the test was repeated at the upper titers (1/640, 1/1280, 1/2560) when positive results were detected. In the test using antigenic red cell suspension, after 2 hours of incubation, a button-like precipitate was considered negative, while a serrated and lenticular appearance was considered positive. According to the manufacturer's recommendations ≥ 1/320 antibody titration were evaluated as positive for CE.

All IHA positive sera with low, mid-range, and high titers were secondly retested with WB method (Anti-*Echinococcus* EUROLINE-WBIgG, Germany), which accepted as confirmation test. The commercially developed WB assay contains electrophoretically separated *E. multilocularis* metacestode vesicle fluid antigens; p25/26 (25-26kDa), p16/18(16-18kDa), p21 and p7 antigens and three membrane chips with recombinant *E. granulosus* antigen AgB8 plus *E. multilocularis* antigens Em18 and Em95. The presence and intensity of antigen bands on the WB strips were assessed using the commercial EUROLine Scan software. The manufacturer reported that the sensitivity of the WB test was 93% and the specificity was 100%.

Consistency analysis of the test results was statistically evaluated by Cohen's Kappa analysis (28).

## RESULTS

Of the 54 serum samples, 44 (81.48%) were positive and ten (18.52%) were negative with IHA test. Forty-six (85.19%) of the samples were positive, seven (12.96%) were borderline and one (1.85%) was negative for *E. granulosus* antibody with WB method.

Six serum samples (11.12%) were detected as positive with WB while they were detected as negative by the IHA test (< 1/320 antibody titer). Two of them were IHA-negative in the titer 1/80, four in the titer 1/160.

Three samples were detected as borderline with WB while they were negative with IHA. One (1.85%) of them was IHA-negative in the titer 1/80, two (3.71%) of them were IHA-negative in the titer 1/160.

One serum sample IHA-negative in the titer 1/160 was found as negative with WB method.

Four samples (7.4%) were found that were borderline with WB but positive with IHA test (≥ 1/320). Two (3.771%) of them were IHA-positive in the titer 1/320, one (1.85%) in the titer 1/1280, and the other one (1.85%) in the titer 1/2560. When the blot test results of these patients were reviewed again, it was seen that all of the IgG and EgAgB bands were positive.

Coherence analysis was performed with Cohen's Kappa analysis, with a Kappa value of 0.22 and a weighted Kappa value of 0.26. In this analysis, it is accepted that values above 0.2 indicate consistency, and in our study it was determined that there was fair (slightly) consistency in the Cohen's Kappa analysis between IHA and WB tests.

A comparison of the results of the IHA and WB methods of serum samples is shown in Table1.

**Table 1.** WB and IHA test results of serum samples

| | | | Western Blot Test | | | |
|---|---|---|---|---|---|---|
| | | | **Negative n(%)** | **Positive n(%)** | **Borderline n(%)** | **Total (%) n(%)** |
| IHA Test | **Negative** | **1/80** | | 2 (3.71) | 1 (1.85) | 3 (5.56) |
| | | **1/160** | 1 (1.85) | 4 (7.41) | 2 (3.71) | 7 (12.96) |
| | **Positive** | **1/320** | - | 8 (14.82) | 2 (3.71) | 10 (18.52) |
| | | **1/640** | - | 10 (18.52) | - | 10 (18.52) |
| | | **1/1280** | - | 15 (27.77) | 1 (1.85) | 16 (29.62) |
| | | **1/2560** | - | 7 (12.96) | 1 (1.85) | 8 (14.82) |
| | **Total** | | 1 (1.85) | 46 (85.19) | 7 (12.96) | 54 (100.00) |

## DISCUSSION

The use of serological tests in the diagnosis of CE and follow-up of patients has not been achieved at the desired level and it remains to be discussed which test should be selected for diagnosis and follow-up. There are a number of diagnostic tests based on ELISA, IHA, and immune-chromatography available for CE diagnosis, including either unprocessed natural antigens of the hydatic fluid or semi-purified fractions of the this antigenic mixture (14). In particular, test methods involving *E. granulosus* natural antigens are not sensitive and their specificity is not at the desired levels due to cross-reactions with cysticercosis, fasciolosis, filariasis and other helminth infections (12). It has been reported in the literature that serology could be detected as positive in 80-94% of the cases of CE while only 65% of the cases in the alveolar echinococcosis (7). The rate of false-positivity in current tests are high, because of the cross reactivity with other parasites, especially in infected patients with cestodes, and even in healthy individuals (29). In addition, serological tests are ineffective to determine inactive (treated or calcific cyst) or active (active and progressive cyst) disease (30). Therefore, a positive serology result should be confirmed by more specific secondary tests in cases where cyst cannot be clearly detected by radiological imaging methods. Secondary assays are immunoblot tests generated with *E. granulosus* antigens, the detection of specific IgG subtypes and arc five precipitation tests, which are generally less sensitive but more specific than the primary test systems (12, 22).

