Clinical Rheumatology (2019) 38:3477–3483
https://doi.org/10.1007/s10067-019-04736-6

ORIGINAL ARTICLE

Evaluation of 12 different assays for detecting ANCA in Chinese patients with GPA and MPA: a multicenter study in China

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Received: 26 May 2019 / Revised: 31 July 2019 / Accepted: 2 August 2019 / Published online: 14 August 2019

Abstract

Objective Due to lack of comprehensive evaluation for various detection methods for antineutrophil cytoplasmic antibody (ANCA) in Chinese population, we evaluate the diagnostic performance of 12 established analysis methods in Chinese patients having granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA).

Methods Sera were collected from 209 patients with GPA or MPA and 243 diseases controls from 15 centers. Twelve different reagents were employed for C-ANCA, P-ANCA, myeloperoxidase (MPO)-ANCA, and proteinase 3(PR3)-ANCA detection. The accuracy, sensitivity, and specificity of each method were analyzed.

Results The accuracy of the two indirect immunofluorescence (IIF) and two line immunoassay (LIA) was 0.838 and 0.874, 0.869, and 0.862, respectively. The accuracy of the eight quantitative antigen-specific immunoassays was varied from 0.867 to 0.967. The sensitivity of ANCA-associated vasculitis (AAV) was 0.770 and 0.761 for the two IIF, 0.727 and 0.718 for the two LIAs, respectively. For the eight quantitative antigen-specific immunoassays, the sensitivity varied from 0.79 to 0.967. The specificity was 0.897 and 0.971 for the two IIF, 0.992 and 0.988 for the two LIAs, respectively. For the eight quantitative antigen-specific immunoassays, the specificity of AAV varied from 0.963 to 0.983.

Conclusion For Chinese patients suspected of having GPA and MPA, both the first-generation enzyme-linked immunosorbent assay (ELISA) and high-quality antigen-specific immunoassay can be used to detect MPO-ANCA and PR3-ANCA alone, without the combined detection with IIF to have good diagnostic performance. The chemiluminescent immunoassay (CLIA) seems to be a method worth recommending.

Key points
- *Quantitative antigen-specific immunoassays can be used to detect MPO-ANCA and PR3-ANCA without IIF in Chinese.*
- *CLIA has the maximum AUC value and the minimum LR (−) value, which seems to be a method worth recommending.*

Keywords Antineutrophil cytoplasmic antibody · Detection · Granulomatosis with polyangiitis · Microscopic polyangiitis · Multicenter studies

Introduction

Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) is a group of autoimmune diseases characterized by multiple-system and multiple-organ damage, including granulomatosis with polyangiitis (GPA; previously known as Wegener's granulomatosis), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA; previously known as Churg-Strauss syndrome). ANCAs are a group of antibodies targeting the cytoplasmic components of neutrophils and monocytes, which are important serological hallmarks for the diagnosis of AAV. They can be divided into P-ANCAs, C-

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10067-019-04736-6) contains supplementary material, which is available to authorized users.

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ANCAs, and atypical ANCAs (A-ANCAs) according to their morphological characteristics under a fluorescence microscope. The main target antigen of C-ANCAs is proteinase 3 (PR3) while that of P-ANCAs is myeloperoxidase (MPO). The main target antigen of A-ANCAs remains unclear; it can develop in association with drug exposure, inflammatory bowel disease (IBD), or rheumatoid arthritis (RA), most often in the absence of vasculitis [1]. Numerous experiments have shown that ANCA plays an important role in the pathogenesis of AAV [2, 3]. AAV can be divided into MPO-ANCA diseases and PR3-ANCA diseases according to ANCA serotype, which can better reflect the characteristics of the disease, better guide the treatment, and predict the prognosis of the disease [4]. ANCA detection is therefore very important in patients with AAV, and the detection methods for ANCAs will undergo constant development.

In 2017, international consensus on ANCA testing [5] suggested that high-quality antigen-specific immunoassays for MPO-ANCA and PR3-ANCA detection could be used for screening and confirmation in patients suspected of having GPA and MPA, instead of the 1999 international consensus on ANCA testing [6] which suggested the serum of patients suspected of having AAV should be screened by indirect immunofluorescence (IIF) to detect C-ANCAs and P-ANCAs, with confirmation using enzyme-linked immunosorbent assay (ELISA) to detect MPO-ANCAs and PR3-ANCAs in case of a positive IIF result.

