



Autoantibody profiling of patients with primary biliary cirrhosis using a multiplexed line-blot assay



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ABSTRACT

Objective: To evaluate the autoantibody profile in patients with primary biliary cirrhosis (PBC) using a new multiplexed line-blot assay specifically designed for the diagnosis of autoimmune liver diseases.

Methods: Sera of 58 consecutive PBC patients and 191 disease controls (144 with autoimmune liver diseases other than PBC, and 67 with non-autoimmune chronic liver diseases) were tested by both the multiplexed line-blot Autoimmune Liver Disease Profile 2 (ALD2) and by IIF on HEp-2 cells and on rat kidney/liver/stomach tissues. ALD2 contains the following PBC-associated antigens: AMA-M2, natively purified from bovine heart; M2-E3, a recombinant fusion protein including the E2 subunits of PDC, BCOADC and OGDC; sp100, PML and gp210 recombinant proteins.

Results: With the ALD2 assay, a positive reaction to AMA-M2, M2-E3, sp100, PML and gp210 in PBC patients was observed in 77.6%, 84.5%, 34.5%, 15.1% and 18.9%, respectively, of the PBC sera. The overall sensitivity and specificity for PBC were 98.3% and 93.7%. Using IIF, positivity rates to AMA, and to antinuclear autoantibodies with membranous/rim-like and multiple nuclear dot patterns were 86.2%, 8.6% and 22.4%, respectively. The overall sensitivity and specificity for PBC of the IIF method were 86.2% and 97.9%, respectively.

Conclusions: The ALD2 line-blot showed a good diagnostic accuracy for PBC and a higher sensitivity than the IIF method to detect sp100 and gp210 autoantibodies.

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1. Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by the progressive destruction of the small intra-hepatic bile ducts, resulting in cholestasis, portal inflammation, and fibrosis that may lead to cirrhosis and over time to liver failure [1]. Anti-mitochondrial autoantibodies (AMA) detected by the indirect immunofluorescence (IIF) method on rodent kidney/liver/stomach tissues are

the classical specific serological markers of PBC and are found in 90–95% of PBC patients [2]. Due to their high diagnostic value, the presence of AMA represents one of the three criteria required for a definite diagnosis of PBC [3–5].

Berg et al. [6] showed that most of the PBC-specific AMA bind in vitro to a trypsin-sensitive antigen of the inner mitochondrial membrane, and this antigen was later designated M2. Subsequently, the target antigens of AMA-M2 were identified as components of the 2-oxo-acid dehydrogenase complex catalyzing the oxidative decarboxylation of keto-acids [7]. More specifically, the autoantibodies are mainly directed to the E2 subunits of the 2-oxo-acid dehydrogenase complex (pyruvate dehydrogenase complex [PDC-E2], 2-oxoglutarate dehydrogenase complex [OGDC-E2], branched-chain 2-oxoacid dehydrogenase complex

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[BCOADC-E2]), and to a lesser extent to the E1 and E3 subunits. Up to 90% of AMA-M2 antibodies are directed against PDC-E2, whereas about 50% react with OGDC-E2 and BCOADC-E2 [8].

Besides AMA, PBC-specific antinuclear autoantibodies (ANA) with membranous/rim-like and multiple nuclear dots (MND) IIF patterns are detected in approximately 25–30% of patients. In the membranous/rim-like pattern the autoantibodies are directed against gp210 and nucleoporin-62 antigens, while in the MND pattern they are directed against sp100, sp140, promyelocytic leukemia nuclear body proteins (PML) and ubiquitin-like modifier (SUMO) proteins. The detection of these PBC-specific ANA can be used to confirm the diagnosis of PBC in AMA-negative cases, thus increasing the sensitivity of serological tests in diagnosing PBC. Moreover, some authors showed that these ANA are associated with a more severe disease course [9–11].

