A comparative study of the diagnostic accuracy of ELISA systems for the detection of anti-neutrophil cytoplasm antibodies available in Japan and Europe

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Abstract

Objectives

Primary systemic vasculitis associated with anti-neutrophil cytoplasm antibodies (ANCA) differs in its frequency and clinical expression between Japan and Europe. We sought to ascertain whether such differences arise from the performance of enzyme-linked immunosorbent assays (ELISAs) for ANCA.

Methods

Plasma samples from 64 consecutive Japanese patients with a clinical and histological diagnosis of primary systemic vasculitis including microscopic polyangiitis (MPA; n=52), Churg-Strauss syndrome (CSS; n=1), and Wegener's granulomatosis (WG; n=11), or those from disease controls with non-vasculitic glomerulonephritis (n=54) and healthy controls (n=55) were tested for the presence of myeloperoxidase (MPO) by ELISAs available in Japan (Nipro and MBL) and compared with those in Europe (Wieslab). The sensitivity and specificity were calculated for each ELISA, and its diagnostic performance was assessed by receiver operating characteristic curve analysis.

Results

The sensitivity and specificity of either MPO-ANCA assays for a diagnosis of MPA were 90.4% and 98.2% (Nipro), 88.2% and 96.3% (MBL), and 86.5% and 99.1% (Wieslab). The overall diagnostic performance, assessed as the area under curve of the MPO-ANCA ELISAs for MPA were 0.946±0.022 (Nipro), 0.970±0.017 (MBL), and 0.971±0.017 (Wieslab), while that of PR3-ANCA ELISAs for WG were 0.986±0.025 (Nipro), 0.993±0.017 (MBL), and 0.916±0.059 (Wieslab).

Conclusions

The MPO-ANCA ELISAs commercially available in Japan exhibited high sensitivity and specificity for the diagnosis of ANCA-associated vasculitides and provided similar diagnostic value to those in Europe. These results facilitate further international comparison of ANCA-associated vasculitides between Japanese and European populations.

Key words

MPO-ANCA, PR3-ANCA, Capture ELISA, streptavidin-coated ELISA, ANCA-associated vasculitides (AAV), systemic vasculitis.

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Introduction

Anti-neutrophil cytoplasm antibodies (ANCA) are found in a high percentage of patients with Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS) and are used as diagnostic markers for these diseases, which are also termed the ANCA-associated vasculitides (AAV). On the indirect immunofluorescence (IIF) test, ANCA usually exhibits either a granular cytoplasmic pattern (C-ANCA) or a peri-nuclear pattern (P-ANCA). C-ANCA positivity is characteristically observed in patients with WG mostly directed against proteinase 3 (PR3-ANCA), while the P-ANCA that frequently occur in MPA are in general directed against myeloperoxidase (MPO-ANCA). ANCA detected by IIF are also apparent in several other inflammatory conditions; in these cases, ANCA as detected by IIF is not specific for vasculitis. Therefore, ANCA should be demonstrated by using a combination of IIF and ELISA (1, 2).

The major problem with the current application of ANCA ELISA systems is the lack of international standard sera and the lack of international standardization of assay systems. Unfortunately, commercially available ELISA systems have a wide range of performance characteristics and employ arbitrary units determined by each manufacturer (3). When interpreting an ANCA test, therefore, the clinician must take into account the differences between the ELISA systems.

In Japan, three kinds of MPO-ANCA and PR3-ANCA ELISA systems are currently available as extracorporeal diagnostic agents authorized by the Ministry of Health and Welfare of Japan. Comparison of these ELISA systems with those commonly used in Europe is essential for international collaboration and for epidemiological and clinical research.

In the present study we compared the sensitivity and specificity of two major commercially available ANCA ELISA systems in Japan and one of the most widely used systems in Europe for MPO-ANCA and PR3-ANCA using plasma obtained from Japanese patients with a clinical and histological diagnosis of WG, MPA, or CSS. We also assessed the correlation of ANCA values among ELISA systems. This study aims to further validate the role of ANCA assays available in Japan and permit comparative international studies involving Japanese patients with AAV.

