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Diagnostic Performance of an Anti-Actin Autoantibody Binding Enzyme Immunodot Blot in Autoimmune Hepatitis Type 1

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Background: A serologic hallmark of autoimmune hepatitis (AIH) type 1 are anti-smooth muscle autoantibodies (ASMA) with specificity for filamentous actin (F-actin; AAA (anti-actin antibodies)), traditionally detected by indirect immunofluorescence (IFT) using rat liver, kidney, and stomach tissue sections as substrates. However, IFT is a subjective method requiring an experienced investigator. Therefore, a more objective technique for the detection of AAA may be a helpful diagnostic tool. *Methods:* In a retrospective study with cross-sectional design, we evaluated AAA detected by an enzyme immunodot blot (IDB; Liver5 IgG BlueDot, D-tek, Mons, Belgium). Serum samples of patients with AIH type 1 ($n = 47$) and specified controls ($n = 142$) were included. For comparison, standard IFT was applied to rat LKS (liver, kidney, stomach) triple tissue sections. *Results:* IDB readings were done by two independent investigators (92% concordance). The diagnostic sensitivity of the AAA-IDB was 70%, compared to 51% of AAA-IFT (n.s.). The diagnostic specificity of AAA-IDB was significantly lower compared to AAA-IFT (76% vs. 94%; $P < 0.0005$). Correspondingly, the positive predictive value (49% vs. 75%; $P < 0.05$) and positive likelihood ratio (2.9 vs. 8.5) differed significantly. Neither prescreening for ANA or ASMA, nor the exclusion of infectious hepatopathies resulted in a significantly better diagnostic performance of the IDB. *Conclusion:* Compared to standard IFT, testing for AAA via IDB did not result in a significantly better diagnostic performance for AIH type 1. A blot with higher antigen binding specificity may be more functional. J. Clin. Lab. Anal. 30:123–129, 2016. © 2014 Wiley Periodicals, Inc.

Key words: autoimmune hepatitis type 1; anti-actin autoantibodies; enzyme immunodot blot

INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic, not strictly organ-specific autoimmune disorder of unknown etiology. With a prevalence of about 17/100.000 in Northern Europe, it is a rather uncommon disease (1). Despite considerable heterogeneity, it is characterized by about 80% female predominance, hypergammaglobulinemia, circulating autoantibodies, a genetic predisposition through association with human leukocyte antigens (HLA) DR3 or DR4, and a favorable response to immunosuppression (2). Its clinical manifestations and course are highly variable, with some patients suffering from acute liver failure, and others in whom it may progress to cirrhosis (3). Variant forms with overlapping features of cholestatic liver disease may occur in up to 20% of the cases, in which nomenclature and diagnostic criteria so far have not been standardized (4).

Glucocorticosteroids and immunosuppressive agents usually are capable of inducing remission in AIH, but long-term maintenance therapy is most often required. With respect to adverse events under immunosuppression and, on the other hand, a potentially fatal outcome without therapy, AIH should be diagnosed thoroughly.

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Diagnosis generally relies on characteristic serologic and histologic findings as well as exclusion of other forms of chronic liver disease (5). Scoring systems have previously been implemented and revised by the International Autoimmune Hepatitis Group (IAHG) (6, 7). With respect to the scoring system of Alvarez et al. (6), the diagnosis of AIH is definite in untreated patients with more than 15 points and probable in patients with 10–15 points. After onset of therapy, diagnosis is considered as definite in patients with more than 17 points, and as probable in patients with 12–17 points. However, with regard to a moderate sensitivity, a limited value has been attributed to such scoring systems in diagnosing individual patients (8).

A diagnostic hallmark for AIH are circulating autoantibodies, which are probably neither involved in pathogenesis, nor predictive of histologic severity or treatment response. However, these antibodies allow for classification of AIH into two major groups (9). AIH type 1 is most frequently characterized by anti-nuclear antibodies (ANA), anti-smooth muscle antibodies (ASMA), and anti-actin antibodies (AAA). Type 2 AIH is primarily seen in children and young adults and is generally characterized by antibodies to a specific cytochrome P450 located in liver and kidney microsomes (LKM-1) and/or liver cytosol antigen (LC1) (10). Antibodies to soluble liver antigen/liver-pancreas-antigen (SLA/LP) are found in 10 to 30 percent of patients with both forms of AIH, benchmarking highest specificity (11). Other autoantibodies are considered to be of minor importance.

