Evaluation of capture ELISA for detection of antineutrophil cytoplasmic antibodies directed against proteinase 3 in Wegener's granulomatosis: first results from a multicentre study

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Objective: To evaluate the performance characteristics of direct and capture ELISA for the detection of PR3-ANCA in Wegener's granulomatosis (WG) in international ANCA reference laboratories.

Methods: Serum samples were derived from patients with histological and clinical diagnosis of WG ($n = 60$), rheumatoid arthritis (RA) ($n = 30$) and healthy controls ($n = 30$). Each of them was tested for the presence of ANCA by indirect immunofluorescence technique (IFT), direct and capture ELISA in six international reference laboratories (Massachusetts General Hospital, Boston; Wieslab AB, Lund; University of Maastricht; University Hospital Groningen; Mayo Clinic, Rochester; Rheumaklinik Bad Bramstedt/University of Schleswig–Holstein Campus Lübeck). Each centre tested the sera according to their house protocols of IFT and ELISA. The diagnostic performance of each test was estimated by receiver operating characteristic curve analysis and sensitivity and specificity in detection of ANCA/PR3-ANCA were calculated for the respective methods.

Results: In patients histologically and clinically known as WG, the detection of ANCA by IFT varied between 52 and 83% among the participating centres. PR3-ANCA positivity with the different ELISAs ranged from 53 to 80% in direct ELISA and from 72 to 76% in capture ELISA. While most capture ELISAs successfully detected PR3-ANCA, there were significant differences between IFT and direct ELISA results between laboratories. ROC curve analysis demonstrated that in five of six laboratories the overall diagnostic performance of capture ELISA was superior to IFT and direct ELISA, respectively.

Conclusion: Capture ELISA is a highly sensitive assay for detection of PR3-ANCA in WG and should be used in conjunction with compatible clinical picture and histological evidence.

KEY WORDS: ANCA, ELISA, IFT, Proteinase 3, Wegener's granulomatosis.

The measurement of ANCA, especially ANCA against proteinase 3 (PR3-ANCA), is important for the diagnosis and follow-up of patients with Wegener's granulomatosis (WG). For routine detection of ANCA, the indirect immunofluorescence technique (IFT) and/or antigen-specific direct ELISA (PR3-ANCA) are the common screening methods. It has already been demonstrated that by inclusion of a standardized antigen-specific ELISA the value of the IFT can be greatly increased [1]. Recently, an international group of ANCA researchers has published a consensus statement on ANCA testing [2]. These guidelines demand that in case of positive IFT-testing for ANCA, a direct ELISA test is obligatory as a minimum requirement. The recommendation for optimal testing includes both IFT and direct ELISA on all samples.

However, there are significant differences in sensitivity, specificity and predictive value among available commercial ELISA kits [3–5]. Moreover, IFT and direct ELISA do not always correlate. The reason might be that proteins are denatured during antigen purification or coating onto the solid phase, thereby hiding or destroying conformational epitopes on PR3. In order to avoid this, capture ELISA has been designed: here the plate is precoated with a monoclonal antibody to capture the antigen (Fig. 1). However, capturing antibodies may block relevant epitopes [6]. Some data suggest an advantage over direct ELISA [5, 7, 8] but the diagnostic value of different capture ELISA is not yet evaluated and there exists no agreed standard on available capture tests. This prompted us to compare the ANCA results obtained by IFT, direct and capture ELISA in clearly defined groups of WG patients, performed in six international ANCA reference laboratories. The specificities of the assays were related to rheumatoid arthritis (RA) sera and normal human sera.