Patients and methods

Patient population and diagnostic criteria

The plasma samples were derived from newly diagnosed patients with primary systemic vasculitis (PSV) including WG, MPA, or CSS in accordance with the American College of Rheumatology (ACR) classification criteria and Chapel Hill consensus criteria (CHCC) with reference of EMEA algorithm method (4-7). We modified the algorithm to be irrespective of positivity of ANCA for the purpose of this study. 64 consecutive patients who had newly diagnosed disease were enrolled (MPA, n=52; CSS, n=1; WG, n=11) in the Department of Nephrology and Cardiovascular Medicine at Kyoto University hospital (Kyoto, Japan) between June 1999 and June 2000, in the Department of Nephrology and Dialysis and in the Department of Clinical Immunology and Rheumatology in Tazuke Kofukai Medical Research Institute Kitano Hospital (Osaka, Japan) between July 2000 and Apr 2008, and in the Department of Internal Medicine and Rheumatology in Juntendo University Hospital (Tokyo, Japan) between January 2005 and January 2006. The same number of plasma samples (n=58) were obtained during the untreated phase when the patients showed an acute exacerbation of symptom of organ involvement consistent with active vasculitis before the start of any immunosuppressive treatment. All patients had active disease at enrollment defined as Birmingham Vasculitis Activity Score (BVAS) of at least 4 (8). Plasma samples were also obtained from these patients in the follow up period between one week and six months after the start of immunosuppressive treatment (MPA, n=80; WG, n=2). All the patients were systematically assessed for potential subclinical granulomatous disease with diagnostic

imaging as well as ENT consultation. Confirmatory organ histological biopsies were available in eight out of 11 patients with WG including four renal biopsies, two lung biopsies, and two biopsies from nodules of paranasal sinuses. Renal biopsies were performed in all the MPA and CSS patients and revealed that all of them showed renal involvement.

For disease controls, we also assayed plasma from 64 consecutive new patients with non-vasculitic glomerulonephritis who had a renal biopsy in Kitano Hospital in this study period; diagnoses included IgA nephropathy (n=18), non-IgA type mesangioproliferative glomerulonephritis (n=5), endocapillary glomerulonephritis (n=1), interstitial nephritis (n=2), hepatitis C virus-related nephritis (n=1), membranoproliferative glomerulonephritis (n=3), membranous nephropathy (n=4), focal glomerulosclerosis (n=2), minimal change nephrotic syndrome (n=3), diabetic nephropathy (n=2), amyloidosis (n=1), malignant hypertension (n=1), nephrosclerosis (n=3), pseudo-Bartter syndrome (n=1), antiphospholipid syndrome (n=1), and lupus nephritis (n=6). None of the disease control patients were receiving immunosuppressive therapy at the time of sampling.

As healthy controls, the plasma samples from 55 people who received regular physical checkup in the clinic of Louis Pasteur Centre for Medical Research and had not have any diseases until 2004 were enrolled in this study. This study was carried out in accordance with the 1975 Declaration of Helsinki of the World Medical Association. The design of the work has been approved by the ethical committee of the hospitals and clinic involved and each patient gave written informed consent for participation in the study. All plasma samples were fully spun down to remove fibrin clots preventing non-specific reaction and were stored at -80°C until tested.

Methods of ANCA detection
All assays for MPO-, PR3-ANCA, and IIF were performed according to the manufacturers' instructions.

Nipro MPO- and PR3-ANCA ELISA. Nipro Nephroscholar MPO-ANC II kit (Nipro, Osaka, Japan; authorization number of diagnostic drugs and medications in Japan No. 212000AMZ00598000) has been developed in Japan. Briefly, The 1:500 diluted samples and biotinylated MPO antigen solution (0.1 mg/well) were applied onto the plate coated with Streptavidine and incubated for 1 hour at room temperature. The binding of MPO-ANCA to MPO antigens purified from human sputum was assessed using alkaline phosphatase (AP)-labeled polyclonal goat anti-human IgG with p-Nitrophenyl phosphate disodium in diethanolamine as a substrate. After adding sodium hydrate as stop solution, the absorbance was measured photometrically at 405 nm. The cut-off value was 20 ELISA Unit/ml.

Nipro Nephroscholar PR3-ANC kit (authorization No. 21000AMY00275000) is imported from Euro-Diagnostica (Arnhem, The Netherlands and Malmö, Sweden) and the kit is equivalent to Immunoscan PR3-ANCA (Euro-Diagnostica). Purified PR3 from human neutrophils were directly coated onto a 96-well microplate. The 1:50 diluted samples were applied onto the plate coated with purified PR3. AP-labeled polyclonal pig anti-human IgG was added followed by p-Nitrophenyl phosphate disodium in diethanolamine as a substrate. The absorbance was measured photometrically at 405 nm. The cut-off value was 10 ELISA Unit/ml.

