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【 ORIGINAL ARTICLE 】

A Comparison of Line Blots, Enzyme-linked Immunosorbent, and RNA-immunoprecipitation Assays of Antisynthetase Antibodies in Serum Samples from 44 Patients

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Abstract:

Objective To determine the differences between anti-aminoacyl tRNA synthetase (ARS) antibodies among line blots, enzyme-linked immunosorbent assay (ELISA) anti-ARS tests, and RNA-immunoprecipitation (IP) assays.

Methods Sera from patients with confirmed or suspected antisynthetase syndrome (ASS) that were positive for either the anti-ARS test or the line-blot assay were used to perform an RNA-IP assay and ELISA to detect individual anti-ARS antibodies.

Results Among the 44 patients, 10 were positive only in line-blot assays, 6 were positive only in the anti-ARS test, and 28 were positive in both assays. We compared the accuracy of these assays against the gold standard RNA-IP assay. The κ coefficient was 0.23 in the line-blot assay, but this increased to 0.75 when the cut-off was increased from 1+ to 2+. The κ coefficient was 0.73 in the anti-ARS test. The κ coefficient was 0.85 for positivity in both assays. Patients with ASS that was positive in an RNA-IP assay more frequently had mechanic's hand (62.1% vs. 20%: p=0.031), myositis (51.7 vs. 10%: p=0.028) and more ASS symptoms than those who were positive only in line-blot assays (3.48 vs. 2.2: p=0.019).

Conclusions Clinicians need to understand the features of each assay and determine diagnoses by also considering clinical presentations. Diagnoses should not be judged based only on the results of line-blot assays due to the risk of a misdiagnosis from false positives.

Key words: antisynthetase syndrome, RNA-immunoprecipitation assay, line-blot assay, anti-synthetase enzyme-linked immunosorbent assay (anti-ARS test)

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Introduction

Anti-aminoacyl tRNA synthetase (ARS) antibodies are myositis-specific autoantibodies associated with clinical characteristics of a condition known as antisynthetase syndrome (ASS). This syndrome has a variety of common clinical symptoms, including polyarthritis, mechanic's hand, myositis, and interstitial lung disease (ILD) (1). Anti-ARS antibodies comprise a common feature of these conditions, but each ARS functions as a specific antigen and thus has specific antibodies. These include anti-Jo-1 (targeting histidyl tRNA synthetase), anti-PL7 (threonyl), anti-PL12 (alanyl), anti-OJ (isoleucyl), anti-KS (asparaginyl), anti-EJ (glycyl), anti-Zo (phenylalanyl), and anti-Ha (tyrosyl) antibodies (2-4). A single patient rarely produces multiple overlapping antibodies, so the antibodies in each patient result in the manifestation of unique clinical symptoms, progression, and a prognosis (5-10).

The gold standard for evaluating various antibodies in connective tissue diseases is the RNA immunoprecipitation (RNA-IP) assay, but only a few facilities can conduct these

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assays, so the adoption of simpler evaluation methods, including the popular semi-quantitative EUROLINE® line blot assay (Euroimmun Medizinische Labordiagnostika, Lübeck, Germany), has become widespread. One such method is the multianalyte EUROLINE® Myositis Profile 3 line-blot assay (Euroimmun) that detects Mi-2, Ku, PM-Scl, Jo-1, SRP, PL-7, PL-12, EJ, and OJ. However, rates of false positives are higher in this assay than in the RNA-IP assay, and the adjustment of the cut-off for positivity is controversial (11-13).

Given the above limitations, a novel anti-synthetase enzyme-linked immunosorbent assay (ELISA), the MESACUP™ anti-ARS test [Medical & Biological Laboratories (MBL)], was developed in Japan to detect the anti-ARS specificities of five (Jo-1, EJ, PL-7, PL-12, and KS) antibodies in a single kit. Five solid-phase recombinant ARS antigens can be detected in the same well, yielding an anti-ARS antibody-positive result for samples that react with any one of the five antigens, indicating the anti-Jo-1, anti-PL-7, anti-PL-12, anti-KS, or anti-EJ phenotype in patients (14). The results of the MESACUP™ anti-ARS test and the RNA-IP assay closely agree, and anti-ARS test are covered in Japan by National Health Insurance (14). However, to our knowledge, the line-blot, anti-ARS test, and RNA-IP assays have never been compared.

We therefore evaluated 44 patients with suspected or previously confirmed ASS using the domestic anti-ARS and line-blot assays. Samples with positive results in either assay were then validated using the RNA-IP assay followed by an ELISA to identify each anti-ARS antibody. Overall, this study compares the results of each test and discusses how to interpret the test results in routine practice.

Materials and Methods

Participants

Among the outpatients who visited the Department of Rheumatology and Clinical Immunology at Toyama University Hospital between February 2013 and June 2015, 270 were assessed using anti-ARS test and line-blot assays due to clinical findings of dermatomyositis (DM), polymyositis (PM), ILD, or polyarthritis. After the results showed that 44 patients were positive for anti-ARS antibodies in either test, these patients provided serum samples for a further evaluation using RNA-IP assays at the Department of Neurology, Keio University. MBL was also asked to run tests on the same sera, and an ELISA was performed to validate the identity of each ARS antibody.