The high-quality antigen-specific immunoassays suggested in 2017 international consensus include the second and third generations of ELISA, chemiluminescent immunoassays (CLIA), fluorescent-enzyme immunoassays (FEIA), and automated multiplex flow immunoassay. However, in most laboratories in China, the combination of the IIF and ELISA is used to detect ANCA. Some laboratories use first generation of the ELISA to detect MPO-ANCAs and PR3-ANCAs; many laboratories use qualitative or semi-quantitative line immunoassays (LIA). Furthermore, there are significant differences in the distribution pattern of ANCA specificity among different regions: PR3-ANCA vasculitis is common in Nordic, American, and Australian countries while MPO-ANCA vasculitis is common in Southern European and Asian countries [4, 7]. Whether antigen-specific immunoassays alone for ANCA detection can also be used for screening and confirmation of AAV patients in China should be further investigated.

In this regard, a questionnaire survey was conducted to investigate the methods currently used for ANCA testing in 19 centers. Then, sera were collected from patients with AAV and disease controls from 15 centers, and 5 methods using 12 different reagents were employed for C-ANCA, P-ANCA, MPO-ANCA, and PR3-ANCA detection with an aim to provide the reliable ANCA detection strategies in China.

Materials and methods

Questionnaire survey on methods used for ANCA testing in China

A questionnaire survey was conducted to investigate the methods used for ANCA testing in 19 centers in China (Supplementary 1). The main questions posed are as follows: Does your laboratory perform ANCA testing? Which methods do you use in your laboratory for ANCA testing? Which manufacturers' reagents do you use for ANCA testing?

Samples and patients

A total of 452 serum samples were collected from 15 centers in China, including 153 samples from patients with MPA, 56 samples from patients with GPA, and 243 samples from disease controls comprising 82 samples of RA, 102 of systemic lupus erythematosus (SLE), 35 of Sjogren's syndrome (SS), 20 of systemic sclerosis (SSc), two of mixed connective tissue disease (MTCD), one of SLE/SS overlap, and one of SS\SSC overlap (Supplementary 2). In the disease control group, doctors considered ANCA testing important for patients and then excluded the diagnosis of ANCA-associated vasculitis. The diagnoses of RA, SLE, SS, SSc, and MPA and GPA were based on the American College of Rheumatology (ACR)/the European League Against Rheumatism (EULAR) classification standard of 2010 or the ACR classification standard of 1987, ACR classification standard of 2009, ACR classification standard of 2012, ACR/EULAR classification standard of 2013, and the Chapel Hill Consensus Conference (CHCC) classification standard of 2012, respectively.

Exclusion criteria Patients in whom IBD, autoimmune liver disease, EGPA, tumors, tuberculosis, hepatitis B, hepatitis C and/or acute infections, and allergic diseases was suspected were excluded. Furthermore, patients who had been vaccinated within 3 months before blood collection were also excluded.

The study was approved by the ethics committee of each participating center, all the participants gave written informed consent.

ANCA detection methods

The five methods using 12 different reagents included five ELISA methods, two CLIA methods, one multiplexed bead assay (MBA), two LIA methods, and two IIF methods, which are shown in Table 1.

INOVA IIF was carried out at the Department of Rheumatology, Xiangya Hospital, Central South University. The other 11 methods were carried out at the Department of

Table 1 ANCA detection methods and related reagent manufacturers

Quantitative methods				Semi-quantitative method (LIA)	Qualitative method	
ELISA		CLIA	MBA		LIA	IIF
First generation	Second generation					
ORGENTEC Diagnostika GmbH (Mainz, Germany) INOVA Diagnostics, Inc. (San Diego, CA, USA) AESKU DIAGNOSTICS GmbH & Co. KG (Wendelsheim, Germany) Shenzhen YHLO Biotech Co. Ltd. (Shenzhen, China)	Euro-Diagnostica AB (Malmö, Sweden)	Shenzhen YHLO Biotech Co., Ltd. (Shenzhen, China) INOVA Diagnostics, Inc. (San Diego, CA, USA)	Zeus Scientific, Inc. (Branchburg, NJ, USA)	Shenzhen YHLO Biotech Co., Ltd. (Shenzhen, China)	AESKU DIAGNOSTICS GmbH & Co. KG (Wendelsheim, Germany)	AESKU DIAGNOSTICS GmbH & Co. KG (Wendelsheim, Germany) INOVA Diagnostics, Inc. (San Diego, CA, USA)