MBL MPO- and PR3-ANCA ELISA. MBL (Nagoya, Japan) has imported the kits from Binding Site (Birmingham, UK). MBL MPO-ANCA test (BS) Code No.BS-031 (Nagoya, Japan; authorization No. 21100AMY00184000) is equivalent to BINDAZYME™ Human Anti-MPO Enzyme Immunoassay kit, Code No.MK031 (Binding Site). MBL PR3-ANCA test (BS) Code No.BS-032 (authorization No. 21000AMY00075000) is equivalent to BINDAZYME™ Human Anti-PR3 Enzyme Immunoassay kit, Code No.MK032 (Binding Site). Each sample, diluted 1:101 in diluent was used. The binding of MPO- and PR3-ANCA to human purified antigens coated on the plate was assessed using

anti-human IgG peroxidase conjugate and 3, 3', 5, 5'-tetramethylbenzidine (TMB) as a chromogen. The absorbance was measured photometrically at 450 nm and the cut-off values for MPO- and PR3-ANCA were 9.0 U/ml and 3.5 U/ml, respectively.

Wieslab MPO-ANCA and Capture PR3-ANCA ELISA. These kits were not permitted for diagnostic purpose in Japan as of Apr 2008. For detection of MPO-ANCA by direct ELISA, Wielisa MPO-ANCA MPO 103 (Wieslab AB, Lund, Sweden) was used. MPO purified from human neutrophils was coated on 96-well plates. Samples were diluted at 1:80 and antibody binding was assessed with AP-conjugated anti-human IgG. Values >25 units were considered to be positive.

Capture PR3-ANCA testing was performed with Wielisa capture PR3-ANCA, Cap-PR3 108X. Each sample, diluted 1:80 in PBS was used. The antigen-antibody complex was detected by AP-labeled anti-human IgG antibodies using p-nitrophenylphosphate as a chromogen with spectrophotometric reading at 405 nm. For this method, the cut-off value was 25 U/ml.

Indirect immunofluorescence (IIF) assay. ANCA detection by IIF was performed on commercially available slides of ethanol-fixed and formalin-fixed purified normal granulocytes. The MBL Fluoro ANCA test Code No.4710 and 4720 (MBL) are equivalent to the Binding Site ANCA ethanol kit Code FK016 and ANCA formalin kit Code FK017 (Binding Site), respectively. If peri-nuclear or nuclear immunofluorescence was detected on ethanol-fixed granulocyte, the IIF was repeated using formalin-fixed neutrophil preparations. Samples were interpreted as P-ANCA positive if they displayed cytoplasmic staining on formalin-fixed slides. Plasma sample of untreated PSV patients (n=64), disease controls (n=54), and healthy controls (n=55) were tested for positivity of IIF-ANCA.

Statistics
Performance characteristics were compared by receiver operating characteristic (ROC) curve analysis according to the method described by Hanley (9).

Table I. Sensitivity and Specificity of MPO-ANCA for MPA using clinical cut off value of each ELISA systems.

ELISA kit	Cut off	Sensitivity (% [95%CI])	Specificity (% [95%CI])	AUC (± SE)	AUC 95% CI	P versus		
						Nipro	MBL	Wieslab
Nipro MPO-ANCA	20 U/ml	90.4 [79.0 - 96.8]	98.2 [93.5 - 99.7]	0.946 ± 0.022	0.899 - 0.976		0.282	0.269
MBL MPO-ANCA	9 U/ml	88.2 [76.1 - 95.5]	96.3 [90.9 - 99.0]	0.970 ± 0.017	0.930 - 0.990	0.282		0.951
Wieslab MPO-ANCA	25 U/ml	86.5 [74.2 - 94.4]	99.1 [95.0 - 99.8]	0.971 ± 0.017	0.932 - 0.991	0.269	0.951	

MPA: microscopic polyangiitis; ROC: receiver operating characteristics curve analysis; AUC: area under the ROC curve; SE: standard error; 95% CI: 95% confidence interval.
Sample size: MPA, n=52; disease controls, n=54; and healthy controls n=55.

Table II. Sensitivity and Specificity of PR3-ANCA for WG using clinical cut off value of each ELISA systems.

