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• *REVIEW* •

Enzyme inhibition assay for pyruvate dehydrogenase complex: Clinical utility for the diagnosis of primary biliary cirrhosis

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Abstract
Primary biliary cirrhosis (PBC) is usually diagnosed by the presence of characteristic histopathological features of the liver and/or antimitochondrial antibodies (AMA) in the serum traditionally detected by immunofluorescence. Recently, new and more accurate serological assays for the detection of AMA, such as enzyme-linked immunosorbent assay (ELISA), immunoblotting, and enzyme inhibition assay, have been developed. Of these, the enzyme inhibition assay for the detection of anti-pyruvate dehydrogenase complex (PDC) antibodies offers certain advantages such as objectivity, rapidity, simplicity, and low cost. Since this assay has almost 100% specificity, it may have particular applicability in screening the at-risk segment of the population in developing countries. Moreover, this assay could be also used for monitoring the disease course in PBC. Almost all sera of PBC-suspected patients can be confirmed for PBC or non-PBC by the combination results of immunoblotting and enzyme inhibition assay without histopathological examination. For the development of a "complete" or "gold standard" diagnostic assay for PBC, similar assays of the enzyme inhibition for anti-2-oxoglutarate dehydrogenase complex (OGDC) and anti-branched chain oxo-acid dehydrogenase complex (BCOADC) antibodies will be needed in future.

Key words: Primary biliary cirrhosis; Enzyme inhibition assay; Antimitochondrial antibody; 2-oxo-acid dehydrogenase complex

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INTRODUCTION
Primary biliary cirrhosis (PBC) is a chronic autoimmune cholestatic liver disease characterized by the destruction of small and medium-sized bile ducts and the presence of antimitochondrial antibodies (AMA) in the serum traditionally detected by immunofluorescence[1,2]. The "gold standard" procedure for the diagnosis of PBC is histopathological examination of liver tissue. However, the characteristic histopathological changes of PBC are not always evident in biopsy specimens. Therefore, serological examination such as AMA is useful for the diagnosis of PBC because this is non-invasive and therefore can be repeated throughout the course of the disease. The major mitochondrial autoantigens recognized in the sera of PBC patients are members of 2-oxo-acid dehydrogenase complex (2-OADC) family, including E2 subunit of pyruvate dehydrogenase complex (PDC-E2), E2 subunit of branched chain oxo-acid dehydrogenase complex (BCOADC-E2), and E2 subunit of 2-oxoglutarate dehydrogenase complex (OGDC-E2)[1,3]. Unfortunately, however, there is so far no "gold standard" assay (i.e., with 100% sensitivity and 100% specificity) for the detection of AMA in PBC.

PBC is present among various ethnic and racial populations, but its incidence and prevalence varies quite widely, from the highest among Northern European populations to vanishingly low in certain parts of Asia[3]. This difference may be due, at least in part, to the diagnostic awareness of physicians for asymptomatic cases. Therefore, reliable and easy-to-use tool for screening PBC in general population is needed.

Serological assays for the detection of AMA
AMA is one of the most diagnostically useful of all autoimmune markers, since both the sensitivity and specificity for the diagnosis of PBC are acceptably high[1]. Indirect immunofluorescence assay using either Hep-2 cells or mouse kidney/stomach sections as the substrate and enzyme-linked immunosorbent assay (ELISA) using semipurified PDC as the antigen source are now widely used in clinical laboratories. Traditional indirect immunofluorescence assay has high sensitivity, and can detect reactivity to all 2-OADC enzymes. However, this assay is non-automated and labor-intensive, and the "readout" is subjective. The reactivity of serum with mitochondrial antigens other than PBC specific 2-OADC enzymes and nonspecific staining or high background could influence its specificity and sensitivity[3]. The

sensitivity, specificity, positive predictive value, negative predictive value, and accuracy determined in our previous study were 89%, 99%, 98%, 94%, and 95%, respectively[4].