In addition, other non-PBC-specific ANA, especially those displaying speckled or anti-centromere IIF patterns, may be found in a subgroup of patients, suggesting the presence of a concurrent autoimmune disease (i.e. systemic sclerosis in patients with anti-centromere antibodies) [12].

Following the identification of the AMA and of the PBC-specific ANA target antigens, a number of different analytical methods such as ELISA, dot- or line-blot, or fluorescent bead-based assays, have been developed. These methods use either a mixture of recombinantly produced human E2 subunits [13], or a fusion protein combining all the three E2 subunits [14], or a mixture of native and recombinant antigens [15]. The aim of our study was to evaluate the autoantibody profile in a cohort of consecutive PBC patients and in patients with other autoimmune and non-autoimmune liver diseases, using a new multiplexed line-blot assay designed for the diagnosis of autoimmune liver diseases.

2. Patients and methods

Sera obtained at the diagnosis of 58 consecutive PBC patients diagnosed following the internationally accepted criteria [1,3,4] (mean age, 59.8 years; range, 37–81; male/female ratio, 1:4.3) and 191 disease controls (133 autoimmune hepatitis [AIH], 11 primary sclerosing cholangitis [PSC] diagnosed by established contrast cholangiography [16] and 67 non-autoimmune chronic liver diseases [including 39 liver steatosis, 11 chronic alcoholic liver diseases, 9 cryptogenic cirrhosis, and 8 toxic non-alcoholic liver diseases]) (mean age, 41.1 years; range, 1–81 years; male/female ratio, 1:1.85) were collected in 10 different hospital and university centers in Italy.

All the sera were tested with the multiplexed line-blot Autoimmune Liver Disease Profile 2 (ALD2) (Euroimmun, Lübeck, Germany), containing the following PBC-associated antigens: AMA-M2-natively purified from bovine heart, containing the 74 kDa E2 subunit of the PDC; the M2-3E recombinant fusion protein comprising the immunogenic domains of the E2 subunits of PDC, of the BCOADC and of the OGDC (MIT3) enriched with native PDC antigen; sp100 and PML recombinant proteins (which are autoantigens associated with MND reactivity); gp210 recombinant protein, an integral component of the nuclear pore complex. The ALD2 profile contains also some non-PBC-specific nuclear autoantigens (Ro52, SSA, CENP-A, CENP-B, Scl70) and some autoimmune hepatitis- (AIH-) specific autoantigens (LKM1, LC1 and SLA/LP). Line-blot assay was performed following the manufacturer's instruction. Blot strips were digitalized using a camera and band intensities were determined by a computer program (EUROLineScan, Euroimmun). Signal strengths >10 arbitrary units (AU) were considered positive, as recommended by the manufacturer.

All the sera were also tested by IIF on HEP-2 cells and on rat kidney/liver/stomach tissues (Euroimmun) at a starting serum dilution of 1:40. The slides were read visually by two expert operators (D.V. and M.G.A.) who worked independently.

Diagnostic sensitivity and specificity were calculated for each PBC-associated autoantibody. Cohen's kappa with 95% confidence interval (95% CI) was used to evaluate the analytical agreement among results

obtained with the IIF methods and with the line-blot assay and between AMA-M2 and M2-3E. MedCalc software (Mariakerke, Belgium) was used for statistical analysis.

The study protocol followed the ethical guidelines of the Declaration of Helsinki and informed consent was obtained from all patients included in the study.

3. Results

The autoantibody profiles of PBC patients and controls are shown in Table 1. Using the IIF method, AMA were positive in 86.2% of PBC patients with a specificity of 97.9%. With the ALD2 assay, reactivity to M2-3E-recombinant fusion protein was higher (84.5%) than that to AMA-M2 natively purified from bovine heart (77.6%). M2-E3 also showed a higher specificity than AMA-M2 (98.4% vs. 97.9%).

