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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

de Joode, A. A. E. (2016). *Improving clinical management in ANCA-associated vasculitis*. [Thesis fully internal (DIV), University of Groningen]. Rijksuniversiteit Groningen.

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Performance of two strategies for urgent ANCA and anti-GBM analysis in vasculitis.

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Eur J Int Med 2014; 25: 182-186

ABSTRACT

Background

In anti-neutrophil cytoplasmic antibodies (ANCA) associated small vessel vasculitis (AAV), rapid testing for ANCA and anti-glomerular basement membrane (GBM) antibodies may be beneficial for therapeutic purpose.

Objective

We analysed the diagnostic performance of two rapid ANCA and anti-GBM test methods in 260 patients with suspected AAV.

Methods

Between January 2004 and November 2010, we analysed 260 samples by qualitative Dotblot (Biomedical Diagnostics); retrospective analysis followed with directly coated highly sensitive automated Phadia ELiA and ELiA anti-GBM.

Results were related to the final clinical diagnosis and compared with routine capture ELISA.

Results

Seventy-four patients had a final diagnosis of AAV (n=62) or anti-GBM disease (n=12). Both Dotblot and ELiA detected all 12 cases of anti-GBM disease; 2 false positive results were found.

Dotblot detected ANCA in 56 of 62 AAV-patients (sens 90%, NPV 97%), and showed 5 false positives (spec 97%, PPV 90%). The Phadia ELiA anti-PR3^s or anti-MPO^s was positive in 57 of 62 AAV patients (sens 92%, NPV 97%), and had 5 false positives (spec 97%, PPV 88%). Routine capture ELISA was equally accurate (sens 94%, spec 97%, PPV 88%, NPV 98%).

Conclusion

The Dotblot and Phadia ELiA on anti-GBM, anti-PR3s and anti-MPOs performed excellent; results were almost identical to routine ELISA. When suspicion of AAV or anti-GBM disease is high and diagnosis is urgently needed, both tests are very powerful for rapid serological diagnosis. Further studies have to confirm the test performances in samples routinely presented for ANCA-testing and in follow-up of positive patients.

INTRODUCTION

In anti-neutrophil cytoplasmic antibody (ANCA) associated small vessel vasculitis (AAV), patients may present with a rapid clinical decline due to life threatening progressive loss of renal or respiratory function. Because of this possible rapid deterioration, patients who are suspected of AAV may benefit from rapid testing for ANCA and anti-glomerular basement membrane (GBM) antibodies to start immunosuppressive therapy as soon as a diagnosis is serologically supported and to differentiate these diseases from other conditions.

The classical method to detect presence of ANCA is indirect immunofluorescence (IIF) on ethanol-fixed neutrophils which may show a C-ANCA (granular cytoplasmatic) pattern or P-ANCA (perinuclear staining) pattern.[1-3] In patients with small vessel vasculitis (AAV), these patterns are usually associated with antibodies against proteinase 3 (PR3) and myeloperoxidase (MPO) which are strongly associated with the presence of granulomatosis with poly-angiitis (GPA) or microscopic poly-angiitis (MPA).[3-5] However, C-ANCA and P-ANCA as detected by IIF are not equivalent to the presence of anti-PR3 and anti-MPO antibodies respectively, as ANCA with different or unknown antigenetic specificities do occur in other diseases as well.[2,4,8] The diagnosis of ANCA-associated vasculitis is of course made by clinical symptoms and biopsy, so presence of C-ANCA or P-ANCA only gives a clue to the diagnosis. [5] Therefore, it has been agreed that after a positive result on IIF, one should confirm these ANCA with antigen specific enzyme linked immunosorbent assay (direct and/or capture ELISA) to improve sensitivity and especially specificity.[3-7,9] Recently, studies on different strategies have been published: screening with ELISA and confirming positive results with IIF could be as valuable and accurate as the other way around and because of the specificity of newer antigen-specific serological tests, there even has been doubt whether testing for ANCA with IIF is still necessary at all.[4,5] Since early treatment can prevent adverse outcome in systemic small vessel vasculitis, a drawback of both IIF and ELISA (or a combination of tests) is the time needed for obtaining results. Although ELISA has the advantage of being automated and requires less technical experience, the time needed to obtain a result still can be substantial due to various logistical reasons.[4,10]