Recently, new and more accurate serological assays for the detection of anti-2-OADC, such as ELISA, immunoblotting, and enzyme inhibition assay, has been developed. ELISA can detect more precisely the reactivity to a single 2-OADC enzyme in each run, and is non-subjective readout. Recently, more sensitive ELISAs using PDC-E2, BCOADC-E2 and OGDC-E2 as coating antigens have been developed[5-8]. In ELISA using commercially available MESACUP-2 Test Mitochondria M2 kit (Medical & Biological Laboratories Co., Nagoya, Japan), the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy are 90%, 98%, 95%, 96%, and 94%, respectively[9]. Immunoblotting has been reported to have almost 100% sensitivity, and can detect individual reactivity to 2-OADC enzymes[10,11]. In our immunoblotting assay condition, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were 99%, 86%, 89%, 99%, and 93%, respectively[11]. However, this assay is labor intensive, and can only be performed in specialized laboratories. Moreover, its specificity has not been well established[12]. The enzyme inhibition assay, which measures the capacity of PBC sera to inhibit the catalytic activity of PDC, is non-subjective compared to immunofluorescence, is more rapid and technically simpler than immunoblotting and ELISA. This assay has almost 100% specificity[6,13-16], but the sensitivity has been reported to be around 80%[6,15,16]. This lower sensitivity can be explained by the fact that this assay does not detect the inhibitory activity of sera to 2-OADC enzymes other than PDC, such as BCOADC or OGDC.

Enzyme inhibition assay

A striking property of AMA in PBC sera is their capacity to rapidly inactivate the catalytic function of 2-OADC *in vitro*[17]. Enzyme inhibition assay has been utilized to demonstrate a population of autoantibodies in PBC sera that inhibit enzyme function, and a miniaturized semiautomated enzyme inhibition assay was developed for the detection of anti-PDC antibodies in PBC in 1991[18] (Figure 1). Several subsequent studies have assessed its objectivity, rapidity, simplicity, and costeffectiveness, by comparing these parameters to other assays such as immunofluorescence, ELISA, and immunoblotting[13,15]. Immunoblotting might be the most expensive, and enzyme inhibition assay might have most cost-effectiveness in many countries. Recently, an automated enzyme inhibition assay kit, TRACE enzymatic mitochondrial Antibody (M2) assay (EMA) kit (Thermo Trace, Victoria, australia), became available commercially. In principle, the "Substrate reagent" (250 μL) containing sodium pyruvate, magnesium acetate, cocarboxylase, coenzyme A, and nicotinamide adenine dinucleotide (NAD), is placed in flat-bottomed microtiter wells. Thereafter, 4 μL of undiluted test serum is added to each well and incubated for 1 min at 37 °C before adding 50 μL of the "Enzyme reagent" containing

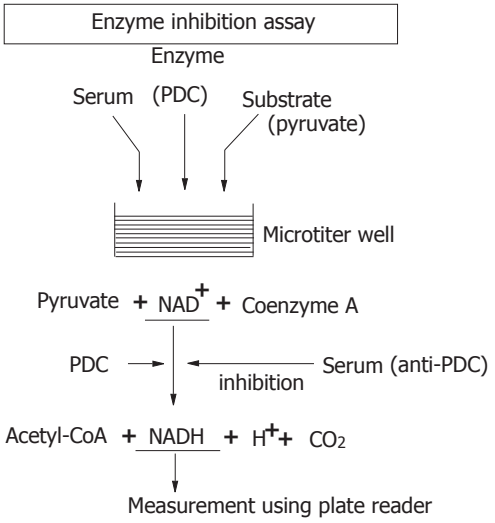

Figure 1 Principle of enzyme inhibition assay.

pyruvate dehydrogenase and dithiothreitol. After 30 s of lag time, the rate of reaction is monitored by measuring the rate of increase in absorbance at 340 nm in a microplate reader. The reaction rate (RR) is calculated using the absorbance values at 0 and 2 min based on the following formula: (final absorbance - initial absorbance)/time. The units of activity (%) are derived from the formula: (test RR/standard RR) x 100. The standard RR is derived from the "Calibrator" wells that contain anti-PDC antibody-free serum (100% activity). The unit of PDC activity of less than 70% is considered as anti-PDC positive with sensitivity and specificity of 82% and 100%, respectively, based on the information provided by the manufacturer[16] (Figure 2). By using this kit, Schmit *et al*[14] tested the enzyme inhibition assay for 23 sera from patients with AMA-positive PBC and 92 sera from non-PBC including healthy controls, and compared the results to those of immunofluorescence and in-house ELISA. They reported that the sensitivity and specificity of EMA were quite sufficient compared to other assays. Our previous