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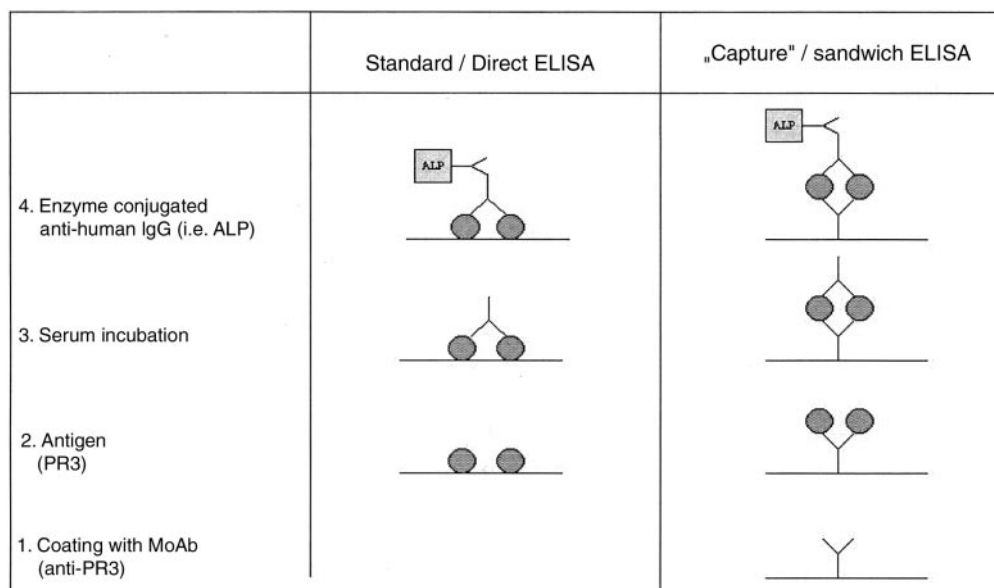
FIG. 1. Principle of PR3-ANCA-ELISAs.

Patients and methods

Patient population and diagnostic criteria

The analysed sera are derived from well-defined patients with a clinical diagnosis of WG that had been made irrespective of serology (i.e. the presence of ANCA in sera was not a criterion for the diagnosis of WG). Sixty consecutive patients diagnosed with WG in the Departments of Rheumatology and Immunology at Bad Bramstedt (Rheumaklinik) and Lübeck (Department of Rheumatology/University of Schleswig–Holstein) between 1997 and 2000 were studied. The diagnosis of WG was established according to international standards by applying the 1990 classification criteria of the American College of Rheumatology [9], and the definitions of the 1992 Chapel Hill Consensus Conference [10]. WG was biopsy-proven in each patient. Biopsies were seen in the German reference centre for vasculitis (Department of Pathology, University of Schleswig–Holstein Campus Lübeck) by two different observers. The disease activity in vasculitis patients was documented by using the Birmingham Vasculitis Activity Score (BVAS) at the time the serum samples were collected [11]. Moreover, sera from 30 age- and sex-matched healthy controls and 30 RA patients were analysed.

This study was carried out in accordance with the 1997 Declaration of Helsinki of the World Medical Association (World Medical Association, 1997). The design of the work has been approved by the ethical committee of the University Schleswig–Holstein, Campus Lübeck, and each patient, or their legal designee, gave informed consent prior to participation in the study.

Methods of ANCA detection

The sera were coded before being sent to the centres. Each centre tested the sera according to their house protocols of IFT, direct and capture ELISA.

Bad Bramstedt/Lübeck. IFT and direct and capture ELISA for PR3-ANCA were performed as described elsewhere [5].

ANCA detection by IFT and direct ELISA were performed according to standardized European guidelines. Briefly, IFT was performed on air-dried, ethanol-fixed leukocytes, which were incubated with diluted patient's sera. Autoantibody binding was detected with FITC-conjugated rabbit anti-human IgG (Dako, Hamburg). For the differentiation of P-ANCA and anti-nuclear antibodies (ANA), sera that gave perinuclear/nuclear staining were further tested on formalin-fixed neutrophils and HEp-2 cells. In our laboratory, a positive ANCA is defined as the titre of antibodies > 1:20. For detection of PR3-ANCA by direct ELISA, 96-well microtitre plates were coated with affinity-purified PR3 at a concentration of $2 \mu g/ml$. Sera were added at a dilution of 1:50 in phosphate buffered saline (PBS). Detection of autoantibodies was performed with anti-human IgG ALP labeled conjugate. Extinction was measured at 405 nm (620 nm). The results were recorded as positive when the concentration was > 20 U/ml.