Positivities to sp100 (34.5%) and gp210 (18.9%) were higher than the corresponding MND (22.4%) and membranous/rim-like (8.6%) ANA pattern positivities observed with the IIF method. Autoantibodies against PML were always associated to autoantibodies to sp100. Autoantibodies to sp100, gp210 and PML, alone or associated, were found in 6/8 (75%) of AMA-IIF-negative PBC patients. Combining the positivity to AMA-M2/ME-E3, sp100, gp210 and PML, the overall sensitivity of the ALD2 assay for PBC was 98.3% and the specificity 93.7%. Combining the results of the PBC-associated IIF patterns (AMA-IIF on rodent tissues and ANA on HEP-2 cells), the overall sensitivity and specificity were 96.6% and 96.3%, respectively.

Among controls, PBC-specific autoantibodies were detected in 10 patients with AIH using the line-blot assay (three AMA-M2 + M2-3E, one AMA-M2, three sp100, one sp100 + PML, two gp210), and in seven patients using the IIF method (four AMA, two MND and one membranous/rime-like positive). Only one of these patients (AMA-M2

Table 1
Demographic and laboratory features of PBC patients and control subjects.

	PBC	Controls
Number	58	191
Mean age (range)	59.8 (37–81)	41.1 (2–87)
Female	47	124
Male	11	67
<i>Indirect immunofluorescence</i>		
AMA	50 (86.2%)	4 (2.1%) [AIH: 2, unclassified: 1; alcoholic: 1]
Multiple nuclear dots	13 (22.4%)	2 (1.0%) [AIH: 1, PSC: 1]
Rim-like/membranous	5 (8.6%)	1 (1.0%) [AIH: 1]
<i>Multiple line immunoblot assay (ALD2)</i>		
AMA-M2	45 (77.6%)	4 (2.1%) [AIH: 2, PSC: 1, steatosis: 1]
M2-3E	49 (84.5%)	3 (1.6%) [AIH: 2, crypto: 1]
sp100	20 (34.5%)	4 (2.1%) [AIH: 2, steatosis: 1, crypto: 1]
gp210	11 (18.9%)	2 (1.0%) [AIH: 2]
PML	9 (15.1%)	1 (0.5%) [AIH: 1]
AMA-M2/M2-3E alone	28 (48.2%)	
sp100 alone	2 (3.4%)	
gp210 alone	1 (1.7%)	
PML alone	0 (0%)	
AMA-M2/M2-3E + sp100	6 (10.3%)	
AMA-M2/M2-3E + gp210	7 (12.1%)	
AMA-M2/M2-3E + sp100 + gp210	2 (3.4%)	
AMA-M2/M2-3E + sp100 + PML	7 (12.1%)	
sp100 + gp210	1 (1.7%)	
sp100 + PML	2 (3.4%)	
CENP-A	3 (5.2%)	3 (1.6%)
CENP-B	3 (5.2%)	8 (4.2%)
Scl-70	1 (1.7%)	4 (2.1%)
Ro60	5 (8.6%)	2 (1.0%)
Ro52	12 (20.7%)	10 (5.2%)

and M2-3E positive) was subsequently diagnosed with AIH-PBC overlap syndrome.

Almost perfect agreement was shown between the IIF and the line-blot ALD2 method for AMA detection, whereas substantial agreement was shown between the MND ANA pattern and sp100 and only moderate agreement occurred between the membranous/rime-like ANA pattern and gp210 (Table 2).

Three patients with PBC (5.2%) resulted positive for anti-centromere (CENP-A and CENP-B) autoantibodies. One of them had an associated diagnosis of limited cutaneous systemic sclerosis and another of isolated Raynaud's phenomenon. No signs or symptoms suggestive of systemic sclerosis were observed in the only one PBC patient who was positive for anti-topoisomerase I (Sc170), nor in the four anti-Sc170-positive controls. However, since in all these cases a low autoantibody level (<15 AU) was detected, the possibility of a false positive reaction cannot be excluded. Finally, in spite of the relevant percentage of PBC patients who were positive for Ro60 or Ro52 antibodies, none of them were shown to be affected by an associated systemic autoimmune rheumatic disease.