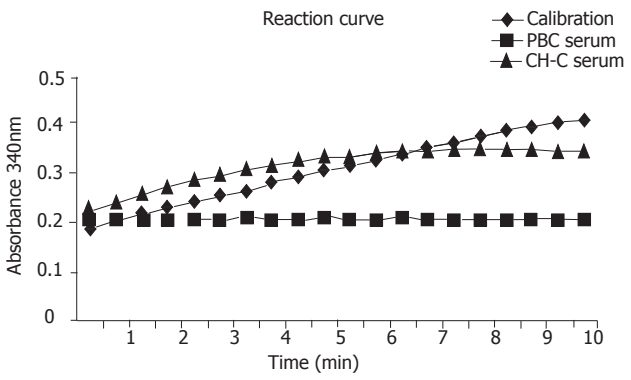

Figure 2 Representative reaction curve of automated enzyme inhibition assay using TRACE enzymatic mitochondrial antibody (M2) assay (EMA) kit. PBC; primary biliary cirrhosis, CH-C; chronic hepatitis C.

Table 1 Detection of enzymatic inhibitory antibody to PDC by enzyme inhibition assay in non-PBC s

First author	Year	Case studied	Antigen source	Serum amount (μL)	Serum dilution	Positivity rate (%)[1]
Teoh	1991	Normal subject, AIH, ALD, RA, SLE	Commercial PDC	100[2]	1:500	0 / 62 (<1.7)
Teoh	1994	Healthy subject, immunopathic diseases	Commercial PDC	100[2]	1:500	0 / 42 (<2.4)
Omagari	1996	Adult blood donors, healthy women	Commercial PDC	2	Undiluted	0 / 186 (<0.6)
Schmit	1999	Healthy controls, viral hepatitis, AIH	TRACE EMA kit	4	Undiluted	0 / 92 (<1.1)
Jois	2000	Normal subject, AIC, AIH, ALD, RA, SLE, etc.	Commercial PDC	2	Undilute	4 / 1055 (0.4)
Hazama	2000	Healthy subject, ALD, viral hepatitis, fatty liver	TRACE EMA kit	4	Undiluted	0 / 50 (<2.0)
Jensen	2000	AIH, abnormal LFT patients, Normal blood donors, etc.	TRACE EMA kit	4	Undiluted	0 / 250 (<0.4)
Masuda	2002	Healthy subject, ALD, viral hepatitis, fatty liver, etc.	TRACE EMA kit	4	Undiluted	0 / 130 (<0.8)
Hazama	2002	Healthy subject, ALD, viral hepatitis, fatty liver, etc.	TRACE EMA kit	4	Undiluted	0 / 97 (<1.1)

[1]When the numerator is zero, the percentage is calculated as a nominal value of <1.
[2]100 μL of doubling dilutions of serum from 1:500 in phosphate-buffered saline solution. PDC, pyruvate dehydrogenase complex; PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; ALD, alcoholic liver disease; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; EMA kit,Enzymatic mitochondrial Antibody (M2) Assay kit; AIC, autoimmune cholangitis; LFT; liver function tests.

study of EMA indicated sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of 72%, 100%, 100%, 87%, and 90%, respectively. We also concluded that EMA is useful for the diagnosis of AMA-positive PBC and could be used to monitor the disease course of PBC, particularly due to the small amount of serum requested, objective read-out, and rapid turnaround time[16,19]. Jensen *et al*[6] also reported that the sensitivity and specificity of EMA were 83% and 100%, respectively, and the EMA compared favorably against commercial ELISA methods.

Clinical utility of enzyme inhibition assay in PBC

Enzyme inhibition assay including commercially available EMA kit has the advantages of few procedural steps, small amount of test serum requested (only 4 μL of undiluted test serum), rapid turnaround time (approximately 6 min for 10 serum samples), and non-subjective readout[14,19]. Moreover, this assay has almost 100% specificity (Table 1). This means that if the serum is positive for enzymatic inhibitory antibody to PDC by enzyme inhibition assay, the diagnosis of PBC is almost confirmed. The result from our laboratory is in line with this finding because none of 245 non-PBC sera was positive for enzymatic inhibitory antibody to PDC by enzyme inhibition assay, whereas 96 (76%) of 127 PBC sera were positive (detailed data are not shown). Therefore, enzyme inhibition assay may have particular applicability in screening the at-risk segment of the population, middle-aged to elderly females[15]. This assay is also applicable in developing countries due to its objectivity, rapidity, simplicity, and low cost. However, this assay may not be suitable for screening in a particular country or area such as Japan. This assay has relatively low sensitivity compared with that of immunofluorescence and immunoblotting due to the lower frequency of autoantibodies to PDC-E2 among the Japanese compared with Caucasian patients with PBC, and a correspondingly higher frequency of antibodies to E2 subunits of the other 2-OADC enzymes[20].