For capture ELISA, monoclonal antibody (MoAb) WGM2 (anti-PR3 MoAb) was coated at a concentration of $2 \mu g/ml$ to microtitre plates for 12 h at 4°C in carbonate buffer at pH 9.6. After washing three times in PBS and 0.05% Tween 20, purified PR3 was incubated at a concentration of $1 \mu g/ml$ in PBS at room temperature (RT) for 1 h and the plates were washed. Sera diluted 1:50 in PBS with 0.1% BSA and 0.05% Tween 20 were incubated for 1 h at RT. After washing, bound IgG was detected by alkaline phosphatase-conjugated anti-human IgG (Dako, Hamburg). To exclude non-specific binding, a control plate was coated with an unrelated mouse MoAb of the same mouse Ig subclass and the absorbance values of this plate were subtracted from the absorbance values obtained on the anti-PR3 coated plates for each individual well. A serum was regarded as positive if the absorbance was > 3 S.D. higher than values obtained with sera ($n = 120$) from healthy donors. Values were related to a set of calibrators to quantify the values in arbitrary units (AU)/ml. The cut-off value was set at 35 AU/ml. Intra-assay variability was 6%, as calculated by testing one sample 10 times in the same run. Inter-assay variability was 9%, as calculated by testing the same sample in five different runs.

Boston. Each serum specimen was examined by IFT, direct ELISA and capture ELISA. Methodologic details about assays have been previously described [12]. Briefly, ANCA were detected by IFT at a 1:16 dilution of serum by using centrifuged, ethanol-fixed normal neutrophils. All sera were also examined for the presence of ANCA using standard techniques. IFT studies were classified as C-ANCA, P-ANCA, atypical (A-ANCA) or negative. The direct PR3-ANCA ELISA was done as previously described [12]. In the capture ELISA, monoclonal antibody 1E8 was adhered to the wells of microtitre plates and used to bind PR3. Subsequent steps were the same as those of the direct ELISA. To control for antibodies to the monoclonal catching antibody, additional wells

were coated with monoclonal anti-PR3 catching antibody 1E8 but were not subsequently incubated with PR3. The reactivity of the serum to the monoclonal 1E8 alone was then subtracted from the reactivity to the 1E8–PR3 complex. The result is the titre for a revised capture ELISA for PR3-ANCA.

Groningen. IFT, direct ELISA and capture ELISA were performed as described elsewhere [13, 14]. Briefly, ANCA detection by IFT was performed on ethanol-fixed granulocytes. Serum samples were diluted in PBS and tested at 2-fold serial dilutions starting at 1:20. Antibody binding was detected with FITC-conjugated F(ab)2 rabbit anti-human IgG (Dako, Copenhagen, Denmark). Titres > 1:40 were considered positive. For detection of PR3-ANCA by direct ELISA, 96-well microtitre plates (Nunc, Maxisorp) were coated at 37°C for 1.5 h with highly purified inactivated human PR3 (1 μg/ml) in coating buffer (0.1 M sodium carbonate). Sera were diluted and applied at a dilution of 1:100 and 1:300. Antibody binding was detected with AP-conjugated goat anti-human IgG (American Qualex Inc, San Clemente CA). Values of > 6 AU/ml according to an in-house standard (mean + 2 S.D. of 65 normal controls) were considered to be positive. The intra- and interassay variations were < 10%.