ANA and ASMA are usually detected by indirect immunofluorescence (IFT). ANA in homogeneous, speckled, or less frequently centromeric, diffuse granular, nucleolar, or mixed patterns may be the only autoantibody present, or in conjunction with ASMA, which are less prevalent. With titres of 1:320 or greater, they are closely related to the presence of more specific AAA. Characteristic renal glomerular and peritubular fluorescence patterns in IFT are indicative of AAA with specificity for filamentous actin (F-actin) (12). However, in routine laboratory, IFT is a time-consuming and investigator-dependent technique, impeding a reliable and efficient identification of patients with AIH. Consequently, alternative test methods have been evaluated, but yielded contradictory results with respect to the relative diagnostic sensitivity and specificity (13–15). Thus, in the present study we compared the diagnostic performance of an immunodot blot (IDB) with liver-specific autoantigens to classic IFT in order to distinguish patients with AIH from patients with other forms of chronic liver disease, systemic rheumatic diseases, or healthy controls. The study focussed on the most prevalent AIH type 1.

MATERIALS AND METHODS

Study Population

After receipt of informed consent, blood sera from 136 patients with chronic hepatopathies, 23 patients with systemic rheumatic diseases, and 30 healthy blood donors were collected at the outpatient clinic of the Münster University Hospital, Germany. Applying the 1999 IAHG scoring system, in 21 patients the diagnosis AIH was classified as definite (17–22, median 18 points), and in 26 patients as probable (12–16, median 14 points) (6). Median patient age was 47 years (17–74), 33 were females. Median disease duration was 6 years (0–16). Thirty-eight patients actually were under therapy (prednisolone 2–25 mg, azathioprine 50–200 mg daily), 45 patients had ever been under therapy before.

The control group consisted of 25 patients with primary biliary cirrhosis, four patients with hepatic overlap syndrome that did not reach a sufficient number of points to be classified as AIH, three patients with primary sclerosing cholangitis, 39 patients with chronic hepatitis C, and eight patients with chronic hepatitis B virus infection. In three controls alcoholic cirrhosis was suspected, seven controls were classified as idiopathic hepatopathy. Further 15 controls suffered from systemic lupus erythematosus, and eight controls from progressive systemic sclerosis. Patients in all control groups met the respective current disease classification criteria. After exclusion of significant illness reviewing medical history, physical examination, and laboratory analysis, sera from 30 healthy blood donors were recruited. Median age of the entire control population (patients and healthy blood donors) was 46 years (21–79), 92 were females. Sera obtained from centrifugated peripheral venous blood samples were refrigerated and immediately stored at −80°C until analysis.

Enzyme IDB

Liver5 IgG BlueDot kits were kindly provided by D-tek sa, Mons, Belgium. The test is based on the classic principle of an enzyme immunoassay (16). Test strips consisting of a membrane fixed on a plastic support were incubated with patients' sera. Existent human antibodies bound to the corresponding five specific antigens attached to the membrane (F-actin: purified, from rabbit skeletal muscle, AMA-M2, LC-1, LKM-1, SLA). Washed and preincubated test strips were loaded with 1.5 ml of 1:151 diluted sera per well and incubated on a rocking platform for 30 min at room temperature. The wells were thoroughly washed (three times for 3 min), afterwards the provided conjugate solution (1.5 ml) containing alkaline phosphatase-conjugated goat anti-human IgG was