ELISA kit	Cut off	Sensitivity (% [95%CI])	Specificity (% [95%CI])	AUC (± SE)	AUC 95% CI	P versus		
						Nipro	MBL	Wieslab
Nipro PR3-ANCA	10 U/ml	100.0 [71.3 - 100.0]	98.2 [93.5 - 99.7]	0.986 ± 0.025	0.945- 0.998		0.729	0.211
MBL PR3-ANCA	3.5 U/ml	100.0 [71.3 - 100.0]	95.4 [88.4 – 97.9]	0.993 ± 0.017	0.957 - 0.998	0.729		0.163
Wieslab PR3 ANCA	25 U/ml	90.9 [58.7 – 98.5]	99.1 [95.0 – 99.8]	0.916 ± 0.059	0.851 - 0.959	0.211	0.163	

WG: Wegener's granulomatosis; ROC: receiver operating characteristics curve analysis; AUC: area under the ROC curve; SE, standard error; 95% CI: 95% confidence interval.
Sample size: WG, n=11; disease controls, n=54; and healthy controls, n=55.

A difference of *p*<0.05 was considered to be statistically significant. The Software MedCalc® version 9.3.0.0. (MedCalc®, Mariakerke, Belgium) was used for statistical analysis. A correlation coefficient between MPO-ANCA ELISA kits was obtained using Statview-J software version 5.0 for Windows (SAS Institute Inc., Cary, NC). A probability value <0.05 was considered significant.

Results
Clinical diagnostic performance of MPO-ANCA for MPA
Table I shows the sensitivity and specificity of MPO-ANCA for the diagnosis of MPA using predetermined cut-off values of each ELISA system. Plasma samples from untreated MPA patients (n=52), disease controls (n=54) and healthy controls (n=55) were used in this study. The sensitivity and specificity were 90.4% and 98.2% (Nipro), 88.2% and 96.3% (MBL), and 86.5% and 99.1% (Wieslab) (Table I).

ROC analysis and AUC, MPO-ANCA for MPA
ROC was analysed with plasma samples from patients with untreated MPA patients (n=52), disease controls (n=54), and healthy control (n=55). The overall diagnostic performance,

assessed as the area under curve (AUC) of the four MPO-ANCA ELISAs were 0.946±0.022 (Nipro), 0.970±0.017 (MBL), and 0.971±0.017 (Wieslab). There were no significant differences among these ELISAs using pairwise comparison of ROC curves (Table I).

Clinical diagnostic performance of PR3-ANCA for WG
Table II shows the sensitivity and specificity of PR3-ANCA for the diagnosis of WG using predetermined cut-off values of each ELISA system. Plasma samples from untreated WG patients (n=11), disease controls (n=54), and healthy controls (n=55) were used in this study. The sensitivity and specificity were 100.0% and 98.2% (Nipro), 100.0% and 95.4% (MBL), and 90.9% and 99.1% (Wieslab) (Table II).

ROC analysis and AUC, PR3-ANCA for WG
ROC was analysed with plasma samples from untreated WG patients (n=11), disease controls (n=54), and healthy control (n=55). The AUC of PR3-ANCA ELISAs were 0.986±0.025 (Nipro), 0.993±0.017 (MBL), and 0.916±0.059 (Wieslab). There were no significant differences among these ELISAs using pairwise comparison of ROC curves (Table II).

Correlation between MPO-ANCA ELISA systems
Correlations between MPO-ANCA ELISAs were analysed using 146 plasma samples from patients with PSV at various stages (MPA, n=132; CSS, n=1; WG, n=13). All data were log_{10} transformed to normalise distributions prior to this analyses. Nipro MPO-ANCA ELISA was positively correlated with MBL MPO-ANCA (*r*=0.891, *p*<0.0001, Fig. 1) and Wieslab MPO-ANCA (*r*=0.879, *p*<0.0001). MBL MPO-ANCA and Wieslab MPO-ANCA were positively correlated with each other (*r*=0.899, *p*<0.0001, Table III).

Percentage of ANCA positivity in patients with PSV
MPO-ANCA positivity in MPA was 86.5% (45/52) in Nipro ELISA, 80.8% (42/52) in MBL ELISA, and 82.7% (43/52) in Wieslab ELISA. PR3-ANCA positivity in MPA was 3.8% (2/52) both in Nipro and Wieslab ELISAs, and 7.7 % (4/52) in MBL ELISAs. Absence of ANCA in MPA was 7.7 % (4/52) both in Nipro and MBL ELISAs, and 9.6% (5/52) in Wieslab ELISA. PR3-ANCA positivity in WG was 100% (11/11) both in Nipro and MBL ELISAs, and 90.9% (10/11) in Wieslab ELISA. A CSS patient showed positive MPO-ANCA only in MBL ELISA (Table IV).

Table III. Correlation coefficient between MPO-ANCA ELISAs.

MPO-ANCA ELISAs	Nipro	MBL [BS]	Wieslab
Nipro	–	0.891	0.879
MBL	0.891	–	0.899
Wieslab	0.879	0.899	–

Samples with PSV before treatment and in the follow up period (n=146).