ELISA, enzyme-linked immunosorbent assay; *CLIA*, chemiluminescent immunoassay; *MBA*, multiplexed bead assay; *IIF*, indirect immunofluorescence; *LIA*, line immunoassay

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Regarding IIF, two reagents were performed on ethanol-fixed neutrophils in combination with additional tests on formalin-fixed neutrophils to better discriminate P-ANCA (or atypical ANCAs). AESKU IIF was performed using HelMed Full Automatic Fluorescence Staining Instrument 1000 and two experienced experimenters read the results manually. INOVA IIF was performed using NOVA View (Inova Diagnostics (a Werfen company)). The instrument automatically read results and manually checked and corrected them simultaneously. Any positive or double-positive results for P-ANCAs/C-ANCAs/MPO-ANCAs/PR3-ANCAs/A-ANCA were judged to be positive, and the A-ANCA result was classified as P-ANCA in the analyses. When the results of all antigen-specific immunological methods were judged, the positive value was determined according to the value provided by the kit, and the gray area was judged to be negative.

Statistical analysis

The questionnaire survey was performed using GraphPad Prism 5.0. Accuracy was determined as follows: (number of controls that tested negative + number of GPA patients that tested positive + number of MPA patients that tested positive) / (number of controls + number of GPA + number of MPA) [8]. Receiver operating characteristic (ROC) curve analysis was performed using SPSS 17.0.

Results

Methods for ANCA testing in China

A survey of ANCA testing methods was conducted in 19 centers in China, and 16 valid questionnaires were collected. Among the laboratories surveyed, 10 (62.5%) used IIF combined with ELISA, four (25%) used IIF combined with LIA, one (6.25%) used ELISA, and one (6.25%) only used LIA (Fig. 1).

Results of 12 methods for ANCA detection

The results of 12 methods for ANCA detection are summarized in Table 2. The sensitivity of AAV were 0.770 and 0.761 for the two IIF and 0.727 and 0.718 for the two LIAs, respectively. For the eight quantitative antigen-specific immunoassays, the sensitivity of AAV varied from 0.79 to 0.967. The specificity of AAV were 0.897 and 0.971 for the two IIF and 0.992 and 0.988 for the two LIAs, respectively. For the eight quantitative antigen-specific immunoassays, the specificity of AAV varied from 0.963 to 0.983. Additionally, the specificity of IIF (97.53–1%) for C-ANCAs was higher than that for P-ANCAs (92.18–97.12%). The specificity of the antigen-specific immunoassay for PR3-ANCA detection (97.94–99.59%) was higher than that for MPO-ANCA detection (96.71–99.59%). Furthermore, the sensitivity of LIA for GPA was higher than that of IIF whereas that for MPA was lower than that of IIF. The LIA showed the highest

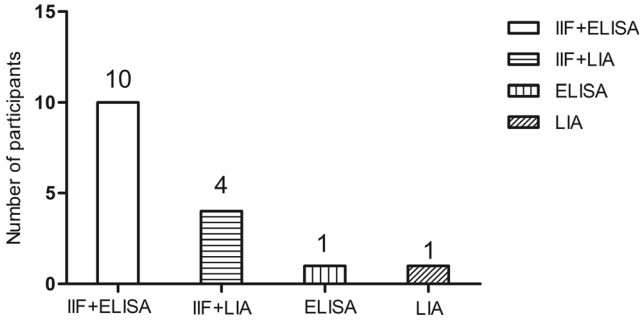

Fig. 1 Methods for ANCA testing in most hospitals in China. Summary of ANCA detection methods in laboratories surveyed in China, 10 hospitals used IIF combined with ELISA, four used IIF combined with LIA, one used ELISA, and one used LIA. IIF, indirect immunofluorescence; ELISA, enzyme-linked immunosorbent assay; LIA, line immunoassay

likelihood ratio (LR) for AAV-positive test results (LR (+)) of 90.87 (YHLO LIA) and 59.83 (AESKU LIA) (Table 2). The LR for an AAV-negative test result (LR (−)) was the lowest with CLIA of 0.03 (Table 2).

The accuracy analysis showed that the accuracy of Euro-Diagnostica ELISA, ORGENTEC ELISA, INOVA ELISA, AESKU ELISA, YHLO ELISA, YHLO CLIA, INOVA CLIA, and Zeus MBA was 0.94, 0.925, 0.867, 0.912, 0.9, 0.967, 0.926, and 0.94, respectively. Except for the accuracy results of INOVA ELISA, the accuracy results of the other seven quantitative antigen-specific immunoassays were not significantly different, and the accuracy of YHLO LIA, AESKU LIA, AESKU IIF, and INOVA IIF was 0.869, 0.862, 0.838, and 0.874, respectively. Further, the accuracy of these four qualitative/semi-quantitative methods was lower than that of the quantitative antigen-specific immunoassays (except INOVA ELISA).