4. Discussion

Accurate detection of AMA is important for establishing diagnosis or predicting disease onset of PBC [17]. The IIF method on tissue sections has been considered for a long time the gold standard for AMA detection; however, the IIF technique is time consuming, observer dependent and cannot be fully automated. In addition, it is unable to provide information regarding the antigenic specificity and, in the presence of concurrent antibody reactivity, the IIF pattern may be difficult to interpret. Recently released ELISA and immunoblot assays may overcome the limitations of the IIF method and combining the use of AMA, sp100, gp210 and PML antigens may represent a more rapid and cost-effective method than IIF to detect PBC-associated autoantibodies. In this study we evaluated the diagnostic accuracy of the multiplexed line blot ALD2 assay and compared the results with those obtained with the IIF assays.

Concerning AMA, the IIF method proved to be the most sensitive, since among the IIF AMA-positive sera, one resulted negative for M2-E3 and five for AMA-M2 with the line-blot assay. The data obtained with the AMA-M2 line-blot are similar to those previously reported by some authors, who showed that ELISA methods based on purified PDC from porcine or bovine heart mitochondria may fail to detect AMA in 4%–13% of cases of PBC, because of the lack of the BCOADC-E2 and/or OGDC-E2 antigens in the solid phase [18–20]. On the other hand, in a large cohort of IIF AMA-positive PBC patients, Liu et al. [21] using an ELISA based on a recombinant fusion protein including the E2 immunodominant portions of PDC, BCOADC and OGDC (MIT3) showed a diagnostic sensitivity of 92.4%, lower than that obtained in our study (98.0%) using a similar antigenic preparation enriched, however, with native PDC antigen. The higher sensitivity of the ALD2 assay compared to the ELISA methods may be thus attributable to the ability of the former to detect antibodies against the subdominant PDC-E1 epitope or other mitochondrial antigenic subunits [15]. However, even considering the higher reactivity to the M2-E3 antigenic preparation compared to ELISA methods, the sensitivity of ALD2 line-blot for AMA detection

remains slightly lower than that obtained with the IIF assay. As in our patient cohort, the diagnosis of PBC was based on the detection of AMA using the IIF technique, we cannot exclude that using the line-blot as the diagnostic test, additional patients could have been classified as AMA positive. This hypothesis seems to be confirmed by the results of the studies of Liu et al. [21] and of Bizzaro et al. [22], in which 28.1% and 11.0%, respectively, of IIF AMA-negative PBC sera were positive with an ELISA assay using the MIT3 antigen.

An advantage of the evaluated multiplexed line-blot is the simultaneous detection of other PBC specific autoantibodies in addition to AMA, increasing the diagnostic sensitivity, especially in AMA-negative cases or when the serological picture is unclear. In our study, 6/8 (75%) patients that were IIF AMA-negative, were positive for sp100 and/or for gp210, a much higher percentage than that found in previous studies [22–24]. This finding may be attributable to the low number of AMA-negative sera included in our series. However, it is important to remark that the ALD2 line-blot was more sensitive than IIF to detect these ANA-associated autoantibodies, with a similar specificity. These data are apparently discordant with the data obtained by Bizzaro et al. [22] that found a concordance of 92% between the MND ANA pattern and sp100, and of 99% between the rim-like/membranous ANA pattern and gp210. However, in the study of Bizzaro et al., only IIF AMA negative patients were evaluated, whereas our study was performed in consecutively enrolled PBC patients (86.2% AMA positive). Therefore, the higher sensitivity of the line-blot may be explained by the difficulty of identifying MND and rim-like/membranous ANA patterns with the IIF method when there is the concurrent presence of AMA. Combining all the PBC-specific autoantibodies, the overall sensitivity and specificity of the ALD2 assay for PBC were 98.3% and 93.7%, respectively, compared to 96.6% and 96.3% of the IIF assays. With the ALD2 multiplexed line-blot, PBC-specific autoantibodies were also detectable in 10/133 (7.5%) patients with AIH. According to the guidelines of the European Association for the Study of Liver (EASL) [4], only one of these patients was classified as affected by AIH-PBC overlap syndrome. The percentage of autoantibody positivity (12.8%) was comparable to that obtained in the study of Liu et al. [21] in 281 AIH patients using an ELISA PBC screen assay containing MIT3, gp210 and sp100 antigens. However, the clinical impact of the presence of PBC-specific autoantibodies and in particular of AMA in AIH patients is uncertain and probably without real clinical significance since studies have reported that clinical features and histological findings of AIH are not influenced by the AMA status over many years of follow-up [25,26].