Table IV. Percentage of ANCA positivity in PSV patients.

	Diagnosis / ELISA positivity	MPA % (n)	WG % (n)	CSS % (n)
Nipro	MPO-ANCA	86.5 (45/52)	0 (0/11)	0 (0/1)
	PR3-ANCA	3.8 (2/52)	100.0 (11/11)	0 (0/1)
	Double positive	1.9 (1/52)	0 (0/11)	0 (0/1)
	Double negative	7.7 (4/52)	0 (0/11)	100.0 (1/1)
MBL	MPO-ANCA	80.8 (42/52)	0 (0/11)	100.0 (1/1)
	PR3-ANCA	7.7 (4/52)	100.0 (11/11)	0 (0/1)
	Double positive	3.8 (2/52)	0 (0/11)	0 (0/1)
	Double negative	7.7 (4/52)	0 (0/11)	0 (0/1)
Wieslab	MPO-ANCA	82.7 (43/52)	0 (0/11)	0 (0/1)
	PR3-ANCA	3.8 (2/52)	90.9 (10/11)	0 (0/1)
	Double positive	3.8 (2/52)	0 (0/11)	0 (0/1)
	Double negative	9.6 (5/52)	9.1 (1/11)	100.0 (1/1)

Double positive, both MPO- and PR3-ANCA positive;
Double negative, both MPO- and PR3-ANCA negative;
MPA: microscopic polyangiitis; WG: Wegener's granulomatosis;
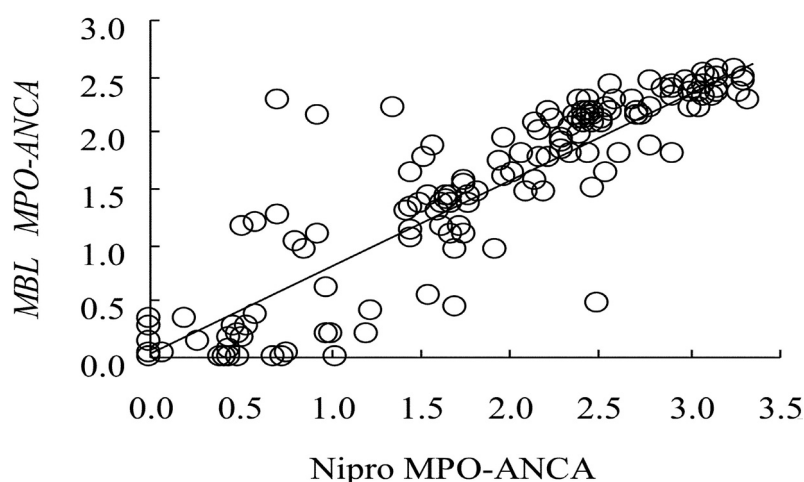CSS: Churg-Strauss syndrome.

Fig. 1. *Correlation between Nipro MPO-ANCA and MBL MPO-ANCA.* Correlations between MPO-ANCA were analysed using 146 plasma samples from patients with PSV in the active and follow-up stages (MPA, n=132; CSS, n=1; WG, n=13). Nipro MPO-ANCA and MBL MPO-ANCA positively correlated with each other ($r = 0.891$, $p<0.0001$). *Estimated Nipro MPO-ANCA* = 1.0146×MBL MPO-ANCA + 0.3545, *Estimated MBL MPO-ANCA* = 0.7829 ×Nipro MPO-ANC II + 0.0265. All data were $\log_{10}$ transformed to normalise distributions prior to these analyses.

Clinical diagnostic performance of IIF for PSV.
Plasma samples of untreated PSV patients (n=64), disease controls (n=54), and healthy controls (n=55) were tested for positivity of IIF-ANCA. IIF showed a sensitivity of 81.3% (95% CI, 69.5–89.9) and 94.5% specificity (88.4–97.9) for active PSV.

P-ANCA and C-ANCA were detected in 46 and 11 out of the total 173 samples. Among the 57 samples with positive P- or C-ANCA by IIF, 56 samples were positive for MPO- and/or PR3-ANCA by Nipro MPO- and PR3-ANCA ELISAs. All the 57 samples with positive P- or C-ANCA were positive for MPO- and/or PR3-ANCA by MBL MPO- and PR3-ANCA ELISAs. The result of ELISAs by Nipro and MBL were well corresponded with ANCA by IIF. P-ANCA positivity in MPA was 82.7% (43/52), while C-ANCA positivity in MPA was 7.7% (4/52). Absence of P- and C-ANCA in MPA was 9.6% (5/52). C-ANCA and P-ANCA positivity in WG is 54.5% (6/11) and 9.1% (1/11) respectively. Absence of P- and C-ANCA in WG was 36.4% (4/11). IIF-ANCA was negative in our CSS patient (Table V).