added and incubated for 30 min on a rocking platform. The solution was discarded from the wells, they were washed again (3 times for 3 min). Subsequently, 1.5 ml of substrate solution (nitroblue tetrazolium/bromo-chloro-indolyl-phosphate with 0.05% NaN3 as preservative) was incubated for 10 min again on a rocking platform. The wells were finally washed once for 3 min to stop the color reaction and dried on an absorbent paper. In each test strip, reaction control and cutoff control were incorporated in order to guarantee for a correct color reaction process and exclude unspecific antibody binding. A sample was considered positive in case that the color intensity of the corresponding antigen dot was higher than the intensity of the cutoff control dot. An external positive control (sera of individuals previously being tested positive for F-actin antibodies) was run with each series. Test evaluation was done immediately thereafter by two independent investigators (B.S. and J.S.), allowing for the assessment of interobserver accordance. In case of divergent results, the strips were reassessed together by both the investigators. When this did not result in concordance, the senior investigator's vote (B.S.) was decisive.

Indirect Immunofluorescence

Test kits for IFT were obtained from BioSystems S.A., Barcelona, Spain, consisting of acetone-fixed rodent tissue slides (rat triple substrate) from kidney, stomach, and liver and allowed, among others, the detection of AAA and ASMA (17). Sera with a dilution of 1:40 were incubated with the tissue substrates according to the manufacturer's instructions. A characteristic renal glomerular and peritubular fluorescence pattern was regarded as an indicative of antibodies with specificity for F-actin (12). Additionally, ANA were examined by VIRGO® ANA/Hep-2 IgG Determination Kits (Hemagen, Columbia MD) according to the manufacturer's instructions, using a cutoff titre of 1:80 (18). Each run included a positive and negative antibody control provided by the manufacturer. All analyses by IFT were performed by two independent investigators (B.S. and J.S.) using a standard fluorescence microscope (Carl Zeiss, Jena, Germany). In case of divergent results, evaluation proceeded analogously to the IDB readings.

Statistical Analysis

Sensitivity (proportion of true positive test results in all individuals affected), specificity (proportion of true negative test results in all individuals not affected), positive predictive value (ratio of true positive results to all positive results), negative predictive value (ratio of true negative results to all negative results), and diagnostic efficiency (proportion of correct positive and negative test results to all test results) were calculated using standard biomathematical methods. Significant differences between the applied classificators in different test methods were evaluated on a personal computer by the Chi-squared test for the comparison of proportions via the MedCalc Software (Version 12 for Windows Vista).

Likelihood ratios (LR) indicate by how much a given diagnostic test result raises or lowers the pretest probability of the target disorder (19). Calculation was done as follows: LR+ = sensitivity/(1 − specificity); LR− = (1 − sensitivity)/specificity. An LR+ greater than 10 or an LR− lower than 0.1 is indicative of large and often conclusive changes, an LR+ of 5–10 or an LR− of 0.1–0.2 of moderate shifts, an LR+ of 2–5 or an LR− of 0.2–0.5 of small, but sometimes important changes, and an LR+ of 1–2 or an LR− of 0.5–1 of small and rarely important from pretest to posttest probability.

TABLE 1. Interinvestigator Concordance in Immunodot Blot Readings

	Investigator #1	Investigator #2	Concordance absolute	(Relative, %)
AAA	67	59	173	92
Anti-LKM-1	2	2	189	100
Anti-LC1	1	1	189	100
Anti-SLA	10	10	189	100
AMA-M2	35	36	188	99

Data represent counts of positive IDB readings of 189 by two independent investigators, absolute and relative concordance. AAA, anti-actin autoantibodies; LKM, liver/kidney microsomal antibody type 1; LC1, liver cytosol type 1; SLA, soluble liver antigen; AMA, anti-mitochondrial antibody

RESULTS

Interobserver Concordance

In order to test for interindividual concordance concerning the IDB results, readings were done by two blinded independent investigators. Although testing for AAA resulted in 92% concordance, values reached up to 100% in testing for anti-LKM-1, anti-LC1, anti-SLA, and AMA-M2 (Table 1). The interobserver concordance of the IFT procedure was 90% (data not shown).

