Evaluation of a Commercial *Echinococcus* Western Blot Assay for Serological Follow-Up of Patients with Alveolar Echinococcosis

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A total of 20 patients with alveolar echinococcosis in different clinical stages according to the WHO-PNM staging system (P, parasitic mass in the liver; N, involvement of neighboring organs; M, metastasis) were followed up serologically with the commercial *Echinococcus* Western Blot IgG assay and a crude antigen extract enzyme-linked immunosorbent assay (ELISA). The cohort included patients after curative resection and patients who had unresectable lesions with stable disease or progressive infection. There were visible correlations of the crude antigen ELISA index and the presence and intensity of diagnostic bands in the Western blot. In most patients after curative resection, bands at 7, 16, and 18 kDa markedly decreased or vanished after 1 to 4 years. In a patient with a nonviable lesion (it died out), bands at 16 and 18 kDa vanished after 4 years. Among individuals with unresectable lesions but stable disease under antiparasitic chemotherapy, a decrease of all diagnostic bands was visible after 2 to 3 years in half of the patients, whereas the other half had unchanged blot results after 4 to 6 years. Patients with progressive disease showed increasing intensities of bands at 16, 18, and 7 kDa. The change of banding patterns was not influenced by the PNM stage in patients after curative surgery or with unresectable lesions. Our data indicate a correlation of the 7-, 16-, and 18-kDa-Western blot bands with disease activity independent of the PNM stage. This study demonstrated the usefulness of the *Echinococcus* Western Blot IgG assay as an additional serological test for the follow-up of patients with alveolar echinococcosis.

Alveolar echinococcosis (AE) is a serious parasitic zoonosis endemic in the northern hemisphere. The larval stage (metacestode) of the fox tapeworm, *Echinococcus multilocularis*, develops predominantly in the liver in infected patients, grows infiltratively, and may spread to distant organ systems. Diagnosis of AE is based on imaging techniques and serology. A PNM clinical staging system (P, parasitic mass in the liver; N, involvement of neighboring organs; M, metastasis) has been proposed by the World Health Organization following the criteria of malignancies, and stages I to IV are recognized (6). Depending on imaging results, single PNM categories are combined and patients are assigned to the respective clinical stage. Recently, a commercialized Western blot assay has been evaluated for the serological confirmatory testing of echinococcosis and differentiation between infections with *E. multilocularis* and *Echinococcus granulosus*. The assay uses a whole larval antigen extract of *E. multilocularis*, and in patients with AE, a particular banding pattern develops (7). In this study, we evaluated the usefulness of the commercialized *Echinococcus* Western Blot IgG assay (LDBIO Diagnostics, Lyon, France) for the serological follow-up of patients with AE in different clinical stages with resected and unresectable parasitic lesions.

**MATERIALS AND METHODS**

**Patients.** A total of 20 patients with AE were included in this study. All had acquired the infection in southern Germany, where the disease is endemic. The patients were grouped according to the WHO-PNM staging system (6), with four patients per stage. The patient groups were also divided into cohorts with unresectable lesions (nine patients) and after curative resection (nine patients); one patient had a nonviable lesion (it died out), and another underwent palliative resection only. All patients were undergoing albendazole therapy. The mean patient age when the first serum sample was taken was 53 years, and the male/female ratio was 0.8:1.2. In cases with resected parasitic lesions, the first blood sample available was drawn at the time of surgery. Three consecutive sera per patient were examined by a crude larval-antigen enzyme-linked immunosorbent assay (ELISA) and a Western blot assay. The follow-up duration was 1 to 7 years, and follow-up intervals were 6 months to 3.5 years (Table 1). The classification of curative resection, stable disease, progressive disease, or presence of a nonviable lesion was assessed by magnetic resonance imaging based on the lesion size and morphology at the respective follow-up intervals.

**Methods.** The *Echinococcus* Western Blot IgG assay (LDBIO Diagnostics, Lyon, France) was used according to the manufacturer’s instructions. 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 immunoglobulin G (IgG) in serum. These bands, as well as those at 12 and 15 kDa, are shared by *E. multilocularis* and *E. granulosus*, whereas antibodies in sera from patients with AE bind specifically to antigens of 16, 17, 18, and 20 kDa as sharp bands. A banding pattern called “P3” develops in patients with AE (7). All blots were developed on the same day in parallel, including a positive and negative control strip for each PNM stage.