All patient information was rendered innominate for the outsourced tests. The Ethics Committee of Toyama University Hospital approved this study (No. R2020154), and the patients provided their written informed consent via an opt-out form on our hospital website.

Anti-ARS test

The MBL MESACUP™ anti-ARS assay determines positivity when antigen reactivity exceeds the cut-off stipulated in the kit. This kit enables the simultaneous evaluation of the anti-ARS antibodies, anti-Jo-1, anti-PL-7, anti-PL-12, anti-KS, and anti-EJ, but not OJ. The five antigens are fixed as a solid phase in the ELISA, and reactivity to serum is determined. Even when results are positive for one of the antibodies, this kit does not define the antibody (14). We therefore added further evaluations to identify specific anti-ARS antibodies in each sample.

Detection of individual anti-ARS antibodies using an ELISA

Each ARS antibody was detected using an ELISA. The antigen comprised purified recombinant ARS coated onto wells of 96-well Maxisorp microtiter plates (Nalge Nunc International, Rochester, USA). The PL-12, EJ, PL-7, KS, and Jo-1 antigens were diluted in phosphate-buffered saline (PBS) to a final concentration of 5 mg/mL, and 100-μL/well portions were incubated overnight at 4℃. The plates were washed twice with PBS, and then non-specific binding was blocked overnight at 4℃ with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose. Sera from patients and healthy donors were diluted 1:100 in PBS containing 0.15% Tween 20 (PBS-T), 1% casein enzymatic hydrolysate, and 0.2 mg/mL *Escherichia coli* extract, and then 100-μL samples were added to each well. After incubation for 60 min at room temperature (RT), the wells were washed 4 times with PBS-T. Thereafter, 100 μL/well of peroxidase-conjugated goat anti-human IgG (Code No. 208, MBL) diluted 1:5,000 in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxyphenylacetic acid (peroxidase stabilizer) was incubated for 30 min at RT. The wells were washed 4 times with PBS-T and incubated for 5 min at RT with 3,3',5,5'-tetramethylbenzidine substrate. The reaction was stopped by adding 100 μL of 0.25 N sulfuric acid, and then absorbance was read at 450 nm (A450). The reference was absorbance from the healthy control, and the highest absorbance for antigen reactions determined the identity of the anti-ARS antibody.

Patient 16 had anti-Jo-1 and anti-KS antibodies, which is unusual. Therefore, we evaluated this sample using suppression tests. We applied Jo-1 and KS as sensitizing antigens (10 μg/mL) to Jo-1/KS solid phase plates, added serum from Patient 16, and then measured changes in absorbance to determine response rates.

Line-blot assays

Myositis-associated autoantibodies were detected using EUROLINE® Blot test kits as described by the manufacturer. This kit detected anti-Jo-1, anti-EJ, anti-PL-7, anti-PL-12, anti-OJ, anti-Mi-2, anti-SRP, anti-Ku, anti-PM-Scl75, anti-PM-Scl100, and anti-Ro-52 antibodies. The respective recombinant proteins were separately coated onto nitrocellulose membranes. The results are arbitrarily defined according to the manufacturer as negative (0), borderline (+), positive (1+ or 2+), or strongly positive (3+), but we determined

(0) and (+) as negative. Sera that were positive (1+, 2+, or 3 +) for anti-Jo-1, anti-EJ, anti-OJ, anti-PL-7, or anti-PL-12 antibodies were selected in this analysis.

RNA-IP assays

Frozen serum samples were stored at -70°C. Autoantibodies were detected using RNA-IP assays and HeLa cell extracts at Keio University by investigators who were blinded to the clinical information of the patients. In brief, serum (10 μg) was incubated for 2 h with 2 mg of protein A-Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) in 500 μg of immunoprecipitation buffer (10 mM Tris HCl, pH 8.0, 500 mM NaCl, 0.1% Nonidet P40). After three washes with immunoprecipitation buffer, antibody-bound Sepharose beads were incubated for 2 h with 100 μg of HeLa cell extract (6×10^6 cell equivalents per sample), and then bound RNA was extracted with 30 μg of 3 M sodium acetate, 30 μg of 10% sodium dodecyl sulfate, and 300 μg of phenol: chloroform:isoamyl alcohol (50:50:1, containing 0.1% 8-hydroxyquinoline). The RNA was precipitated with ethanol, resolved by 7 M urea-8% polyacrylamide gel electrophoresis, and then stained with silver (Bio-Rad Laboratories, Hercules, USA). The antisynthetase antibodies were considered positive if serum samples produced lines with immunological identity to the six reference sera according to RNA-IP assays. We distinguished the anti-ARS antibodies, anti-Jo-1, anti-PL-7, anti-KS, anti-EJ, anti-OJ, and anti-PL-12 using RNA-IP assays.