This assay could also be used for monitoring the disease course in PBC. In our previous study, we determined the serial changes in enzymatic inhibitory antibody to PDC by enzyme inhibition assay using EMA kit in Japanese patients with PBC[19]. The units of PDC activity by EMA correlated significantly and inversely with AMA titers by immunofluorescence, and serum reactivity to PDC-E2 by immunoblotting, respectively. Indeed, in three patients who showed a decrease in AMA titers by immunofluorescence, AMA titers correlated more with EMA results than immunoblotting. Moreover, in a patient with fluctuating AMA titers by immunofluorescence, the units of PDC activity by EMA paralleled AMA titers[19] (Figure 3). These data suggested that PBC disease course might influence the EMA results.

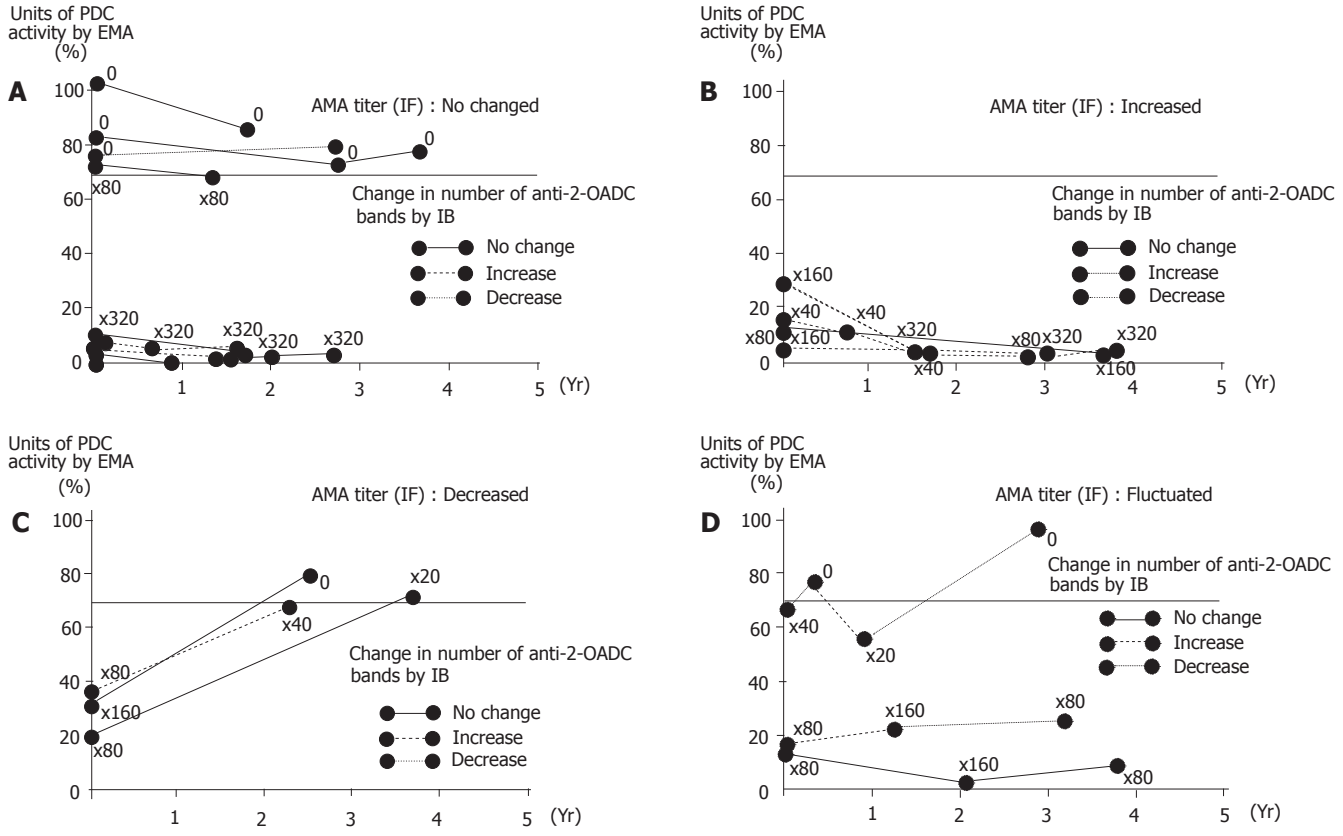

Figure 3 The units of pyruvate dehydrogenase complex (PDC) activity by enzymatic mitochondrial antibody (M2) assay (EMA) did not significantly vary in all the 9 patients in whom the titer of antimitochondrial antibodies (AMA) by immunofluorescence (IF) did not change during the course of follow-up. However, the number of anti-2-oxo-acid dehydrogenase complex (anti-2-OADC) bands by immunoblotting (IB) changed in 4 of these patients (2 increased and 2 decreased) (**A**). The units of PDC activity by EMA decreased in all 4 patients whose AMA titers by IF had increased, but the number of anti-2-OADC bands by IB did not change in 1 patient (**B**). The units of PDC activity by EMA increased in all 3 patients in whom AMA titers by IF had decreased, but the number of anti-2-OADC bands by IB in these patients did not decrease (**C**). Of the 3 patients in whom AMA titers by IF showed some fluctuation, one showed fluctuation in the units of PDC activity by EMA and two showed fluctuation in the number of anti-2-OADC bands by IB (**D**).

Table 2 Interpretation of detection of anti-2-OADC by immunoblotting and enzyme inhibition assay

Immunoblotting (IgG/IgM/IgA)	Enzyme inhibition assay	Interpretation	Estimated percentage (%)
Positive	Positive	PBC	72 - 83
Positive	Negative	Non-anti-PDC positive PBC	10 - 25
		Immunoblotting false positive?	10 - 15
Negative	Positive	Enzyme inhibition assay false positive?	Very rare
Negative	Negative	Non-PBC	Very rare

These data are based on our results that the sensitivity of immunoblotting for PBC is almost 100%, and the specificity of enzyme inhibition assay for PBC is nearly 100%, i.e., when the result by immunoblotting is negative, the serum is not from PBC, and when the result of enzyme inhibition assay is positive, the serum should be from the patient with PBC. 2-OADC, 2-oxo-acid dehydrogenase complex; PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase complex.