Discussion
In this study we investigated ANCA ELISA systems authorized by the Japanese Ministry of Health and Welfare for diagnostic purpose: a streptavidin coated capture ELISA and a direct ELISA for MPO-ANCA and direct ELISAs for PR3-ANCA.

The present study shows that the ELISA systems widely used in Japan are highly sensitive and specific for detection of MPO-ANCA in MPA. There were no detectable differences in diagnostic performance between ELISA systems as analysed by ROC curve analysis.

The incidence of WG among the ANCA-associated systemic vasculitides is higher than MPA in northern Europe (10-13). By contrast, nationwide Japanese surveys demonstrated that the prevalence of patients with WG is very low compared with that of patients with MPA (14). Fujimoto *et al.* shows that the estimated annual incidence of MPA in Miyazaki Prefecture, the Southern part of Japan, is 14.8/million, which is as frequent as that of ANCA-associated systemic vasculitides identified in several European studies. They also demonstrate that MPA are more common than WG among the ANCA-associated vasculitides in Japan (15). MPO-ANCA was identified in 79 to 93% of patients with MPA in Japan, whereas reports from Europe described the ratio as being 44 to 69% (12-19).

Table V. Percentage of indirect immunofluorescence (IIF) test positivity in primary systemic vasculitis patients.

Diagnosis IIF positivity	MPA % (n)	WG % (n)	CSS % (n)
P-ANCA	82.7 (43/52)	9.1 (1/11)	0 (0/1)
C-ANCA	7.7 (4/52)	54.5 (6/11)	0 (0/1)
Negative	9.6 (5/52)	36.4 (4/11)	100 (1/1)

P-ANCA: perinuclear ANCA; C-ANCA: cytoplasmic ANCA;
Negative: both P- and C-ANCA negative.

Although differences in several clinico-epidemiological manifestations among vasculitides have been identified between Japan and European countries, it was suspected that such difference may result from different ELISA systems employed in Japan and Europe. However, this study shows MPO-ANCA predominance in Japan is not due to the different ELISA system employed in Japan and Europe. Therefore, the ANCA-associated systemic vasculitides epidemiologically and serologically differ between Japan and European countries.

Nevertheless, we should still bear in mind that there are significant differences in sensitivity and specificity among commercially available ELISA systems (3, 20-25). The reason might be that in ELISAs, proteins are denatured during antigen purification or coating onto the solid phase, thereby hiding or destroying conformational epitopes on PR3 or MPO. In order to avoid this, capture ELISA in which the plate is pre-coated with a monoclonal antibody to capture the antigen has been designed. Csernok et al. reported that capture PR3 ELISA was a highly sensitive assay in WG in several international laboratories (23). Recently Hellmich et al. reported sensitivity of a new anchor PR3-ELISA for WG was superior to those of usual direct or capture PR3-ELISA. They used a new technique to immobilize PR3 on the ELISA plate by using a bridging molecule as an "anchor" preventing direct adhesion to the plastic surface and thereby preserving all epitopes for binding with ANCA (24).

Another important issue is that the lack of the standard sera and international unit makes it difficult to compare ELISAs from different manufacturers. For example, Nipro MPO-ANCA and MBL MPO-ANCA are widely used in reference laboratories or university hospitals in Japan. Although sensitivity and specificity were not different between these two systems and they were positively correlated with each other (Fig. 1, Table III), the absolute values obtained from these two ELISA systems cannot be compared because they do not use the same standard serum.

We should also discuss cut-off values employed in the ELISA systems. Holle et al. analysed ANCA ELISAs from 11 manufacturers and reported that applying the manufacturers' cut off values results in great variation in sensitivity. Lowering the cut off values increased the sensitivity and reduced specificity but increased overall diagnostic performance (3). They concluded that the low sensitivity of some commercial ELISA systems reflects the high cut off values rather than methodological problems in the assays.

For standardization of ANCA testing, the BCR office of the European Union sponsored a large international standardization project of monitoring different antigen purification methods (26), suitability of purified antigens for solid-phase assays and standardization (27), and clinical utility of the developed tests (28). In the meantime, before the results become clear, it is important that laboratories should understand the difference and limitations of the assays in use.