Data collection

The following information about the patients was extracted from electronic medical records at the time of the anti-ARS antibody tests: the age, sex, final diagnosis based on the overall clinical course, glucocorticoid treatment, pulmonary function test results, prognosis, malignancies within two years before or after a diagnosis of suspected/confirmed ASS, a disease-related fever, arthritis, Raynaud phenomenon, mechanic's hand, myositis, or ILD, results for anti-Jo-1 antibodies using an ELISA or Ouchterlony tests of serum samples obtained at different times.

Statistical analyses

The sensitivity and specificity of each test were compared with those of the RNA-IP assay, which is the gold standard for detecting anti-ARS antibodies. Inter-rater agreement was calculated using the Cohen kappa coefficient: values of 0-0.4, 0.41-0.6, 0.61-0.8 and 0.81-1 indicate poor-to-fair, moderate, substantial, and almost perfect agreement, respectively. The prevalence of each ASS symptom in patients with positive or negative RNA-IP assay results or with positive RNA-IP and single-positive line-blot or anti-ARS test results were compared using Fisher's exact test, and the mean numbers of symptoms were compared using Wilcoxon's rank sum test. Data were statistically analyzed using the EZR (15) and JMP software programs (SAS Institute, Cary, USA).

Results

Results of anti-ARS antibody tests

Table 1 and Figure summarize the test results and clinical presentations of the 44 evaluated patients (mean age, 58 years old; men, n=9; women, n=35; Table 1). The test results were as follows: 10 patients were positive in line-blot assays (patients 1-10), 6 were positive in anti-ARS assays (patients 11-16), and 28 were positive in both tests (double-positive).

Results of RNA-IP assays in line-blot assay single-positive patients 1-10

Of the 10 patients who were positive only in line-blot assays (patients 1-10), 2, 4, 2, 1, and 1 were positive for anti-Jo-1, anti-PL-7, anti-PL12, anti-EJ, and anti-PL-12/anti-OJ antibodies, respectively. Furthermore, the scores of all patients were 1+, except for one with anti-OJ antibodies, who scored 2+. Two patients each in this group who were negative in RNA-IP assays were diagnosed with Sjögren syndrome (SS), rheumatoid arthritis (RA), and ILD, and one each was diagnosed with mixed connective tissue disease (MCTD), systemic sclerosis (SSc), overlap syndrome (PM with SSc), and amyopathic dermatomyositis (ADM). Patients 1 and 2 were anti-Jo-1 antibody-positive on ELISAs repeated at different times, and their clinical courses were not typical of ASS. Patient 1 was diagnosed with primary SS, and patient 2 initially presented with myositis and digital ulcers, and then the finding of SSc led to a diagnosis of overlap syndrome. Anti-MDA5 antibody was later found in patient 6, who had ADM that presented with ILD. However, the first test result suggested that this patient was anti-PL-7 antibody-positive, so the patient was successfully treated with corticosteroids and cyclosporin A without the need for combined intravenous cyclophosphamide pulse therapy (IVCY).

Results of RNA-IP assays in anti-ARS test single-positive patients 11-16

The patients in this group were diagnosed with PM (n=3), RA, MCTD, and immune-mediated necrotizing myopathy (IMNM) (n=1 each). Only two (33%) of these patients were positive in the RNA-IP assay, with one each having anti-Jo-1 (patient 11) and anti-KS (patient 12) antibodies that matched in the anti-ARS and RNA-IP assays. However, the results for four of these six patients were contradictory. Patient 13 was positive for anti-Jo-1 antibodies in the ELISA and Ouchterlony assays and had a typical ASS clinical presentation; thus, a false negative in the RNA-IP assay could not be ruled out. Patient 14 was U1-RNP antibody-positive and initially diagnosed with MCTD based on clinical symptoms of Raynaud phenomenon, myositis, and ILD. However, the results of a later anti-ARS test were positive for anti-PL-7 antibodies. Whether these symptoms were due to MCTD

Table 1. Test Results of Antisynthetase Antibodies Measured Using the Line-blot Assay, Anti-ARS Test, and RNA-IP Assay and Clinical Diagnosis of 44 Cases.