Today, the gold standard in the serological diagnosis of the disease is the detection of IgG antibodies by either ELISA or immunoblotting, using natural or recombinant antigen B subunits originating from cyst fluid (31). However, the difficulty of the standardization of the preparation techniques of the antigens and the limitations of the use of the appropriate source of antigen material significantly influence the performance of these assays (32). It was reported that the combined use of immune-based tests in CE diagnosis will increase the diagnostic sensitivity (7, 19, 20).

In our study, we used IHA test as a screening test and WB assay as a confirming test in patients, who were prediagnosed CE. The aim was to determine whether there is consistency between the two tests. The IHA test was preferred because it is an easy, reliable, and short-term result, especially in serological diagnosis. IHA (Fumouze Laboratories, France) was tested by various investigators and reported with a sensitivity ranging from 34.9-88% and specificity ranging from 44-70% (20, 33, 34).

Of the 54 cases, we found that 44 (81.48%) were positive with IHA test while 46 (85.19%) of them were positive with WB method. Although the WB test used for CE diagnosis had a higher sensitivity than the ELISA and IHA tests (20, 22, 35), there were also studies reporting different results in the literature (36, 37). In a study in which 1323 patients with suspected CE disease were screened, only 48 (37.7%) were found to be positive by the WB method from 127 sera, which were found to be positive by the IHA method, while the others were found to be negative (36). We found that 40 (90%) of 44 IHA positive patients were positive and 4 patients were negative with WB, for CE. Of the four patients (7.4%), two (3.771%) of them were IHA-positive in the titer 1/320, one (1.85%) in the titer 1/1280, and the other one (1.85%) in the titer 1/2560. When the blot test results of these patients were reviewed again, it was seen that all of the IgG and EgAgB bands were positive. But according the kit procedure they considered as “borderline” due to the presence of only one of the p7, p21 or p25/26 patterns. It is thought that, the incompatibility of the WB and IHA tests in these patients may be related to the immunity of the patients, the location of the cyst, the size of the cyst, the number of cysts and even the genotype of the parasite (7, 12, 14, 19, 24, 25, 27, 38). Therefore, the detection of EgAB and specific IgG bands in the blot results may be

indicative of disease diagnosis, even if the WB test is detected at the borderline. It was indicated that AgB is a polymeric lipoprotein with a molecular weight of 120 kDa and had an important role in relation to parasite biology and parasite host (39). We found six patients (11.12%) were positive with WB while they were negative by the IHA test (<1/320 antibody titer). Two of them were IHA-negative in the titer 1/80, four in the titer 1/160. False negativity in IHA tests may be related to patient-related factors such as the number of cysts, localization, size, age, immunity of the patient, and treatment (40). Akçam et. al. (41), reported that 23.1% of 134 patients, with extra-hepatic cysts reported as negative with IHA; ten of them were found to be as positive with WB (41). In another study, nine patients with a negative IHA test were diagnosed as CE by clinical and imaging methods (42). Therefore, the use of a single test in the serological diagnosis of CE may be insufficient for diagnosis, so it is recommended to evaluate it after studying more than one serological method (20, 22, 35, 37).

In our study, a fair consistency between the IHA and WB tests was statistically determined by Cohen's Kappa analysis. For this reason, although IHA was negative according to the kit instruction manual, it was suggested that all of the samples which have IHA-negative in the titer in ≥1/80 should be re-evaluated with the WB test.

As a result, the IHA and WB tests show little consistency. It should not be forgotten that CE diagnosis cannot be excluded as a result of a single negative serological test because of the possibility that some patients' diagnoses may not be detected by the IHA test alone. In clinical laboratories, if the WB test is used only for confirmation, it is recommended to evaluate all sera that have a positive titration with the IHA test, even if it is below the limit value. There is a continuing need for the development of test systems that are sensitive and specific, low cost, and easy to implement, which can be used today in the diagnosis of CE.

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