Routinely, in our laboratory ANCA are tested by IIF followed by an in house antigen-specific capture ELISA.[6] IIF is performed twice a week and the antigen-specific capture ELISA once a week; the results are thus usually not available on the day the sample is taken from the patient. For rapid detection of ANCA, we therefore use a qualitative Dotblot detecting PR3-ANCA, MPO-ANCA and anti-GBM (Biomedical Diagnostics), which has the results available within 2 hours. We prospectively analysed the diagnostic performance of our qualitative Dotblot for rapid assessment of the presence of PR3-ANCA, MPO-ANCA and anti-GBM in a cohort of patients with suspected AAV. We compared the results of this rapid qualitative

test with the final clinical diagnosis and with our standard IIF (C- or P-ANCA), capture ANCA-ELISA, and anti-GBM direct ELISA.[7, 8] In addition, we retrospectively analysed the performance of the novel quantitative Phadia high sensitive ELiA anti-PR3^s and anti- MPO^s anchor tests which have recently become available for rapid detection of ANCA and anti-GBM antibodies. Also these data were compared to the final clinical diagnosis and our routine serological analysis with IIF and antigen-specific capture ELISA.

METHODS

Patients

Consecutive samples sent to our Laboratory for urgent analysis of ANCA and anti-GBM between January 2004 and November 2010 were included in this study. The requests came from seven different clinical centres, including our own university hospital. The study population included 260 serum samples, taken from 260 patients who were suspected for AVV or anti-GBM related pulmonal-renal syndrome based on various clinical grounds (Table 1). The physician requesting the urgent analysis had to contact the coordinator of the laboratory to discuss the need for urgent determination. We recorded the reasons for these requests. A few weeks later, the physician requesting the urgent ANCA and anti-GBM determination was contacted and asked to provide the diagnosis that had been made. The final clinical diagnosis was based on biopsy results and other additional test results and clinical symptoms and signs. Different forms of small vessel vasculitis were classified using the Chapel Hill Classification criteria with the modification by Watts et al.[12] Finally, at the time of the analysis of the stored samples by the Phadia ELiA the physicians were asked to re-assess the diagnosis at the time the sample for ANCA and anti-GBM was taken and to see whether during follow up a diagnosis of AAV or anti-GBM had been made.

Methods

At the time of request, all samples were tested immediately after arrival at the laboratory by commercially available Dotblot for PR3-ANCA, MPO-ANCA and anti-GBM (MBG Dot, Biomedical Diagnostics, Antwerp, Belgium) performed according to the manufacturer's instructions.[2] Results were available within 2 hours after receipt of the patient's blood sample and reported back by telephone.

In the following days results were confirmed with our standard in house combination of IIF on ethanol-fixed neutrophil slides as described earlier and capture ELISA for anti-PR3 and anti-MPO (all human native antigens PR3 and MPO antigen) and remaining samples were stored (-20 °C).[11,13-16,18] Anti-GBM antibodies were measured in an external diagnostic laboratory (Sanquin, Amsterdam, The Netherlands) using an in house direct ELISA.[17]

Retrospectively, all stored samples were tested with the novel anchor coated highly sensitive (hs) Phadia ELiA (Thermo Fisher Scientific/Phadia, Freiburg, Germany) using human native antigens, performed on a Phadia250 analyser. Reference values were as recommended by the manufacturer. This ELiA-test method was calibrated on international standards available for MPO-and PR3-ANCA and results are in IU/ml. In addition, all samples were tested for ELiA anti-GBM on the Phadia250 analyser (human recombinant antigen). A Cohen's kappa was used to compare interrater agreement for Dotblot and ELiA data with routine ELISA. For calculation of sensitivity, specificity and likelihood ratios, we compared the serological results obtained by the different methods to the final clinical diagnosis. In case of double positivity for ANCA and anti-GBM antibodies, the best fitting test result of the two in view of the final diagnosis was considered true positive and the other result as false negative.