Finally, the finding of some specific antinuclear autoantibody markers of connective tissue diseases using the ALD2 line-blot, may be regarded as false positive reactions or may suggest the likelihood of the presence of another concurrent autoimmune disease. Indeed, PBC is frequently associated with other autoimmune diseases such as systemic sclerosis, Sjögren's syndrome, rheumatoid arthritis, autoimmune thyroiditis and celiac disease. In our study, one of the three PBC patients that were anti-centromere positive, was affected by limited systemic sclerosis and another one had Raynaud's phenomenon. In addition, recent studies have indicated that anti-centromere may have prognostic value. In particular, anti-centromere positive PBC patients seem to progress more rapidly to liver failure [27], have higher serum alkaline phosphatase levels [28] and greater frequency of portal hypertension [29].

Table 2

Agreement among results obtained with the IIF methods and with the line-blot assay and between AMA-M2 and M2-3E.

	Overall agreement (95% CI)	Positive agreement (95% CI)	Negative agreement (95% CI)	Cohen's kappa (95% CI)
AMA-M2 vs M3-E3	0.964 (0.935–0.983)	0.911 (0.853–0.968)	0.977 (0.962–0.992)	0.888 (0.817–0.959)
AMA-IIF vs AMA-M2	0.952 (0.917–0.975)	0.882 (0.816–0.945)	0.969 (0.952–0.986)	0.852 (0.770–0.934)
AMA-IIF vs M2-E3	0.976 (0.948–0.991)	0.942 (0.896–0.988)	0.984 (0.973–0.997)	0.927 (0.869–0.984)
ANA (MND) vs sp100	0.9558 (0.922–0.977)	0.717 (0.558–0.878)	0.976 (0.961–0.990)	0.695 (0.527–0.863)
ANA (membranous/rim-like) vs gp210	0.968 (0.938–0.986)	0.556 (0.279–0.831)	0.983 (0.971–0.994)	0.543 (0.265–0.817)

We are unable to confirm these data, because of the low number of anti-centromere positive PBC patients. Anti-Ro60- or anti-Ro52-positive PBC patients did not present signs or symptoms of systemic autoimmune diseases, but we cannot exclude their onset during the follow-up.

In conclusion, the multiplexed ALD2 line-blot showed good diagnostic accuracy for PBC and higher sensitivity than the IIF method to detect sp100 and gp210 autoantibodies. The recombinant fusion protein (M2-E3) tested as more sensitive than the natively purified bovine heart antigenic preparation for AMA detection. In general laboratories, where the prevalence of PBC-associated autoantibodies is usually low, the IIF method may still represent a cost effective first-line technique. The ALD2 multiplexed line-blot would then be an excellent assay to confirm the IIF results or to evaluate cases where the clinical presentation and/or serological picture is unclear. In specialized referring laboratories, where the prevalence of PBC-associated autoantibodies is higher, the chance to test simultaneously all the relevant PBC-specific antibodies and to better define the AMA sub-specificities likely makes the use of the ALD2 line-blot as a first-line assay a more advantageous and rapid option.

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