IDB Compared to Standard IFT

Screening for AIH by testing for AAA-IDB yielded a sensitivity of 70% and specificity of 76% (Table 2). The expansion of the IDB analysis to further liver-specific antigens (LC-1, SLA, LKM-1) and the exclusion of AMA-M2 did not result in a significantly better diagnostic performance than the isolated search for AAA. Comparing the IDB to IFT, the positive predictive value was significantly

TABLE 2. Immunodot Blot Compared to IFT Procedures to Diagnose AIH

Procedure	No. of patients	No. of controls	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Eff. (%)	LR+	LR−
AAA-IDB	33	34	70	76	49	89	75	2.9	0.39
Comb.-IDB	34	38	72	73	47	89	73	2.7	0.38
AAA-IFT	24	8	51	94[5)]	75[1)]	85	84[1)]	8.5	0.52
ASMA-IFT	36	54	77	62[1)]	40	89	66	2.0	0.37
ANA-IFT	39	82	83	42[5)]	32[1)]	88	52[5)]	1.4	0.40
AAA/ASMA/ANA-IFT	46	100	98[4)]	30[6)]	32[1)]	98	47[5)]	1.4	0.07

Data represent number of patients ($n = 47$) and controls ($n = 142$) tested positive for the respective autoantibodies and characterize the test-specific diagnostic performances. Significant differences between AAA-IDB and the other tests are indicated as follows: [1)] $P < 0.05$, [4)] $P < 0.001$, [5)] $P < 0.0005$, [6)] $P < 0.0001$. AAA, anti-actin antibodies; ASMA, anti-smooth muscle antibodies; ANA, anti-nuclear antibodies; IDB, immunodot blot; IFT, indirect immunofluorescence; Comb.-IDB refers to AAA, anti-LC-1, anti-SLA, anti-LKM-1, or AMA-M2; PPV, positive predictive value; NPV, negative predictive value; Eff., diagnostic efficiency; LR+, positive likelihood ratio; LR−, negative likelihood ratio.

higher for AAA-IFT, whereas the best negative prediction was obtained from the combined negativity of autoantibodies (AAA, ASMA, ANA) by IFT. By contrast, the IDB provided relatively low diagnostic evidence as positive and negative LRs only resulted in small changes from pretest to posttest probability.

Diagnostic Algorithm I: Prescreening for ANA or ASMA, Followed by Testing for AAA

ANA and ASMA are the most prevalent autoantibodies in AIH type 1. Thus, a reasonable diagnostic algorithm for AIH type 1 would first screen for ANA or ASMA to achieve highest sensitivity, followed by a test for AAA to gain specificity (Table 3). The prescreening for ANA or ASMA was highly sensitive and resulted in the loss of only one patient (2%) that had definitively been diagnosed with AIH. One hundred controls (70%) were tested positive and consecutively further examined for AAA. In the case of AAA-IFT, this approach resulted in a specificity of 94%, for AAA-IDB of 82%, both highly significant better than ANA/ASMA alone at $P < 0.0001$. However, AAA-IFT reduced sensitivity to one-half ($P < 0.0005$), AAA-IDB to two-thirds ($P < 0.001$). The direct comparison of AAA-IDB and AAA-IFT for the completion of the prescreening revealed a higher specificity of the IFT algorithm at $P < 0.005$, whereas the sensitivity of both algorithms did not differ significantly.

Diagnostic Algorithm II: Exclusion of Infectious Hepatopathies, Followed by Testing for Autoantibodies

Based on the established serologic and PCR tests, diagnosing infectious hepatopathies in the majority of cases is unproblematic. Discrimination of AIH from toxic, metabolic, or chologenic liver diseases is the clinically more relevant problem. We therefore reevaluated the diagnostic performance of the IDB and IFT after serologic exclusion of hepatitis B and C infection (Table 4). Test specificity of the IDB hereby increased from 76% to 83% (n.s.), and AAA-IFT from 94% to 99% ($P < 0.05$), resulting in an LR+ of 51.0. This increase reflects that nine patients with hepatitis C (23%) and two patients with hepatitis B (25%) were tested positive for AAA-IDB, whereas five patients with hepatitis C (13%) and one patients with hepatitis B (13%) were tested positive for AAA-IFT. On the other hand, after exclusion of infectious hepatopathies, only two persons of 142 not affected from AIH were tested positive for AAA-IFT. Specificity of the IDB remained lower than that of the IFT procedure. We therefore analyzed the diagnostic performance of the IDB after exclusion of infection and prescreening for ANA or ASMA via IFT. This procedure resulted in a further gain of specificity to 89% ($P < 0.01$). With an LR+ of 6.4 this algorithm provided a moderate shift from pretest to posttest probability in diagnosing AIH, whereas diagnostic performance of AAA-IFT was not further improved.