For the crude larval-antigen ELISA, *E. multilocularis* metacestode tissue harvested from the peritoneal cavities of laboratory-kept Mongolian jirds (*Meriones unguiculatus*, a desert rodent) was mechanically homogenized and centrifuged. The supernatant was used to coat microtiter plates at a concentration of 2 ng/μl. Patients’ sera were tested at a dilution of 1:300 after preabsorption of the wells with 2% skim milk (Merck, Germany). Serum antibodies bound to echinococcal antigens were detected by secondary peroxidase-conjugated anti-human IgG antibody (Dako, Denmark) using ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-sulfonic acid)] (Roche, Germany) as a chromogenic substrate. Absorbance was measured after 60 min at 410 nm with a reference wavelength of 490 nm. For the calculation of the cutoff, the mean value of the absorbances of 12 sera from healthy blood donors was added to three times the standard deviation. The index of an individual serum sample was calculated by dividing the sample’s absorbance by the cutoff, resulting in a standardized threshold index of 1.0 for each sample. All ELISAs were run on the same day in parallel.

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RESULTS

Among patients diagnosed with clinical stage I, three of four individuals exhibited the P3 profile typical for AE in the first serum sample, with genus-specific bands at 26 to 28 kDa and species-specific sharp bands at 16 and 18 kDa (patients 1, 3, and 4). Two of them also had bands at 7 kDa (patients 1 and 4) and one also at 12 kDa (patient 4). In one patient, only bands at 26 to 28 kDa were present (P4 profile; patient 2). After curative resection, one patient (patient 1) lost the 7-kDa band after 2 years and the bands at 16 and 18 kDa decreased in intensity but remained very faintly visible after 2 and 4 years. The ELISA index fell below the threshold index after 2 years. In the other patient (patient 2) after curative resection, no changes in the P4 profile were visible, but the ELISA index also fell below the threshold after 2 years. In the patient with lesions that died out (patient 3), bands at 16 and 18 kDa decreased after 2 years and vanished after 4 years (P4 profile). The ELISA index markedly decreased but remained above the threshold. In this case, species differentiation by the Western blot assay was no longer possible. In the patient with progressive disease (patient 4), bands at 16 and 18 kDa increased markedly after 3 years and the preexisting additional band at 12 kDa disappeared completely. The ELISA index increased over time (Fig. 1).

All patients diagnosed with clinical stage II exhibited the P3 profile in the first serum sample, and all primary sera additionally showed the 7-kDa band. In patients after curative resection, bands at 7, 16, and 18 kDa disappeared after 1.5 and 2 years. Moreover, in patient 9, bands at 26 to 28 kDa also vanished, leading to a completely negative profile. In this case, the ELISA index fell to the threshold level after 2 years. In patient 10, the profile changed to P4, and the ELISA showed a decreasing index, which was still above the threshold after 3 years. Among the patients with stable disease, the profile remained unchanged in patient 11 after 6 years, while a decrease in the intensities of bands at 7, 16, and 18 kDa was visible in patient 12 after 2 years. In the latter, the ELISA index showed a prominent reduction but remained above the threshold. In the former, no significant changes in the index were observable (Fig. 2).

Among patients diagnosed with clinical stage IIIb, three of four individuals exhibited the P3 profile in the first serum sample. One patient (no. 13) expressed the P5 profile. All primary sera showed the 7-kDa band. In patient 13 with palliative resection (debulking surgery), decreased intensities of the bands at 7 and 26 to 28 kDa were measured after 6 years, but the profile remained unchanged. The ELISA index showed a slight decrease over time but remained above the threshold. In one patient with stable AE (no. 14), the profile remained unchanged, whereas in the other patient (no. 15), bands at 7, 16, 18, and 26 to 28 kDa began to fade after 2 years. In both patients, the ELISA index decreased but remained above the threshold after 2 years. In patients 7 and 8, with stable disease, the profile did not change. The intensities of the 16- and 18-kDa bands decreased in one individual (patient 8). ELISA indices slowly decreased but remained above the threshold in both patients (Fig. 1).