For capture ELISA, microtitre plates (Greiner) were coated subsequently with F(ab)2 goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Westgrove PE, 3 μg/ml, 48 h at 4°C), mouse monoclonal anti-PR3 (12.8) or control mouse IgG (2 h at 37°C), and a crude granular extract of human polymorphonuclear leukocytes (PMNs) (overnight at 4°C). Sera were diluted in medium containing normal goat serum and were tested at 2-fold serial dilutions starting at 1:100. Antibody binding was detected as described for the direct ELISA.

Lund. IFT, direct ELISA and capture ELISA were performed as described elsewhere [8]. In short, ethanol-fixed leukocytes were incubated with patient's sera diluted 1:20 in PBS for 30 min at RT and bound IgG was detected by FITC-conjugated rabbit anti-human IgG (Dako, Denmark). For PR3-ANCA direct ELISA, microtitre plates coated with isolated human neutrophil PR3 were obtained from Wieslab AB (Lund, Sweden) and used according to the manufacturer's instructions.

For capture PR3-ANCA ELISA, MoAb 4A3, at a concentration of 1 μg/ml was coated to microtitre plates for 16 h at RT. After washing, purified PR3 at a concentration of 1 μg/ml in PBS was incubated at RT for 1 h. Sera, diluted 1:80 in PBS were incubated in duplicate wells for 1 h at RT, and after washing, bound IgG was detected by ALP-conjugated anti-human IgG (Orion Diagnostik, Helsinki). To exclude non-specific binding, a control plate was coated with an unrelated MoAb of the same mouse IgG subclass and the absorbance values of this plate were subtracted from the absorbance value obtained on anti-PR3 coated plate for each individual well. A serum was regarded as positive if the absorbance was > 3, i.e. higher than values obtained with healthy donors (*n* = 80). Values were related to a set of calibrators to quantitate the values in AU/ml. Cut-off was at 8 AU/ml.

Maastricht. IFT was performed as previously described [15]. Briefly, IFT was performed on ethanol-fixed granulocytes. A titre > 1:40 was considered positive. PR3-ANCA direct and capture ELISA as described elsewhere [16]. In short, 96-well microtitre plates were coated at 37°C for 1.5 h with 5 mM PMSF-inactivated PR3 (1 μg/ml). After washing, duplicate samples, diluted 1:100, were incubated for 1 h at 37°C, washed, and incubated with affinity purified Fab$_2$ goat ALP-conjugated anti-human IgG (American Qualex) for 1 h at 37°C. The optical density was measured at 405 nm. Values of 6 AU (mean + 2 S.D. of 65 normal controls) or more were considered to be positive. In the capture ELISA, mouse monoclonal anti-PR3 (12.8) or control mouse IgG were coated on microtitre plates and used to bind PR3. Subsequent steps were the same as those of the direct ELISA. Antibody binding was detected as described for the direct ELISA.

Mayo Clinic, Rochester. IFT, direct ELISA and capture-ELISA were performed according to the protocols published previously [7].

Briefly, standard IFT was performed using ethanol-fixed neutrophils. Sera were screened at a dilution of 1:4. If perinuclear or nuclear immunofluorescence was detected, the IFT was repeated using formalin-fixed neutrophil preparations. Samples were interpreted as P-ANCA positive if they displayed cytoplasmic staining on formalin-fixed slides. Commercially available PR3-ANCA direct ELISA kit (Scimedx Corporation, Denville, NJ) was used for PR3-ANCA detection, according to the manufacturer's instructions. For PR3-ANCA capture ELISA, cell culture supernatant of 293 cells stably expressing recombinant PR3 (rPR3-S176A) was used as target antigen. Microtitre wells were incubated with MoAb MCPR3-2 (4 μg/ml) at 4°C overnight. After washing, cell lysates from rPR3-S176A were incubated in coated wells for 1 h at RT. Control wells were incubated in parallel with lysis buffer alone. After washing, aliquots from serum dilution (1:20) were incubated for 1 h at RT, followed by three washes and incubation of ALP-conjugated goat anti-human IgG (Sigma). The absorbance was determined at a wavelength of 405 nm. Net absorbance values calculated by subtraction of the background value from the value obtained from wells containing capture antigen are reported. Serum samples yielding net absorbance values of $\geq$0.100 were considered to be PR3-ANCA positive.