Interpretation of detection of anti-2-OADC by immunoblotting and enzyme inhibition assay in PBC

Clinically, the serological diagnosis of PBC is in most instances based on the detection of AMA by indirect immunofluorescence and/or ELISA. As mentioned above, however, these two assays are not yet the "gold standard" (i.e., with 100% sensitivity and 100% specificity) for the detection of AMA in PBC, although both the sensitivity and specificity of these two assays are acceptably high. Based on the fact that immunoblotting has almost 100% sensitivity, and enzyme inhibition assay has almost 100% specificity, interpretation of anti-2-OADC results by combination of these two assays in PBC sera can be established. For example, since the negative predictive value of immunoblotting is 99%, a negative result by immunoblotting means that the serum is not from a patient with PBC. Since a positive predictive value of enzyme inhibition assay is 100%, a positive result by enzyme inhibition assay means that the serum should be from a patient with PBC. When the serum sample is positive for anti-2-OADC by immunoblotting but negative for anti-PDC by enzyme inhibition assay, it can be from non-anti-PDC positive PBC or the result of immunoblotting may be false positive. Conversely, when the serum is negative for anti-2-OADC by immunoblotting but positive for anti-PDC by enzyme inhibition assay, the result of enzyme inhibition assay may be false positive (Table 2). Thus, almost all sera from PBC-suspected patients can be confirmed for PBC or non-PBC by the combination results of immunoblotting and enzyme

inhibition assay without histopathological examination. For the development of a "complete" or "gold standard" (100% sensitivity and 100% specificity) diagnostic assay for PBC, similar assays of the enzyme inhibition for anti-OGDC and anti-BCOADC antibodies (or one-step assay for anti-PDC, OGDC, and BCOADC antibodies) will be needed in the future.

Conclusions

For the diagnosis of PBC, enzyme inhibition assay may have particular applicability in screening the at-risk segment of the population since this assay has almost 100% specificity. This assay is also applicable in developing countries due to its objectivity, rapidity, simplicity, and low cost.

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