All patients diagnosed with clinical stage IIIa exhibited the P3 profile in the first serum sample, and all primary sera additionally showed the 7-kDa band. In patients after curative resection, bands at 7, 16, and 18 kDa disappeared after 1.5 and 2 years. Moreover, in patient 9, bands at 26 to 28 kDa also vanished, leading to a completely negative profile. In this case, the ELISA index fell to the threshold level after 2 years. In patient 10, the profile changed to P4, and the ELISA showed a decreasing index, which was still above the threshold after 3 years. Among the patients with stable disease, the profile remained unchanged in patient 11 after 6 years, while a decrease in the intensities of bands at 7, 16, and 18 kDa was visible in patient 12 after 2 years. In the latter, the ELISA index showed a prominent reduction but remained above the threshold. In the former, no significant changes in the index were observable (Fig. 2).

Among patients diagnosed with clinical stage IIIa, three of four individuals exhibited the P3 profile in the first serum sample. One patient (no. 13) expressed the P5 profile. All primary sera showed the 7-kDa band. In patient 13 with palliative resection (debulking surgery), decreased intensities of the bands at 7 and 26 to 28 kDa were measured after 6 years, but the profile remained unchanged. The ELISA index showed a slight decrease over time but remained above the threshold. In one patient with stable AE (no. 14), the profile remained unchanged, whereas in the other patient (no. 15), bands at 7, 16, 18, and 26 to 28 kDa began to fade after 2 years. In both patients, the ELISA index decreased but remained above the threshold.

The ELISA index fell but still remained above the threshold after 2 years. In patients 7 and 8, with stable disease, the profile did not change. The intensities of the 16- and 18-kDa bands decreased in one individual (patient 8). ELISA indices slowly decreased but remained above the threshold in both patients (Fig. 1).

Among patients diagnosed with clinical stage IIIb, three of four individuals exhibited the P3 profile in the first serum sample. One patient (no. 13) expressed the P5 profile. All primary sera showed the 7-kDa band. In patient 13 with palliative resection (debulking surgery), decreased intensities of the bands at 7 and 26 to 28 kDa were measured after 6 years, but the profile remained unchanged. The ELISA index showed a slight decrease over time but remained above the threshold. In one patient with stable AE (no. 14), the profile remained unchanged, whereas in the other patient (no. 15), bands at 7, 16, 18, and 26 to 28 kDa began to fade after 2 years. In both patients, the ELISA index decreased but remained above the threshold after 2 years. In patients 7 and 8, with stable disease, the profile did not change. The intensities of the 16- and 18-kDa bands decreased in one individual (patient 8). ELISA indices slowly decreased but remained above the threshold in both patients (Fig. 1).

The ELISA index fell but still remained above the threshold after 2 years. In patients 7 and 8, with stable disease, the profile did not change. The intensities of the 16- and 18-kDa bands decreased in one individual (patient 8). ELISA indices slowly decreased but remained above the threshold in both patients (Fig. 1).