RESULTS

Patients, reasons for request and diagnoses.

Two hundred sixty patients from 7 different centres were suspected for AAV or anti-GBM, based on one or more clinical findings. Median age of patients enrolled was 64.9 years, interquartile range 45.3-73.6. The following clinical symptoms and signs were the main reasons for the requests for rapid testing: in renal involvement, main cause was loss of or decline in kidney function combined with haematuria, or suspicion of renal disease combined with systemic complaints like myalgia, tiredness or fever. For pulmonary involvement, main reason was respiratory insufficiency, most of the time combined with lesions on chest X-ray or pulmonary CT. Second reason was haemoptysis combined with dyspnoea and coughing and/or systemic complaints.

In addition to specific signs or symptoms suggestive of vasculitic organ involvement, in many patients a presumed non-infectious systemic inflammatory syndrome was the reason for request of rapid testing. Table 1 shows numbers and summary of reasons of request for rapid testing.

Seventy-four patients (28.5%) had a final diagnosis of anti-GBM-disease and AAV. Twelve of these patients had anti-GBM disease, 35 patients were diagnosed with GPA, 21 patients with MPA, 4 with RLV and 2 patients with eosinophilic granulomatosis with poly-angiitis. In one hundred eighty-six patients no diagnosis of AAV and anti-GBM disease was made. The final diagnosis in this latter group varied from specific solitary organ diseases like minimal change disease, Alports syndrome or alveolar haemosiderosis to sepsis and hematologic cancer or solid tumours (Table 2). During follow up, no diagnosis of AAV or anti-GBM-disease in any of these patients was made.

Table 1 Main reasons for rapid testing according to organ involvement: clinical indications for testing ANCA and anti-GBM (most requests were based on combination of symptoms)

Renal	N= 200
Systemic symptoms	N= 126
Pulmonary	N= 94
Neurological	N=29
ENT	N=26
Abdominal	N= 18
Dermal/soft tissue	N= 24
Otherwise (f.i biochemical abnormality)	N=16

ANCA= anti-neutrophil cytoplasmic antibody

Anti-GBM= anti-glomerular basement membrane

Table 2 Clinical outcome other than AAV: definite diagnosis (specified in supplementary material)

Renal	N= 62
Connective tissue disorders	N= 9
Urological	N= 2
Pulmonary	N=19
Infectious disorders	N= 26
Carcinoma	N= 19
Cardial	N= 9
Neurological	N= 2
Unknown	N= 38

AAV=ANCA-associated vasculitis.

Results of Dotblot, IIF, ELISA and Phadia ELiA

With the qualitative Dotblot test, six patients in whom a diagnosis of AAV was made, tested negative for anti-PR3 and anti-MPO antibodies (8%). The final diagnosis was GPA in 2, MPA in 3 and RLV in 1 patient. In the 186 patients in whom no diagnosis of AAV or anti-GBM disease was made, Dotblot was positive in 5 for ANCA (3%): 2 patients were positive for anti-PR3 and 3 patients for anti-MPO. These patients were diagnosed with sarcoidosis (n=2), IgA-nephropathy (n=1), endocarditis (n=1) and renal failure without a final diagnosis (n=1). In addition, one patient with anti-GBM disease was also positive for anti-MPO by the Dotblot test and as this patient did not show findings compatible with additional AAV,

Table 3 Clinical outcomes compared to outcomes of all test methods

	Dotblot	ELISA	IIF	Phadia hs	Phadia anti-GBM
GPA (n=35)	33	33	32	33	
MPA (n=21)	18	20	21	19	
RLV (n=4)	3	3	3	3	
CSS (n=2)	2	2	2	2	
Anti-GBM (n=12)	12	12			12
Others (n=186)	5	6	41	5	

GPA= granulomatosis with poly-angiitis.