No.		Line-blot assay*	Anti-ARS test†	RNA-IP Assay	Age/Sex	Clinical diagnosis
1	Line-blot assay single positive cases	Jo-1: 1+	4.9 (negative)	negative	80 F	SS
2		Jo-1: 1+	7.6 (negative)	negative	44 F	overlap (PM and SSc)
3		PL-7: 1+	5.9 (negative)	negative	69 M	IIP
4		PL-7: 1+	9.3 (negative)	negative	49 F	RA
5		PL-7: 1+	4 (negative)	negative	42 F	MCTD
6		PL-7: 1+	1.7 (negative)	negative	70 F	ADM (anti-MDA5)
7		PL-12/OJ: 1+/2+	8.9 (negative)	negative	74 F	SS
8		PL-12: 1+	4.6 (negative)	negative	58 F	SSC
9		PL-12: 1+	6.7 (negative)	negative	74 F	IIP
10		EJ: 1+	13.8 (negative)	negative	56 M	RA
11	Anti-ARS test single positive cases	negative	112.7 (Jo-1)	Jo-1	48 F	PM
12		negative	103.6 (KS)	KS	67 F	RA
13		negative	83.4 (Jo-1)	negative	39 M	PM
14		negative	81.8 (PL-7)	negative	63 F	MCTD
15		negative	28.7 (PL-7)	negative	42 F	PM
16		negative	87 (Jo-1/KS) anti-GST ‡	negative	72 F	IMNM (anti-SRP)
17	Line-blot assay and anti-ARS test double positive cases	Jo-1: 2+	169.5 (Jo-1)	Jo-1	71 F	IIP
18		Jo-1: 3+	169.6 (Jo-1)	Jo-1	42 F	IIP
19		Jo-1: 2+	106.7 (Jo-1)	Jo-1	58 F	SS
20		Jo-1: 2+	148.1 (Jo-1)	Jo-1	33 F	PM
21		Jo-1: 3+	160.6 (Jo-1)	Jo-1	41 F	PM
22		Jo-1: 3+	140.5 (Jo-1)	Jo-1	66 F	PM
23		Jo-1: 3+	153.3 (Jo-1)	Jo-1	49 M	PM
24		Jo-1: 3+	165.8 (Jo-1)	Jo-1	59 F	PM
25		Jo-1: 3+	137.2 (Jo-1)	Jo-1	51 F	PM
26		Jo-1: 2+	161.1 (Jo-1)	Jo-1	52 F	DM
27		Jo-1: 3+	124.5 (Jo-1)	Jo-1	67 M	DM
28		Jo-1: 3+	192.4 (Jo-1)	Jo-1	70 F	DM
29		Jo-1: 3+	152.3 (Jo-1)	Jo-1	58 M	DM
30		Jo-1: 3+	114.6 (Jo-1)	Jo-1	66 F	DM
31		Jo-1: 1+	156.2 (Jo-1)	Jo-1	71 F	DM
32		PL-7: 2+	168.4 (PL-7)	PL-7	51 F	IIP
33		PL-7: 2+	164.2 (PL-7)	PL-7	66 F	RA
34		PL-7: 2+	189 (PL-7)	PL-7	54 M	DM
35		PL-7: 2+	171.1 (PL-7)	PL-7	68 F	DM
36		PL-12: 2+	122.1 (PL-12)	PL-12	65 F	IIP
37		PL-12: 3+	190.1 (PL-12)	PL-12	56 M	IIP
38		PL-12: 3+	165.5 (PL-12)	PL-12	50 F	SS
39		PL-12: 3+	150.3 (PL-12)	PL-12	61 F	DM/RA
40		PL-12: 2+	153.3 (PL-12)	PL-12	40 M	ADM
41		EJ: 3+	118.2 (EJ)	EJ	70 F	DM
42		EJ: 3+	86.1 (EJ)	EJ	65 F	RA/SS
43		EJ: 3+	199.4 (EJ)	EJ	67 F	PM
44		Jo-1: 2+	150.3 (Jo-1)	negative	42 F	RA

*The result of the line blot assay indicates the type of antisynthetase antibody and the semi-quantitative test results, defined as negative (0), borderline [(+)], positive (1+ or 2+), or strong positive (3+). †The result of the anti-ARS test indicates the titer of the index and type of anti-synthetase antibody determined by ELISA for the detection of individual anti-ARS antibodies. ‡Case 16 was found to produce two anti-ARS antibodies but the results from a suppression test suggested that this was caused by the presence of autoantibodies against the GST-tagged protein.

Anti-synthetase enzyme-linked immunosorbent assay (anti-ARS test), RNA-immunoprecipitation (RNA-IP) assay, Sjogren syndrome (SS), rheumatoid arthritis (RA), interstitial lung disease (ILD), mixed connective tissue disease (MCTD), systemic sclerosis (SSc), polymyositis (PM), amyopathic dermatomyositis (ADM), anti-MDA5 antibody (anti-MDA5 ab), idiopathic interstitial pneumonia (IIP), immune-mediated necrotizing myopathy (IMNM).