The PR3-ANCA positivity in the sera positive in the capture ELISA, but negative in direct ELISA were analysed by immunoblotting to confirm the positive ELISA results. Immunoblotting was performed by Lüdemann and Utecht (Utecht & Lüdemann GmbH, Raisdorf, Germany).

Statistical analysis

Receiver operating characteristic (ROC) curves were analysed to estimate the diagnostic performance of the respective tests at the different laboratories, as previously described [17]. The cut-off points for calculation of sensitivity and specificity were determined by ROC curves. The diagnostic sensitivity and specificity of each test were calculated by 2×2 tables. The SPSS 11.0 software package (SPSS Inc., Chicago, IL) was used for statistical analysis.

Results

Patient characteristics are outlined in Table 1. Patients were categorized into two groups according to disease activity at the time of sampling. Forty-four patients with various degrees of disease activity were classified as having active disease. Their median

TABLE 1. Characteristics of the patient population

	Total	Active disease	Inactive disease
WG (*n* = 60)			
No. of males	26	19	7
No. of females	34	25	9
Age (years)			
Range	15–81		
Median	58		
Organ involvement[a]	Number[b]		
ENT	35		
Lung	8		
Kidney	9		
Eye	3		
Arthritis	11		
CNS	3		
PNS	4		

[a]ENT, ear, nose, throat; CNS, central nervous system; PNS, peripheral nervous system.
[b]Number with active organ involvement at time of serum sampling.

BVAS was 9.0 (range 2–27). The inactive or remission phase was defined by the absence of clinical activity and by the absence of symptoms or signs attributable to active vasculitis (BVAS: 0). Sixteen patients had no sign of disease activity at the time their serum samples were obtained; their BVAS was 0. Fifty patients had systemic involvement, only a small proportion of patients had 'limited' WG (8.3%). All patients with localized WG were in partial remission with some symptoms still persisting due to granulomatous damage. In this study, all ANCA positive WG patients were C-ANCA and/or PR3-ANCA positive.