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| Patient no. | Stage | PNM code | Status | Age (yr) | Sex | Examination interval/follow-up duration (yr) |
|-------------|-------|----------|--------|---------|-----|--------------------------------------------|
| 1           | I     | P1N0M0   | Curative resection | 25      | F   | 2/4                                        |
| 2           | I     | P1N0M0   | Curative resection | 62      | M   | 2/4                                        |
| 3           | I     | P1N0M0   | Lesion died out    | 58      | M   | 2/4                                        |
| 4           | I     | P1N0M0   | Unresectable, progressive disease | 62      | M   | 1.5/5                                      |
| 5           | II    | P2N0M0   | Curative resection | 67      | M   | 0.5/1                                      |
| 6           | II    | P2N0M0   | Curative resection | 38      | F   | 1/2                                        |
| 7           | II    | P2N0M0   | Unresectable, stable disease | 71      | M   | 3/6                                        |
| 8           | II    | P2N0M0   | Unresectable, stable disease | 60      | F   | 3/6                                        |
| 9           | IIIa  | P3N0M0   | Curative resection | 25      | F   | 2/4                                        |
| 10          | IIIa  | P3N0M0   | Curative resection | 62      | M   | 1.5/3                                      |
| 11          | IIIa  | P3N0M0   | Unresectable, stable disease | 69      | F   | 3/6                                        |
| 12          | IIIa  | P3N0M0   | Unresectable, stable disease | 39      | F   | 2/4                                        |
| 13          | IIIb  | P3N1M0   | Palliative resection | 32      | M   | 3/6                                        |
| 14          | IIIb  | P3N1M0   | Unresectable, stable disease | 49      | F   | 2/4                                        |
| 15          | IIIb  | P4N0M0   | Unresectable, stable disease | 60      | F   | 2/4                                        |
| 16          | IIIb  | P4N0M0   | Unresectable, progressive disease | 53      | M   | 3/6                                        |
| 17          | IV    | P4N1M0   | Curative resection | 56      | F   | 3.5/7                                      |
| 18          | IV    | P4N1M0   | Curative resection | 30      | M   | 1.5/3                                      |
| 19          | IV    | P4N1M0   | Curative resection | 72      | F   | 1/2                                        |
| 20          | IV    | P4N1M0   | Unresectable, stable disease | 71      | F   | 3/6                                        |

a As assessed by imaging (lesion that died out, progressive disease, and stable disease) or imaging and histology (curative resection).
b Age when first blood sample was drawn.
c M, male; F, female.
threshold. In the patient with severe progressive disease (patient 16), the intensities of bands at 7, 16, and 18 kDa increased over 6 years. This patient was intolerant of benzimidazole therapy, and he progressed to PNM stage IV. The ELISA index increased slightly during that time (Fig. 2).

Among patients diagnosed with clinical stage IV, three of four individuals exhibited the P3 profile in the first serum sample and also showed the 7-kDa band. One patient (no. 17) expressed the P4 profile. In all patients after curative resection, a decrease in the intensities of bands at 26 to 28 kDa was

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**FIG. 1.** Western blot results for patients with AE in stages I and II. Three consecutive sera from individuals after curative resection or with lesions that had died out, progressive disease, and stable disease were examined. Patients after curative surgery or with lesions that had died out showed decreasing intensities or loss of bands at 7 and/or 16 and 18 kDa within 0.5 to 4 years. Patients with stable disease undergoing antiparasitic chemotherapy had slowly decreasing intensities of bands at 7, 16, and 18 kDa or had an unchanged pattern for a period of 6 years. One patient with progressive disease had intensified 16- and 18-kDa bands and loss of a 12-kDa band. The positive control strip shows the classical P3 pattern of AE; the masses of bands indicative of echinococcosis are shown. The results of the crude larval-antigen ELISA are shown below each blot strip; the threshold index was 1.00. Table 1 shows patient characteristics.

**FIG. 2.** Western blot results for patients with AE in stages IIIa and IIIb. Three consecutive sera from individuals after curative resection or palliative resection, with stable disease, or with progressive disease were examined. Patients after curative surgery lost bands at 7, 16, and 18 kDa and had decreasing or vanishing bands at 26 to 28 kDa after 1.5 to 2 years. Patients with stable disease undergoing antiparasitic chemotherapy had slowly decreasing intensities of bands at 7, 16, 18, and 26 to 28 kDa after 2 years or had an unchanged pattern. A patient with palliative resection showed a decrease in bands at 7 and 26 to 28 kDa after 6 years. In a patient with progressive disease, the intensities of bands at 7, 16, and 18 kDa increased after 6 years. The layout is the same as in Fig. 1.
visible after 2 to 7 years. In two patients (no. 18 and 19), bands at 16 and 18 kDa faded after 2 to 3 years, and in one patient (no. 19), the 7-kDa band also faded after 2 years. In these two patients, the ELISA index decreased constantly but remained above the threshold level. In patient 17, the ELISA index fell below the threshold after 3.5 years. In the patient with stable disease (no. 20), bands at 7, 16, 18, and 26 to 28 kDa showed a very slight decrease over 6 years, concomitant with a minute decrease in the ELISA index (Fig. 3).