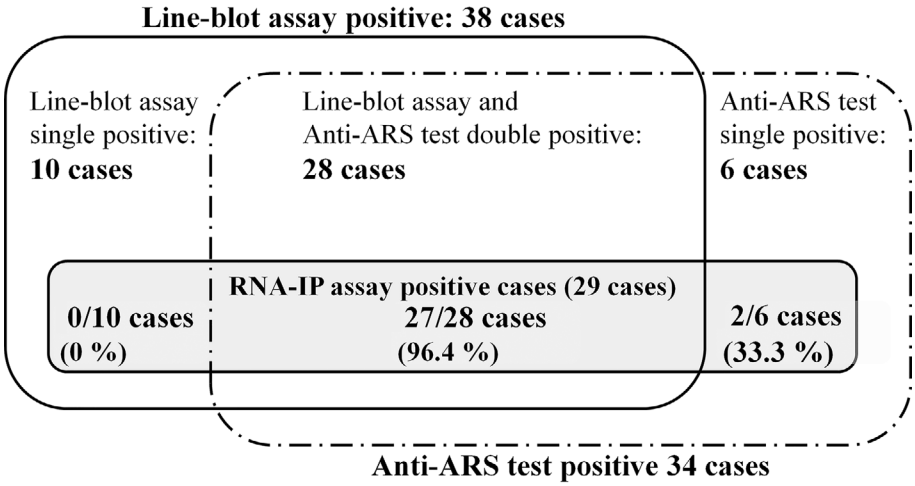

Figure. **Test results of antisynthetase antibodies measured by line-blot, anti-ARS, and RNA-IP assays in serum samples from 44 patients. Anti-synthetase enzyme-linked immunosorbent assay (anti-ARS test), RNA-immunoprecipitation (RNA-IP) assay.**

or ASS was difficult to determine. Hamaguchi et al. diagnosed 2 (1.2%) of 166 patients with coexisting ASS and anti-U1-RNP antibodies (6). We therefore suspect that the present patient had concurrent MCTD and ASS with a false-negative result from the RNA-IP assay. Patient 15 had been diagnosed with ASS two years previously, based on anti-Jo-1 antibodies detected by an ELISA and classical PM and ILD symptoms. However, the results of the anti-ARS test performed herein were positive for anti-PL-7 antibodies, although the results from the RNA-IP assay were negative. The cause of the discrepancy in the test results remains unknown, but different antigens included in the ELISAs might be one explanation. Patient 16 had significant losses in muscle strength, and the findings of a muscle biopsy suggested IMNM. This patient was positive for anti-SRP (1+) and anti-Mi-2 antibodies (1+) in the line-blot and anti-ARS assays and for anti-Jo-1 and anti-KS antibodies in an antigen-specific ELISA but was positive in the RNA-IP assay for anti-SRP antibody, which matched the clinical presentation and pathological findings. Two different anti-ARS antibodies are rarely detected in a single patient (6), so we determined antigen specificity using suppression tests. Jo-1 or KS antigens were added to Jo-1 and KS antigen solid-phase plates. When these antigens were added to the Jo-1 plates, absorbance was suppressed to 78.1%/75.6%, and when added to the KS plates, absorbance was suppressed to 73.8%/77.0%. The Jo-1 and KS antigens were not homologous except for GST-tagged protein. The suppression test results indicated that GST-tagged protein bound to each antigen, rather than either of the Jo-1 or KS antigens, thus suppressing the ability of the patient's antibodies to bind to the antigens coated on the plates. The antibodies detected in this patient were thus not directed against either Jo-1 or KS antigens and might have recognized the GST-tagged protein used in the purification of both antigens.

Results of RNA-IP assays in line blot and anti-ARS test double-positive patients 17-44

The diagnoses of 28 patients who were positive in both evaluations comprised DM (n=9), PM (n=7), ILD (n=5), RA with SS (n=2 each), DM/RA, ADM, and RA/SS (n=1 each). Among the 28 patients, 27 (96.4%) were also positive in the RNA-IP assays for anti-Jo-1 (n=15; patients 17-31), anti-PL-7 (n=4; patients 32-35), anti-PL-12 (n=5; patients 36-40), and EJ (n=3; patients 41-43) antibodies. In addition, the results of antigen assays for all 27 patients were identical (Table 1). Patient 44, who was negative in the RNA-IP assay, presented with ILD and positive anti-Jo-1 antibodies in the anti-ARS and line blot assays and later developed seropositive RA. Considering the clinical presentation, this patient was diagnosed with RA complicated with ASS, and the RNA-IP assay results were taken as false negative.

The comparison of the line-blot and RNA-IP assay results

We compared the accuracy between the line-blot and gold standard RNA-IP assays. The sensitivity and specificity of the line-blot assay were 93.1% and 26.7%, respectively, and the κ coefficient was 0.23, which was extremely low (Table 2a) compared with the RNA-IP assay. However, when the cut-off was increased from 1+ to 2+, the sensitivity fell to 89.7%, the specificity improved to 86.7%, and the κ coefficient increased to 0.75 (Table 2b).

The comparison of the anti-ARS test and RNA-IP assay

The sensitivity and specificity of the anti-ARS assays were 66.7% and 100%, respectively, with a κ coefficient of 0.73 (Table 2c).

Table 2. Comparison of Each Test Result (line-blot Assay and Anti-ARS Test) with the Gold Standard RNA-IP Assay.