In order to determine the diagnostic performance of IFT, direct ELISA and capture ELISA, ROC curves for the respective diagnostic tests were analysed for each of the participating centres separately (Fig. 2). According to a method described by Hanley and McNeil [17], the 95% confidence interval (CI) for the area under the ROC curve was used to test the hypothesis that the theoretical area is 0.5. If the CI does not include the 0.5 value, then there is evidence that the test under investigation has the ability to distinguish between disease (here WG) and controls. Table 2 shows that each of the methods had a high diagnostic power for
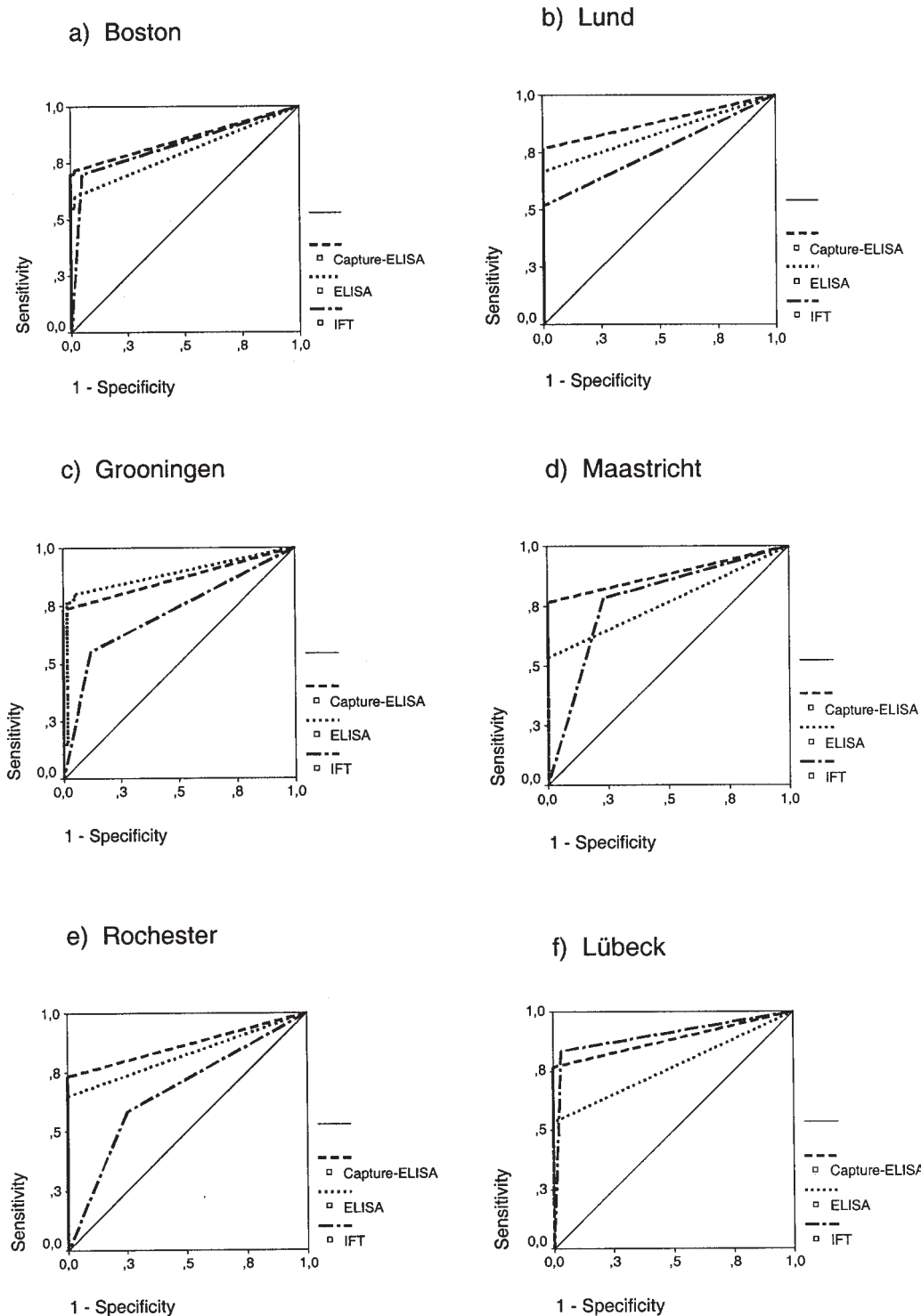

FIG. 2. ROC curves for the detection of anti-PR3-ANCA by IFT, ELISA and capture ELISA by the six participating laboratories.

TABLE 2. ROC curve analysis: interlaboratory variation of diagnostic performance of IFT, direct ELISA and capture ELISA for the determination of ANCA for the diagnosis of WG in six laboratories

Laboratory	IFT	Direct ELISA	Capture ELISA
Boston	0.825 (0.746, 0.904)*	0.796 (0.712, 0.879)*	0.856 (0.783, 0.929)*
Lund	0.758 (0,670, 0,847)*	0.833 (0.756, 0.911)*	0.883 (0.817, 0.950)*
Groningen	0.717 (0.623, 0.810)*	0.883 (0.816, 0.950)*	0.867 (0.796, 0.937)*
Maastricht	0.775 (0.688, 0.862)*	0.767 (0.679, 0.854)*	0.883 (0.817, 0.950)*
Rochester	0.667 (0.569, 0.764)**	0.825 (0.746, 0.904)*	0.867 (0.796, 0.937)*
Lübeck	0.900 (0.838, 0.962)*	0.767 (0.679, 0.854)*	0.883 (0.817, 0.950)*

Data are displayed as area under the ROC curve with 95% CI for each participating centre.
*$P < 0.001$, **$P = 0.002$ *vs* an AUC $= 0.5$.