DISCUSSION

AIH is a non-organ specific autoimmune disorder predominantly diagnosed in females. Its clinical course is highly variable, ranging from subclinical to chronic or even rapid progressive forms, sometimes resulting in cirrhosis or acute liver failure. Usually it responds favorably to immunosuppressants. However, diagnosis is still challenging, as typical histologic findings are not mandatory, and in many cases the clinical and laboratory constellation is not conclusive. Moreover, liver biopsy remains a diagnostic procedure with a low, but perceivable risk. The present study investigated the diagnostic performance of an IDB applied to sera of AIH type 1 patients who had been diagnosed on the basis of the 1999 IAHG scoring system (6).

TABLE 3. Diagnostic Algorithm I: Consecutive Testing for ANA/ASMA-IFT and AAA

Procedure	No. of patients	No. of controls	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Eff. (%)	LR+	LR−
ANA/ASMA-IFT	46	100	98	30	32	98	47	1.4	0.07
ANA/ASMA-IFT + AAA-IDB	33	25	70	82	57	89	79	3.9	0.37
ANA/ASMA-IFT + AAA-IFT	24	8	51	94	75	85	84	8.5	0.52

Data show diagnostic performance of serologic tests for AAA-IDB or AAA-IFT in 47 patients and 142 controls that had previously shown positivity for ANA or ASMA. AAA, anti-actin antibodies; ASMA, anti-smooth muscle antibodies; ANA, anti-nuclear antibodies; IDB, immunodot blot; IFT, indirect immunofluorescence; PPV, positive predictive value; NPV, negative predictive value; Eff., diagnostic efficiency; LR+, positive likelihood ratio; LR−, negative likelihood ratio.

The IDB appeared as a diagnostic procedure that was quickly done and easy to perform with a considerable interobserver concordance. Under practical aspects, this feature would qualify the IDB for screening purposes to stratify individuals with elevated liver enzymes to further diagnostic procedures. Unfortunately the IDB under study revealed a moderate sensitivity of 70%, implying that nearly one-third of the patients would be missed under screening conditions. Inclusion of further liver-specific antigens as LKM-1, LC1, and SLA as well exclusion of AMA-M2 did not significantly improve the overall diagnostic performance of the IDB. These antigens may be of greater importance in younger patient populations in which AIH type 2 is more frequent. Particularly, the exclusion of AMA-M2 may be of special interest to rule out primary biliary cirrhosis.

Direct comparison of the IDB to standard IFT procedures revealed a higher sensitivity of the IFT testing for ANA or ASMA that was significantly better than that of AAA-IDB. On the other hand, specificity of IDB and IFT testing for AAA was significantly better than the search for ANA or ASMA alone. Particularly the specificity of AAA-IFT with 94% was excellent, resulting in a remarkable positive predictive value of 75%.

The different diagnostic performance of IDB and IFT in testing for AAA has to be further analyzed. Our results provide evidence that the antigens used comprise substantially different antibody binding specificities, first due to different origins. The IDB antigen was obtained from rabbit skeletal muscle, whereas the IFT incorporates fixed tissue slides from rat organs. Contamination with monomeric G-actin, which may cause nonspecific reactivity with low specificity, had previously been assumed in ELISA procedures (13, 20). Furthermore, the fixation process in IFT with acetone is different from the fixation process in IDB. Denaturation of F-actin conformation may reduce binding sensitivity of actin autoantibodies (21, 22). For the IDB under study, preservation of the biologic active polymeric conformation in filaments of the F-actin epitope had been predicted by the manufacturer, which is now challenged by our findings (23). Compared to a commercially available ELISA, the IDB provided a comparable sensitivity of about 70%, whereas the specificity was significantly inferior (76% vs. 98%; $P < 0.0005$) (13).