(a)	RNA-IP assay			Line-blot assay positive (1+, 2+, 3+)		
	Positive	Negative	Total	Sensitivity	Specificity	Cohen's κ
Line-blot assay positive (1+, 2+, 3+)	27	11	38	93.10%	26.70%	0.23
Line-blot assay negative [-, (+)]	2	4	6			
Total	29	15	44			

(b)	RNA-IP assay			Line-blot assay positive (2+, 3+)		
	Positive	Negative	Total	Sensitivity	Specificity	Cohen's κ
Line-blot assay positive (2+, 3+)	26	2	28	89.70%	86.70%	0.75
Line-blot assay negative [-, (+), 1+]	3	13	16			
Total	29	15	44			

(c)	RNA-IP assay			Anti-ARS test positive		
	Positive	Negative	Total	Sensitivity	Specificity	Cohen's κ
Anti-ARS test positive	29	5	34	100%	66.70%	0.73
Anti-ARS test negative	0	10	10			
Total	Positive	Negative	44			

(d)	RNA-IP assay			Double positive		
	Positive	Negative	Total	Sensitivity	Specificity	Cohen's κ
Double positive	27	1	28	93.10%	93.30%	0.85
Single positive	2	14	16			
Total	29	15	44			

(a) Comparison of line-blot assay with RNA-IP assay when the positive cut-off value was defined above 1+.
(b) Comparison of line-blot assay with RNA-IP assay when the positive cut-off value was defined above 2+.
(c) Comparison of anti-ARS test with RNA-IP assay.
(d) Comparison of line-blot assay/anti-ARS test double-positive result with RNA-IP assay.
RNA-immunoprecipitation (RNA-IP), anti-synthetase enzyme-linked immunosorbent assay (anti-ARS test).

The comparison of the anti-ARS test/line-blot assay double-positive and RNA-IP assays

The sensitivity and specificity of the combined anti-ARS and line-blot assays were 93.3% and 93.1%, respectively, with a reasonable κ coefficient of 0.85 (Table 2d).

The comparison of the clinical symptoms between anti-ARS antibody-positive and antibody-negative patients by each method

A comparison of the most prevalent symptoms of ASS, namely a fever, mechanic's hand, arthritis, Raynaud phenomenon, ILD, and myositis, between the 29 and 15 patients with respectively positive and negative results in RNA-IP assays showed that a fever was more prevalent in the positive group than in the negative group (41.4% vs. 6.7%: p=0.034). However, the rates of mechanic's hand (62.1% vs. 26.7%: p=0.055), arthritis (62.1% vs. 53.3%: p=0.75), Raynaud phenomenon (31% vs. 40%: p=0.74), ILD (100% vs. 93.3%: p=0.34), and myositis (51.7% vs. 33.3%: p=0.32) did not significantly differ between these groups (Table 3a). We found significantly more symptoms of ASS in patients with positive results than in those with negative results in RNA-IP assays (3.48±1.45 vs. 2.53±1.35, p=0.049; Table 3a). A comparison of the 6 symptoms among the 29, 10, and 4 patients who were respectively positive in the RNA-IP assay, only in the line-blot assay, and only in the anti-ARS assay revealed the following respective rates: a fever, 41.4% vs. 10% vs. 0%; mechanic's hand, 62.1% vs. 20% vs. 50%; arthritis, 62.1% vs. 50% vs. 50%; Raynaud phenomenon, 31% vs. 40% vs. 50%; ILD, 100% vs. 90% vs. 100%; and myositis, 51.7% vs. 10% vs. 100%. The total numbers of symptoms were 3.48±1.45, 2.2±1.32, and 3.5±1.29 for patients who were respectively positive in the RNA-IP assay, only in the line-blot assay, and only in the anti-ARS assay (Table 3b). The prevalence of mechanic's hand (p=0.031), myositis (p=0.028), and the number of symptoms (p=0.019; Table 3b) significantly differed between patients who were positive in the RNA-IP and line-blot assays.

Malignancies

Five of 44 patients developed malignances within two years before or after being diagnosed with suspected or confirmed ASS, including two cases in the line-blot single-positive group (gallbladder cancer in RA, malignant lymphoma in SSc) and 3 in the double-positive group (gastric

Table 3. Comparison of the Prevalence of the Typical Clinical Symptoms of Antisynthetase Syndrome Based on the Test Results of the Antisynthetase Antibody.

(a)

	Fever	Mechanics hand	Arthritis	Raynaud phenomenon	ILD	Myositis	Number of symptoms (mean)
RNA-IP assay positive (29 cases)	12 (41.4)	18 (62.1)	18 (62.1)	9 (31)	29 (100)	15 (51.7)	3.48±1.45
RNA-IP assay negative (15 cases)	1 (6.7)	4 (26.7)	8 (53.3)	6 (40)	14 (93.3)	5 (33.3)	2.53±1.35
p value	0.034	0.055	0.75	0.74	0.34	0.32	0.049

(b)

	Fever	Mechanics hand	Arthritis	Raynaud phenomenon	ILD	Myositis	Number of symptoms (mean)
RNA-IP assay positive (29 cases) *	12 (41.4)	18 (62.1)	18 (62.1)	9 (31)	29 (100)	15 (51.7)	3.48±1.45
Line-blot assay single positve (10 cases) †	1 (10)	2 (20)	5 (50)	4 (40)	9 (90)	1 (10)	2.2±1.32
Anti-ARS test single positive (4 cases) ‡	0 (0)	2 (50)	2 (50)	2 (50)	4 (100)	4 (100)	3.5±1.29
p value * vs. †	0.12	0.031	0.71	0.7	0.26	0.028	0.019
p value * vs. ‡	0.271	1.0	1.0	0.59	1.0	0.12	0.98
p value † vs. ‡	1.0	0.5	1	1	1	0.005	0.144

(a) The number (%) of patients with each clinical symptom and the mean number of total symptoms in the RNA-IP assay positive and negative groups.