TABLE 3. Sensitivity and specificity of IFT, direct ELISA and capture ELISA in WG patients

Centres	IFT		Direct ELISA		Capture ELISA	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Boston	70	100	60	100	72	100
Lund	52	100	68	100	76	100
Groningen	55	100	80	95	73	100
Maastricht	77	100	53	100	76	100
Rochester	58	100	65	100	73	100
Lübeck	83	100	58	100	76	100

All figures are percentage values.

the diagnosis of WG ($P = 0.002$ for IFT in Rochester, $P < 0.001$ for all other tests). Comparison of area under curve (AUCs) between IFT, direct ELISA and capture ELISA demonstrated that, in four of the six centres, capture ELISA yielded the highest serologic accuracy (Table 2, Fig. 2). In one laboratory (Lübeck) IFT was slightly superior to capture ELISA, while in another laboratory direct ELISA showed a modestly better performance compared to capture ELISA (Table 2). Compared to ELISA, capture ELISA yielded a better diagnostic performance in five of the six laboratories, which was quite considerable in three of the five centres (Boston, Maastricht, Lübeck), as expressed by the low overlap of CI values (Table 2). In five of the six laboratories, capture ELISA was also superior to IFT, especially in Lund, Groningen, Maastricht and Rochester, as reflected by the largely different AUC values.

The specificity for IFT and capture ELISA was 100% in each laboratory, specificity for direct ELISA was also 100% in each lab, except for Groningen where the figure was 95% (Table 3). The sensitivities showed a greater variation concerning all testing methods. In IFT the sensitivity ranged from 52% (Lund) to 83% (Lübeck); direct ELISA gave sensitivities between 53% (Maastricht) and 80% (Groningen). The capture ELISA produced a more homogenous result with sensitivities ranging from 72 to 76%. Three of the six centres achieved the highest sensitivity using capture ELISA. In IFT, the Bad Bramstedt/Lübeck laboratory showed the best results with a sensitivity of 83% and a specificity of 100%. Moreover, the average sensitivity of direct ELISA was lower than that of capture ELISA. IFT yielded a lower sensitivity in comparison to capture ELISA due to the great variation in IFT-sensitivity between the different laboratories. The positivity of PR3-ANCA in the sera was confirmed by immunoblotting. All the PR3-ANCA positive sera in capture ELISA were positive in western blot.

Discussion

Solid phase assays for PR3-ANCA testing have been developed in order to allow antigen-specific reader-independent, quantitative

PR3-ANCA testing. Various methods of antigen preparation have been reported and clinical evaluation of several of these methods has been investigated [1, 3, 12]. These studies demonstrated that the sensitivity of most PR3-ANCA ELISA, based on direct coating of the purified antigen to the plastic plate, is equivalent to the standard IFT of ANCA detection using neutrophil cytospin preparations. However, the specificity of the assays was lower. Capture ELISA methods using anti-PR3 monoclonal antibodies to capture the antigen, promises a sensitivity equivalent to the standard C-ANCA detection method by IFT [8]. Data comparing the analytical sensitivity of the various capture ELISA used for PR3-ANCA detection are not available to date. To address the question of whether PR3-ANCA capture ELISA is more effective for the detection of ANCA in WG, we started a multicentre study (six international ANCA reference centres) in which we first evaluated the diagnostic performance of direct and capture PR3-ANCA ELISA and compared it with the IFT. Each centre tested blinded sera from well characterized patients with the diagnosis of WG, RA and healthy controls according to their house-protocols of IFT and ELISA. In this study we selected our patients on the presence of biopsy-proven WG and not based on ANCA positivity by IFT.