DISCUSSION

The demonstration of stable disease, progressive infection, cured disease, or recurrence after surgery is of major importance for patients with AE, as this parasitosis is often fatal in its natural course. Imaging tools are most widely accepted for follow-up purposes, but serology with ELISAs applying crude antigen preparations, affinity-purified antigens (Em2plus), or recombinant antigens (Em18), as well as a Western blot assay using Em18, has shown correlation with disease activity (10, 3, 8, 2). The present study was carried out in order to evaluate for the first time the suitability of the commercial LDBIO Echinococcus Western Blot IgG assay for the serological follow-up of patients with AE. Moreover, the patients examined were grouped according to the WHO-PNM clinical stage of AE for the first time.

Since the Western blot is not a quantitative tool per se, all sera were tested in parallel in order to demonstrate changes in banding pattern intensities and thus obtain semiquantitative results. Moreover, a crude antigen ELISA was chosen to generate comparable quantitative data on a similar antigenic composition. There was a visible correlation of the height of the crude antigen ELISA index and the presence and intensity of diagnostic bands. In patients with indices below the threshold level, bands at 7, 16, and 18 kDa had either vanished or were only very faintly visible. In patients with decreasing indices, the intensity of the banding pattern also decreased, whereas in patients with increasing indices, the intensities of bands also increased. The crude antigen ELISA uses a full larval extract very similar to the antigenic preparation of the Echinococcus Western Blot IgG assay. Both tests thus cover a wide antigenic spectrum and are able to measure a multitude of different anti-Echinococcus antibodies. Banding patterns and kinetics were independent of the PNM stage, but not of the treatment the individual patients underwent. In sera of patients with AE after curative resection, bands at 16 and 18 kDa could disappear after only 1 year, rendering species differentiation by the remaining Western blot pattern difficult or even impossible. Similarly, in sera of patients with cystic echinococcosis due to E. granulosus, a previous analysis of patterns by the same commercial Western blot assay had demonstrated the disappearance of bands at 16, 18, and 26 to 28 kDa within 8 months after complete resection of hydatids (9). In our study, however, bands at 26 to 28 kDa only decreased in intensity in most cases, whereas the 7-kDa band vanished. This might be a species-specific effect.

Antigenic compounds of E. multilocularis at 16 and 18 kDa seem to be good candidates for the serological differentiation of AE and cystic echinococcosis (4), and the 18-kDa antigen was designated a suitable marker for active AE in ELISA and Western blotting techniques using recombinant Em18 (2, 5, 8). In our study, the presence of the 16- and 18-kDa bands correlated with active disease, whereas a loss or decrease correlated with inactive infection or resection. The 7-kDa band showed a similar correlation, but it was not
always present in all primary sera. In a study evaluating the Echinococcus Western Blot IgG assay for primary diagnosis, however, active and inactive-abortive AE could not be distinguished by the 18-kDa band (7), paralleling observations of a study investigating subclass-specific serological reactivity in an in-house Western blot assay that showed that IgG4 from AE patients uniformly recognizes low-molecular-weight antigens independent of the clinical status (1). In contrast, results from a different study using another in-house Western blot assay revealed that IgG4 subclass antibody levels detecting 17.5-kDa and higher-mass antigens became negative within 1 year after successful treatment of AE (11). Moreover, antibodies directed against Em18 fell below the threshold level in patients undergoing long-term antiparasitic chemotherapy or after curative resection (2). In our study, patients undergoing benzimidazole therapy and with stable disease showed a slow decline of all diagnostic bands or had an unchanged pattern. At the moment, it is unclear if the parasite was being killed in those with a reduction of band intensity. In a patient with progressive AE, bands at 16 and 18 kDa increased. This result parallels previous reports of increasing Em18 ELISA indices (2) and IgG4 antibodies against the 17.5-kDa antigen in such cases (11).

In conclusion, the Echinococcus Western Blot IgG assay is suitable as an additional test for the serological follow-up of patients with AE in different clinical PNM stages who undergo different treatments. Bands at 7, 16, and 18 kDa indicate disease activity independent of the patient’s PNM stage. However, the Western blot results should be interpreted with caution and in conjunction with complementary serological tests and imaging results.

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