(b) RNA-IP assay negative groups were divided into line-blot assay single positive group (10 cases), anti-ARS test single positive group (4 cases) and a case with double positive but negative by RNA-IP assay (This case was excluded from the following analyses). The number (%) of patients with each clinical symptom and the mean number of total symptoms in the RNA-IP assay positive, line-blot assay single positive, and anti-ARS test single positive groups. Regarding the prevalence of typical clinical symptoms, p-values were calculated using Fisher's exact test, and the mean number of symptoms was compared using a Wilcoxon rank sum test.

RNA-immunoprecipitation (RNA-IP), anti-synthetase enzyme-linked immunosorbent assay (anti-ARS test), interstitial lung disease (ILD).

cancer in IIP, lung cancer in DM, colorectal cancer in PM).

Treatment and the prognosis

Thirty-nine of the 44 patients were treated with glucocorticoids; four were not treated because of low disease activity, and the subsequent treatment of one who was transferred to another hospital was unknown. We examined responses to treatment with glucocorticoids in 34 patients (line-blot positive, n=5; anti-ARS test positive, n=6; positive in both tests, n=23) without malignancies. We concurrently treated 25 patients (line-blot positive, n=3; anti-ARS test positive, n=5; positive in both tests, n=17) with the immunosuppressants cyclosporine A, tacrolimus, azathioprine, mycophenolate mofetil, cyclophosphamide, or methotrexate. The criteria for a poor prognosis comprised persistent restrictive pulmonary dysfunction with vital capacity (VC) <80% of the predicted value, introduction of home oxygen therapy, or death caused by respiratory failure. Ten of the 34 patients had a poor prognosis, including 2 (40%) of 5 who were line-blot positive, 0 (0%) of 6 who were positive in the anti-ARS assay, and 8 (34.7%) of 23 who were positive in both assays. However, these findings did not significantly differ among the groups.

Discussion

ASS is a chronic, recurrent autoimmune disease that presents with myositis, Raynaud's phenomenon, mechanic's hand, polyarthritis, and ILD (1). At least 90% of patients also develop ILD during the disease course that can progress to respiratory failure, thus requiring appropriate treatment with corticosteroids and immunosuppressive agents as early as possible (4, 16). Measuring anti-ARS antibodies is important for diagnosing ASS, but the RNA-IP assay, which is considered the gold standard for these disorders, can be performed at only a few facilities. Given these limitations, Japanese National Health Insurance covers anti-ARS tests, and outsourced line-blot assays are alternatives. Therefore, we compared the results of these three tests using the same serum samples.

We found that all sera from the 10 patients who were positive only in line-blot assays were negative in RNA-IP assays. The main reason for this might be differences in interpreting the semi-quantitative scores used in these kits, especially when the cut-off was 1+. For example, patient 7 was 1+ for anti-PL-12 and 2+ for anti-OJ antibodies. However, the reported rate of positivity in line-blot assays of sera that were confirmed positive for anti-OJ antibodies by RNA-IP is 0%, and only RNA-IP can detect anti-OJ antibodies (17-19). When anti-OJ antibody results were not included, all line-blot-positive sera were semi-quantitatively scored as 1+. Comparisons of the line-blot results with RNA-IP assay revealed a high sensitivity but low specificity and a low Cohen κ coefficient of 0.23. When 1+ was considered negative, the κ coefficient reached "substantial agreement" at 0.75, coinciding with the fact that the clinical interpretation of results that are 1+ also involves the simultaneous consideration of other diseases. Cavazzana et al. also

compared line-blot and RNA-IP assays and found a low agreement for anti-ARS antibodies, with many samples being simultaneously positive for multiple myositis-related autoantibodies in line-blot assays (20). Espinosa-Ortega et al. also reported a somewhat low agreement rate and called attention to the possibility of misinterpretation in these assays (21). In a comparison of line-blot and RNA-IP assay results among patients with myositis-related autoantibodies and SSc-related autoantibodies, Hamaguchi et al. found favorable agreement with a cut-off of ≥ 1+ for Jo-1 antibodies but suggested that the cut-off should be raised to 2+ for evaluating other anti-EJ, anti-PL-7, anti-PL-12, and anti-SRP antibodies to improve overall agreement with RNA-IP assays. Substantial disagreement was found between the two evaluation methods for anti-Mi-2 and anti-Ku antibodies, and clinical decisions based only on line-blot results confers a risk of a misdiagnosis (12). This was obvious for patient 6 in the present study, who presented with ILD complicated with ADM. This patient was 1+ positive for anti-PL-7 antibodies in the line-blot assay, suggesting ASS and thus prompting treatment with CsA and PSL without IVCY. Although the patient improved, later tests revealed anti-MDA5, not anti-PL-7 antibodies. The coexistence of these antibodies in one patient is extremely rare, as only one such patient has been described to date (22). The final diagnosis for patient 6 was anti-MDA5 antibody-positive ADM, with the anti-PL-7 positive result in the line-blot assay being judged as false positive. This situation suggests the danger of diagnosing ASS based solely on line-blot assays. The results from this test should be interpreted with caution, as such assays are a popular research tool that are not intended for clinical diagnostic purposes.