MPA= microscopic poly-angiitis

RLV= renal limited vasculitis

CSS= Churg Strauss Syndrome

IIF= indirect immunofluorescence

Table 4 Sensitivity, specificity, positive and negative predictive value for all methods of testing in 260 serum samples of patients suspected for AAV.

	Dotblot	ELISA	IIF	ELiA anti-PR3	ELiA anti-MPO
Sensitivity	90%	94%	94%	93%	92%
Specificity	97%	97%	78%	97%	98%
PPV	90%	88%	59%	86%	87%
NPV	97%	98%	97%	98%	97%

AAV= ANCA-associated vasculitis

it was also deemed to be a false-positive (Table 3 and 4). This resulted in a sensitivity of 90%, specificity of 97%, positive predictive value (PPV) of 90% and negative predictive value (NPV) of 97% for the diagnosis of AAV for ANCA detected by the Dotblot test (Table 4). The likelihood ratio for a positive test was 33.6, for a negative test 0.099.

Dotblot detected all 12 cases of anti-GBM-disease. In one patient, anti-MPO antibodies were also positive (see above). In two patients with MPA, Dotblot showed double-positivity for MPO-ANCA and anti-GBM while no evidence for renal anti-GBM disease was found on renal biopsy, resulting in two false positives. Sensitivity for anti-GBM disease was therefore

100%, specificity 99%, positive predictive value 86% and negative predictive value 100%. Likelihood ratio for positive test 124, for a negative test 0.0. Interrater agreement between Dotblot and ELISA by Cohen's kappa was 0.89 for anti-PR3, 0.85 for anti-MPO and 0.92 for anti-GBM.

Routine standard IIF for ANCA and capture ELISA for anti-PR3 and anti-MPO antibodies showed three and four false negative results, respectively, in patients in whom a final diagnosis of AAV was made. Three of the four patients that were negative in capture ELISA were also negative in Dotblot: these patients were diagnosed as GPA (n=2) and RLV (n=1). IIF was also negative in two of these three patients; the last false negative result in both IIF and capture ELISA was found in a patient who was diagnosed with GPA but was positive for anti-PR3 in the Dotblot (Table 3 and 4)

Capture ELISA tested false positive in 8 patients (4 anti-PR3, 4 anti-MPO): five of these patients were also positive in Dotblot (two with sarcoidosis, one renal failure without final diagnosis, one endocarditis, one anti-GBM disease). The other three patients were diagnosed with tubulo-interstitial nephritis (n=2) and anti-GBM disease (n=1), respectively. For capture PR3- and MPO-ELISA, the sensitivity was 94%, specificity 97%, PPV 88%, and NPV 98% (Table 5).

Although we found a high sensitivity of 94% for IIF, specificity was only 78% (false positive P-ANCA in 18 patients, atypical ANCA in 23 patients, no false positive C-ANCA), resulting in a PPV of 59% and a NPV of 97% (Table 5). Likelihood ratios for positive test were 29 and 4.2 for capture ELISA and IIF respectively, for a negative test 0.067 and 0.083 respectively. The direct ELISA for anti-GBM was positive in all twelve patients with anti-GBM-disease. In two of these patients, anti-MPO antibodies were also found by capture ELISA (see discussion earlier). In one of the same patients with MPA in whom Dotblot detected anti-GBM antibodies a positive anti-GBM by ELISA was found. This results in a likelihood ratio for a positive test of 248, for a negative test of 0.0.

The retrospective results of the highly sensitive Phadia ELiA test method showed five false negative results; these patients were diagnosed with GPA (n=2), MPA (n=2), RLV (n=1). These were the same patients who were negative in the Dotblot and three that were also negative by capture ELISA and IIF.