Hagen *et al.* [1], have demonstrated that a significant proportion of patients with WG have MPO-ANCA and not PR3-ANCA, with only rare patients having both. In our cohort of WG patients none of the patients had P-ANCA and/or MPO-ANCA. This can be explained by the small number of WG patients with MPO-ANCA which are found in the patient population (1.8%) seen at the combined unit of Rheumatology of Bad Bramstedt and the University of Schleswig–Holstein Campus Lübeck [19].

The absence of MPO-ANCA positive sera in the cohort studied can be explained by the low percentage of renal involvement in this study (15%, in other series around 60%). Renal disease patients tend to have more MPO-ANCA.

In general, all different methods show maximum or near maximum specificity with good correlation between the different centres; only in the group of direct ELISA tests was specificity lower than 100%—in the Groningen laboratory, which was therefore able to attain the highest sensitivity of this group. The other

laboratories reported significantly lower sensitivities with a 100% specificity in each laboratory. Standardization of cut-off level in the different assays, i.e. using a generally accepted standard, was not the objective of this study, but may explain the differences in the results between the participating laboratories.

ROC curve analysis demonstrated that, based on the investigation of 60 sera from WG patients and 60 sera from disease control (RA) and healthy controls to detect ANCA/PR3-ANCA, the diagnostic performance of the capture ELISA was considerably better compared to IFT in four of the six participating centres and comparable to IFT in the two other centres. Furthermore, diagnostic performance of the capture ELISA was considerably better compared to direct ELISA in two of the six participating centres, and at least similar to direct ELISA in the other four centres.

With regard to sensitivity, we found that this might be further increased in all three test methods. Capture ELISA seems to be a promising tool in achieving this, as it is a test with the potential to be improved. In fact, ROC analysis revealed that capture ELISA compared to IFT and direct ELISA showed the best diagnostic performance in five of the six laboratories, respectively. All borderline PR3-ANCA positive sera in direct ELISA were positive in all capture ELISA. Interestingly, the results from a comparable study concerning detection of MPO-ANCA by different assays in pauci-immune crescentic glomerulonephritis demonstrated that the sensitivity in both direct and capture MPO-ANCA ELISA was comparable, and that the specificity of capture ELISA was much higher versus direct ELISA [19]. The major findings of this study are the following: (i) capture ELISA is a more sensitive technique to detect PR3-ANCA in WG, compared with direct ELISA and IFT; (ii) concerning the sensitivity in IFT and direct ELISA, there was a great variability between the results of participating laboratories; (iii) capture ELISA results showed a significant correlation between all participating centres. In our study, the interlaboratory variation in the capture ELISA was surprisingly small considering the differences in antigen preparations, 'catching' anti-PR3 antibodies, substrates and conjugates.

Based on our preliminary data, we conclude that capture ELISA seems to be the superior method of PR3-ANCA detection in Wegener's granulomatosis, which was confirmed in the majority of the participating laboratories. Thus, in patients with WG, capture ELISA could be the preferred method for detection of PR3-ANCA, and should be used in conjunction with a compatible clinical picture and histological evidence. However, the diagnostic specificity and clinical utility of the capture PR3-ANCA ELISA as a screening or confirmatory test for certain types of vasculitis remains to be investigated. Furthermore, testing for ANCA by capture ELISA may occasionally yield false-positive results as PR3-ANCA from some patients may recognize an epitope on PR3 that is occupied by the capturing monoclonal antibody [6]. However, as confirmed by the present study this does not seem to materially affect the sensitivity for WG.

Further studies are needed to evaluate the capacity of capture ELISA; moreover it seems worthwhile to examine the correlation between disease activity and capture ELISA scores, to study their ability to detect clinical relapse/remission and to analyse if the quantification of PR3-ANCA by capture ELISA can be used as a therapeutic guideline for antibody-directed treatment of patients with WG.

Acknowledgements

	Key points
Rheumatology	**Key messages:** This study addresses the question of the sensitivity and specificity of different laboratory tests (immunofluorescence, direct and capture PR3-ANCA ELISA) for the measurement of ANCA in patients with Wegener's granulomatosis in international ANCA reference laboratories.

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