A limitation of our study is the small number of WG samples. It was difficult to collect samples from patients with active and untreated WG because MPA with renal involvement predominates in Japan but we do not see patients with WG presenting to renal units. Therefore the PR3-ANCA ELISA kit available in Japan is not manufactured in Japan. Nipro PR3-ANCA is equivalent to Immunoscan PR3-ANCA (Euro-Diagnostica) and MBL PR3-ANCA is equivalent to BINDAZYME™ Human Anti-PR3 Enzyme Immunoassay kit (Binding Site). These kits are used internationally and already well screened in the world. Trevisin et al. reported that sensitivity of Euro-Diagnostica Immunoscan PR3-ANCA was 90% sensitivity and 96% specificity (25). Binding Site BINDAZYME RP3-ANCA ELISA were reported 60-96% sensitivity and 88-100% specificity. (3, 25, 29). Further study should be required to investigate the performance of the ELISA systems using larger samples from Japanese patients with active and untreated WG. Then, it would be possible to more directly compare the performance of ELISAs for WG between Europe and Japan.

Another limitation is that we used plasma and not serum samples. However, Lee et al. reported that ANCA positivity between serum and plasma as measured by commercially available ELISA systems was concordant, and that ANCA levels in serum and plasma measured by solid phase assays correlated well (30). Therefore, our results could be compared with those tested with serum samples.

In summary, the two major MPO-ANCA ELISA systems commercially available in Japan exhibited high sensitivity and specificity that provided similar diagnostic value with the ELISA systems used in Europe. Thus, the results of ANCA testing in Japan may be compared to those from other countries. This will facilitate future international surveys exploring differences in the epidemiology of PSV, and aetiological factors contributing to their pathogenesis.

References

1. SAVIGE J, GILLIS D, BENSON E *et al.*: International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA). *Am J Clin Pathol* 1999; 111: 507-13.

2. SAVIGE J, DIMECH W, FRITZLER M *et al.*: Addendum to the International Consensus Statement on testing and reporting of antineutrophil cytoplasmic antibodies. Quality control guidelines, comments, and recommendations for testing in other autoimmune diseases. *Am J Clin Pathol* 2003; 120: 312-8.

3. HOLLE JU, HELLMICH B, BACKES M *et al.*: Variations in performance characteristics of commercial enzyme immunoassay kits for detection of antineutrophil cytoplasmic antibodies: what is the optimal cut off? *Ann Rheum Dis* 2005; 64: 1773-9.

4. LEAVITT RY, FAUCI AS, BLOCH DA *et al.*: The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. *Arthritis Rheum* 1990; 33: 1101-7.

5. MASI AT, HUNDER GG, LIE JT *et al.*: The American College of Rheumatology 1990 criteria for the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis). *Arthritis Rheum* 1990; 33: 1094-100.

6. JENNETTE JC, FALK RJ, ANDRASSY K *et al.*: Nomenclature of systemic vasculitides. Proposal of an international consensus conference. *Arthritis Rheum* 1994; 37: 187-92.

7. WATTS R, LANE S, HANSLIK T *et al.*: Development and validation of a consensus methodology for the classification of the ANCA-associated vasculitides and polyarteritis nodosa for epidemiological studies. *Ann Rheum Dis* 2007; 66: 222-7.

8. LUQMANI RA, BACON PA, MOOTS RJ *et al.*: Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis. *QJM* 1994; 87: 671-8.

9. HANLEY JA, MCNEIL BJ: The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 1982; 143: 29-36.

10. HAUGEBERG G, BIE R, BENDVOLD A *et al.*: Primary vasculitis in a Norwegian community hospital: a retrospective study. *Clin Rheumatol* 1998; 17: 364-8.

11. WATTS RA, LANE SE, BENTHAM G *et al.*: Epidemiology of systemic vasculitis: a ten-year study in the United Kingdom. *Arthritis Rheum* 2000; 43: 414-9.

12. LANE SE, SCOTT DG, HEATON A *et al.*: Primary renal vasculitis in Norfolk-increasing incidence or increasing recognition? *Nephrol Dial Transplant* 2000; 15: 23-7.

13. REINHOLD-KELLER E, HERLYN K, WAGNER-BASTMEYER R *et al.*: Stable incidence of primary systemic vasculitides over five years: Results from German vasculitis register. *Arthritis Rheum* 2005; 53: 93-9.