The Phadia ELiA hs anti-PR3^s and hs anti-MPO^s showed 5 false positive results (3 and 4 respectively): again, these were the same patients who were false positive as tested by ELISA and Dotblot (diagnosed with sarcoidosis, endocarditis, tubulo-interstitial nephritis and anti-GBM disease).

All twelve patients with anti-GBM-disease tested positive by Phadia ELiA. In three of these patients, Phadia found a double positivity for anti-MPO antibodies also. In two patients with MPA (the same as were mentioned at results of Dotblot), Phadia was positive for MPO-ANCA and anti-GBM as well. Sensitivity, specificity, PPV and NPV for Phadia ELiA hs anti-PR3^s and hs anti-MPO are given in Table 5. The likelihood ratio for positive ANCA was

34.2, for anti-GBM 124; for a negative test, it was 0.083 and 0.0 respectively. Interrater agreement between Phadia and ELISA by Cohen's kappa was 0.88 for anti-PR3, 0.85 for anti-MPO and 0.93 for anti-GBM.

DISCUSSION

ANCA and anti-GBM tests are used to support or dismiss a diagnosis of primary anti-neutrophil cytoplasmic antibody associated systemic small vessel vasculitis, and anti-GBM pulmonary renal syndrome, respectively. Patients with these diseases often present with acute severe disease manifestations and may exhibit rapidly progressive and life threatening organ failure. A rapid diagnosis and instalment of effective therapy may be very important for optimal outcome in these circumstances. In the international consensus statement in 1999, it was agreed that for ANCA testing in "new" patients, screening should be with IIF and confirmation by ELISAs that detect ANCA specific for PR3 or MPO. [3-7,9] In common practice like in our laboratory, a drawback for these methods is the time needed for obtaining results, which is especially important in acute situations. Rapid serologic assays identifying patients who may benefit from more targeted diagnostic procedures and early immunosuppressive treatment can be helpful in these circumstances, given that these rapid assays have sufficient sensitivity and especially specificity. Different assays, including Dotblot or line blot, rapid ELISAs and assays on automated random access analysers are commercially available[10,19,20].

We have tested the performance of two systems available for rapid testing in a clinically highly relevant context, i.e. consecutive patients in whom the physician in charge asked for urgent determination of the presence of ANCA. First of all, our results show that physicians are capable of defining on clinical grounds a sample of patients with a high a priori chance of ANCA associated small vessel vasculitis with a prevalence of nearly 24%, despite an annual incidence of this diseases of only 10-20 per million.[21] In a previous study on 1434 new patient samples routinely send to our laboratory from roughly the same hospitals as the current study, only 51 samples (3.5%) were positive for anti-PR3 and anti-MPO antibodies. [8] Second, our study indicates that both a qualitative Dotblot and a novel quantitative Phadia ELiA anchor ANCA tests perform excellently with high sensitivity and specificity in this population highly suspected of having systemic small vessel vasculitis. The clinical utility of both ELiA and Dotblot was concordant with our standard capture-ELISA and clearly superior to IIF, especially with regard to specificity. It can be concluded that both the Dotblot and the Phadia ELiA both perform excellently in an acute setting with a substantial a priori prevalence of AAV and can be used for diagnostic purposes. Although a negative ANCA-IIF has a high negative predictive value, lack of specificity makes this test, outside screening, only of limited value in this setting. [4,13] However, recently, automated reading

of IIF by pattern recognition software has demonstrated high diagnostic performance for the assessment of ANCA and subjective interpretation or poor laboratory reproducibility seems to be accurate and at least comparable to visual scoring. This may shed a new light on the promising debate about the role of IIF in future ANCA-diagnostics [10,22]

Both Dotblot and ELiA detected all 12 cases of anti-GBM disease, as did the standard direct ELISA. Since specificity (100%) and positive predictive value (86-92%) were high, we considered both ELiA and Dotblot of high clinical use for urgent testing in suspected anti-GBM disease. Our findings of double serum positivity are in concordance with prior literature, since it is known that up to 30% of patients with anti-GBM disease have serum positivity for MPO-ANCA as well.[2,23] As none of the MPO-ANCA/anti-GBM double positive patients as detected by the different serological tests in the 12 patients with anti-GBM disease displayed additional proof of AAV, these cases were considered false positive with respect to the detection of MPO-ANCA. Likewise, MPO-ANCA/anti-GBM double positive patients with a final diagnosis of AAV without features of additional anti-GBM disease were considered anti-GBM false positives, resulting in a specificity and positive predictive value of less than 100% for anti-GBM testing.