By two different approaches, we addressed the question whether the incorporation of the IDB into diagnostic algorithms would be able to improve the diagnostic performance of established serologic tests for AIH type 1. In order to receive highest sensitivity, we initially prescreened for the most prevalent ANA or ASMA, followed by, in theory, the more specific test for AAA either by IDB or IFT. This prescreening for ANA or ASMA did not improve the overall diagnostic performance of AAA-IFT, whereas specificity of the AAA-IDB increased from 76% to 82%, which admittedly was not significant. Thus,

TABLE 4. Diagnostic Algorithm II: Exclusion of Infectious Hepatopathies, Consecutive Testing for ANA/ASMA and/or AAA

Procedure	No. of patients	No. of controls	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Eff. (%)	LR+	LR−
øinfect.+AAA-IDB	33	23	70	83	59	89	80	4.1	0.36
øinfect.+AAA-IFT	24	2	51	99[1)]	92	86	87	51.0	0.49
øinfect.+ASMA/ANA-IFT+AAA-IDB	33	15	70	89	69	90	85	6.4	0.34
øinfect.+ASMA/ANA-IFT+AAA-IFT	24	2	51	99	92	86	87	51.0	0.49

Data represent numbers of patients with AIH ($n = 47$) and controls ($n = 142$) in which infectious hepatopathies were serologically excluded, which were tested positive for the respective (combination of) autoantibodies, and the procedure-specific diagnostic performance. Significant differences between the diagnostic procedures are indicated as follows: [1)] $P < 0.05$. øinfect., serologic exclusion of hepatitis B and C; AAA, anti-actin antibodies; ASMA, anti-smooth muscle antibodies; ANA, anti-nuclear antibodies; IDB, immunodot blot; IFT, indirect immunofluorescence; PPV: positive predictive value, NPV: negative predictive value, Eff.: diagnostic efficiency, LR+: positive likelihood ratio, LR−: negative likelihood ratio.

integration of the IDB into the serologic diagnostic algorithm did not allow either for the affirmation, or for the reliable exclusion of AIH in our study population. Furthermore, under practical aspects the restriction to only one laboratory method is, of course, more efficient.

Due to established tests in the routine laboratory, differentiation of AIH from chronic infectious hepatopathies is of inferior clinical importance. Thus, in a second approach, we applied a diagnostic algorithm that first ruled out hepatitis B or C, followed by testing for autoantibodies. Using these procedures, specificity of the AAA-IFT increased significantly. Although this algorithm yielded a positive LR of 51 and gave rise to a conclusive change from pretest to posttest probability, still nearly half of the patients was missed. Thus, exclusion of infection and detection of AAA-IFT is of use only as a positive predictor, the procedure is not sensitive enough to rule out AIH type 1 in otherwise unclassified patients. Comparing this algorithm to exclusion of infection and testing for AAA-IDB, the IDB was neither sensitive nor specific enough to result in conclusive changes from pretest to posttest probability, although a slight, but not significant increase in specificity was noted. Insertion of a prescreening for ANA or ASMA into the diagnostic algorithm did not further improve the diagnostic performance neither of IDB nor IFT. Contradictory to previous reports, AAA were detected in a significant proportion of hepatitis C infected patients, challenging the antigen binding specificity especially of the IDB under study (24). By contrast, in patients with chronic hepatitis B infection, AAA were less prevalent than previously reported, most probably due to a lower and less-specific cutoff that was previously used (25).

CONCLUSIONS

In conclusion, the IDB under study did neither qualify as a reliable screening tool for AIH type 1, nor as a confirmatory test in individuals in whom AIH was suspected. Blots with higher antigen binding specificity may be more functional.

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