14. SAKAI H, KUROKAWA K, KOYAMA A *et al.*: [Guidelines for the management of rapidly progressive glomerulonephritis] *Nippon Jinzo Gakkai Shi* 2002; 44: 55-82.(in Japanese)

15. FUJIMOTO S, UEZONO S, HISANAGA S *et al.*: Incidence of ANCA-associated primary renal vasculitis in the Miyazaki prefecture: the first population-based retrospective, epidemiologic survey in Japan. *Clin J Am Soc Nephrol* 2006; 1: 1016-22.

16. GONZALEZ-GAY MA, GARCIA-PORRUA C, GUERRERO J *et al.*: The epidemiology of the systemic vasculitides in northwest Spain: Implications of the Chapel Hill Consensus Conference Definitions. *Arthritis Rheum* 2003; 49: 388-93.

17. TIDMAN M, OLANDER R, SVALANDER C *et al.*: Patients hospitalized because of small vessel vasculitides with renal involvement in the period 1975-1995: organ involvement, ANCA patterns, seasonal attack rates and fluctuation of annual frequencies. *J Intern Med* 1998; 244: 133-41.

18. HAUER HA, BAJEMA IM, VAN HOUWELINGEN HC *et al.*: Renal histology in ANCA-associated vasculitis: Differences between diagnostic and serologic subgroups. *Kidney Int* 2002; 61: 80-9.

19. FRANSSEN CF, STEGEMAN CA, KALLENBERG CG *et al.*: Antiproteinase 3- and antimyeloperoxidase-associated vasculitis. *Kidney Int*. 2000; 57: 2195-206.

20. SPECKS U, WIEGERT EM, HOMBURGER HA: Human mast cells expressing recombinant proteinase 3 (PR3) as substrate for clinical testing for anti-neutrophil cytoplasmic antibodies (ANCA). *Clin Exp Immunol* 1997; 109: 286-95.

21. LIM LC, TAYLOR JG 3RD, SCHMITZ JL *et al.*: Diagnostic usefulness of antineutrophil cytoplasmic autoantibody serology. Comparative evaluation of commercial indirect fluorescent antibody kits and enzyme immunoassay kits. *Am J Clin Pathol* 1999; 111: 363-9.

22. CSERNOK E, AHLQUIST D, ULLRICH S *et al.*: A critical evaluation of commercial immuno- assays for antineutrophil cytoplasmic antibodies directed against proteinase 3 and myeloperoxidase in Wegener's granulomatosis and microscopic polyangiitis. *Rheumatology* (Oxford) 2002; 41: 1313-7.

23. CSERNOK E, HOLLE J, HELLMICH B *et al.*: Evaluation of capture ELISA for detection of antineutrophil cytoplasmic antibodies directed against proteinase 3 in Wegener's granulomatosis: first results from a multicentre study. *Rheumatology* (Oxford) 2004; 43: 174-80.

24. HELLMICH B, CSERNOK E, FREDENHAGEN G *et al.*: A novel high sensitivity ELISA for detection of antineutrophil cytoplasm antibodies against proteinase-3. *Clin Exp Rheumatol* 2007; 25: S1-5.

25. TREVISIN M, POLLOCK W, DIMECH W *et al.*: Antigen-Specific ANCA ELISAs Have Different Sensitivities for Active and Treated Vasculitis and for Nonvasculitic Disease. *Am J Clin Pathol* 2008; 129: 42-53.

26. HAGEN EC, ANDRASSY K, CHERNOK E *et al.*: The value of indirect immunofluorescence and solid phase techniques for ANCA detection. A report on the first phase of an international cooperative study on the standardization of ANCA assays. EEC/BCR Group for ANCA Assay Standardization. *J Immunol Methods* 1993; 159: 1-16.

27. HAGEN EC, ANDRASSY K, CSERNOK E *et al.*: Development and standardization of solid phase assays for the detection of anti-neutrophil cytoplasmic antibodies (ANCA). A report on the second phase of an international cooperative study on the standardization of ANCA assays. *J Immunol Methods* 1996; 196: 1-15.

28. HAGEN EC, DAHA MR, HERMANS J *et al.*: Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. EC/BCR Project for ANCA Assay Standardization. *Kidney Int* 1998; 53: 743-53.

29. POLLOCK W, DUNSTER K, ROLLAND JM *et al.*: A comparison of commercial and in-house ELISAs for antineutrophil cytoplasmic antibodies directed against proteinase 3 and myeloperoxidase. *Pathology* 1999; 31: 38-43.

30. LEE AS, FINKIELMAN JD, PEIKERT T *et al.*: Agreement of anti-neutrophil cytoplasmic antibody measurements obtained from serum and plasma. *Clin Exp Immunol* 2006; 146: 15-20.