Although we included only 260 serum samples, the 74 patients diagnosed with AAV and anti-GBM disease represents a rather large population of PR3-ANCA and MPO-ANCA positive patients, although the prevalence of anti-GBM disease is low due to its rare nature. Still, because of the high sensitivity and specificity demonstrated by the serological tests, our conclusion that rapid serological screening is valuable applies not only for the diagnosis of GPA and MPA, but also for anti-GBM disease. There are more commercially available PR3-ANCA, MPO-ANCA and anti-GBM assays than tested in this study. All these assays may show differences in sensitivity and specificity and therefore, interpretation of test results is dependent on the test system used. International standards for PR3-ANCA and MPO-ANCA are available and the ELiA test method is calibrated on these standards.

A potential problem of comparing our serological data to the final clinical diagnosis is circular reasoning, i.e. the serological results as detected by the Dotblot test were taken into consideration by the clinicians to make a diagnosis. This is especially important since accurate clinical diagnostics are critically important in determining the analytical and clinical importance of a serological test. To rule out as much as possible the possibility that reporting positive or negative test results have led to incorrect diagnoses, we have again contacted all physicians months and sometimes years after serological testing and requested them to review the patient chart and data and to verify and confirm the diagnosis. No cases were encountered where during follow up the diagnosis of AAV or anti-GBM was overturned, nor where any patients initially judged not to have AAV or anti-GBM disease who were later on diagnosed with AAV or anti-GBM disease found.

Finally, it should be mentioned that this study is only relevant for the rapid requests for serologic diagnosis of AAV and anti-GBM disease in new patients presenting with symptoms

suggestive of AAV. Both the positive and negative predictive value of a laboratory test for an uncommon disease vary tremendously among patients with different clinical manifestations and for patient populations with different a priori prevalence of the disease in question.[3,8,21] It is of utmost importance that assays used in clinical practice are validated for their performance in relevant samples and that interpretation of the serological results is done with acknowledgement of the possible differences in disease prevalence. This means that in a population with high pretest-probability for AAV or anti-GBM disease, both rapid tests and the routine tests studied here perform excellently and can be used as a diagnostic tool. However, in a population with an AAV prevalence of 1%-2% or less and for anti-GBM disease even lower, the predictive positive value will be less as even a high specificity in these circumstances will lead to a numerical increase in false positives. The negative predictive value, however, will remain largely intact due to the high sensitivity as indicated by the extreme negative likelihood ratios for a negative test result. Therefore, a clinician has to be critically aware of who is being tested as well as to have insight in the significance of the test results. This will highly improve understanding and interpreting test results and therefore improve treatment of patients suspected for AAV.[8,24]

In conclusion, we show that in a cohort of patients highly suspected for AAV and anti-GBM disease both a qualitative Dotblot assay and a quantitative automated high sensitive ELiA system for the serological detection of anti-PR3, anti-MPO and anti-GBM antibodies have a high positive and negative predictive value and are comparable to standard ELISA systems. Therefore, these rapid serological assays should enable clinicians to make or dismiss a well-founded clinical diagnosis of AAV and anti-GBM disease and lead to rapid institution of appropriate therapy in case of a positive result and further diagnostic evaluation in case of a negative result, respectively.

SUMMARY

We analysed diagnostic performance of two rapid test methods for ANCA and anti-GBM determination in a relevant patient group presenting with suspected small-vessel vasculitis (n=260). Both test methods provided excellent positive and negative predictive value.

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