The clinical presentation of patients who were positive in line-blot assays included significantly lower rates of mechanic's hand and myositis and fewer total symptoms than in those who were positive in RNA-IP assays. Line-blot false positives are more likely in clinical presentations without PM/DM, as well as in instances where line-blot assays do not produce an obvious positive result (23). Thus, interpreting test results should involve comprehensive considerations, including the clinical presentation and similarities to ASS.

A comparison of anti-ARS and RNA-IP assays reported by Nakashima et al. showed 97.1% sensitivity and 99.8% specificity, values that were 100% and 99.8%, respectively, when anti-OJ antibodies were excluded (14). We also noted 100% sensitivity but only 66.7% specificity in the present study. This was because we included four patients who were positive in anti-ARS assays and negative in RNA-IP assays (patients 13-16) and one who was double-positive but RNA-IP-negative (patient 44). Among them, patients 13, 15, and 44 presented with anti-Jo-1 antibodies detected by anti-Jo-1 antibody ELISAs of different serum samples and were thus deemed false negative in RNA-IP assays, considering their clinical presentation. Sasai et al. analyzed the clinical presentation of 16 patients who were positive in an anti-ARS ELISA but negative in RNA-IP assays and suggested that the frequency of the characteristic clinical presentation for ASS is quite low in such patients (24). They also suggested that the potential causes of this difference in results might be that these antibodies inhibit RNA binding to ARS proteins or recognize denatured ARS antigens. The rate of a fever was low among our patients who were positive only in the anti-ARS assay. However, this issue requires further investigation due to the small sample size in the present study. Patient 16 had discordant results in all three tests, but the results of a suppression test suggested that this was caused by autoantibodies against GST-tagged protein. The anti-ARS assay uses recombinant PL-12 and EJ antigens that include His-tagged proteins, the Jo-1 and KS antigens include GST-tagged proteins, and the PL-7 antigens include cMyc-His-tagged proteins (14). Autoantibodies against GST have also been identified in autoimmune hepatitis (25) and renal transplantation (26). Furthermore, analyzing autoantibodies using an ELISA with recombinant protein antigens can lead to slight underestimation (27). We were unable to examine a direct reaction to the GST-tagged protein by the autoantibodies from the patients, but high-titer anti-GST antibodies might cause false positives in anti-ARS tests.

Positive results in both the anti-ARS and line-blot tests provided the best degree of sensitivity, specificity, and agreement with RNA-IP assays. Therefore, combining these tests in clinical practice may facilitate an accurate diagnosis in lieu of RNA-IP assays.

Several limitations associated with the present study warrant mention. Our small pool of patients included only one who was positive for KS antibodies. Because these antibodies can be detected by the anti-ARS assay but not line-blot assays, RNA-IP assays are required to confirm the anti-ARS results as true positives. This is particularly problematic, as anti-KS antibody-positive patients often develop ILD without myositis, leading to a misdiagnosis of IIP (6, 28). The frequency of ILD was high (97.7%) among our patients compared with previous studies (6, 19). Our department collaborates with the Department of Respiratory Medicine, where patients with ASS complicated by ILD tend to accumulate. In contrast, patients with myositis-predominant ASS are treated in the Department of Neurology and were thus not included in this study, which might have resulted in case selection bias. In addition, we did not compare the results of anti-nuclear antibody tests (indirect immunofluorescence assays) in the tested serum samples. Anti-Jo-1, anti-EJ, anti-PL-7, and anti-PL-12 antibodies are stained in the cytoplasm during anti-nuclear antibody tests; therefore, uncertain results may be able to be clarified using this method (12). The Myositis Profile 3 kit detects antibodies against Jo-1, PL-7, PL-12, and EJ as well as those against Mi-2, Ku, PM-Scl 100, PM-Scl75, SRP, and Ro-52, and we did not evaluate or compare the results with those of the RNA-IP assay. This specific limitation should be addressed in future studies.

Conclusion

Line-blot and anti-ARS assays, which are easy to perform and accessible, can be used to measure anti-ARS antibodies, but clinicians must understand the features of each assay and consider them appropriately in order to avoid misjudgment. The results of line-blot assays should be interpreted with caution, as this method is a research tool. Disease states should not be definitively diagnosed based on the results of this assay alone due to the risk of a misdiagnosis with false positives. These test results should be considered in conjunction with clinical presentations to accurately diagnose diseases.

The authors state that they have no Conflict of Interest (COI).

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