To further compare the diagnostic efficiency of these methods, we drew ROC curves. The MPA and GPA groups were combined into the AAV group. We considered the higher positive data for P-ANCAs/C-ANCAs afforded by IIF and for MPO-ANCAs/PR3-ANCAs afforded by antigen-specific immunology methods for the ROC curves (Fig. 2). The area under the curve (AUC) values and the corresponding 95% confidence intervals are shown in Table 2.

The AUC values for IIF were 0.891 (95% CI, 0.831–0.951; AESKU) and 0.886 (95% CI, 0.826–0.946; INOVA), and the difference between the two values was not significant ($p = 0.072$). For the eight quantitative antigen-specific immunoassays, the AUC values varied between 0.950 (95% CI, 0.910–0.990) and 0.981 (95% CI, 0.959–1), which was significantly higher than the AUC values for IIF ($p < 0.01$ for all assays). For the two LIAs, the AUC values were 0.846 (95% CI, 0.779–0.913) and 0.892 (95% CI, 0.834–0.950), and the difference between the two values was significant ($p < 0.001$). The maximum AUC values were obtained by CLIA, which was 0.979 and 0.981.

Discussion

In view of the epidemiological characteristics, gene type, clinical manifestations, and prognosis of AAV; distribution pattern of ANCA specificity among different regions; and the status of the ANCA detection methods and various detection reagents in China, we conducted this multicenter study.

Our results showed that the AUC values for eight quantitative antigen-specific immunoassays (ELISA, CLIA, and MBA) were higher than 0.95 and the accuracy, GPA sensitivity, MPA sensitivity, and AAV sensitivity, and LR (+) AAV of the eight quantitative antigen-specific immunoassays were higher than those of IIF (except INOVA ELISA). The accuracy of the INOVA ELISA was 0.867, but the AUC value was 0.961. This is because the calculation of accuracy is based on the cutoff value while the AUC value is independent of the cutoff value. These results were similar to those of the European Vasculitis Study Group (EUVAS) multicenter studies in 2016 which the 2017 ANCA international consensus was based [8, 9], suggesting that antigen-specific immunoassays can be used to detect ANCAs in China for the purpose of screening and confirmation. In this study, ELISAs included the first generation of direct ELISA (YHLO, AESKU, INOVA, and Orgentec) and the second generation of capture ELISA (Euro-Diagnostica) and the results showed good consistency. The GPA sensitivity, MPA sensitivity, and AAV sensitivity of the second-generation ELISA, CLIA, and MBA were higher than those of the first-generation ELISA, and the specificity did not change. But we found that even the first-generation ELISA was more sensitive, more specific and accurate compared to IIF. The 5 ELISA test kits could distinguish active AAV from the control-group diseases. LIA is more sensitive to GPA than IIF and MPA is less sensitive to IIF. Therefore, the combination of LIA and IIF is needed to prevent missed diagnosis. Negative detection results inconsistent with clinical manifestation still require confirmation by other high quality antigen-specific immunoassays. CLIA has been used for autoantibodies and ANCA detection for many years and has important diagnostic value [10, 11]. In our study, we found that the CLIA had the maximum AUC value and the minimum LR (−) value, which seemed to be a method worth recommending.

This study also included patients with ANCA-negative AAVs. The IIF revealed negative results for 22.97–23.92% of patients with AAV, while antigen-specific immunoassays revealed negative results for 3.35–28.23% of patients with AAV. However, it should be noted that ANCAs are also positive in patients with IBD, autoimmune liver disease, primary sclerosing cholangitis, drug-induced autoimmune diseases, and infectious diseases, which needs to be tested with IIF but is beyond the scope of this study. In addition, some patients with AAV might test positive for ANCAs by IIF but negative by antigen-specific immunoassay. Hence, the IIF

Table 2 Results of 12 methods for detecting ANCAs in patients with GPA and MPA

			ELISA					CLIA		MBA	LIA		IIF		
	MPO	PR3	1	2	3	4	5	6	7	8	9	10		11	12
Controls	Neg	Neg	229	237	57	236	238	235	58	234	241	240	Neg	218	236
$n = 243$	Pos	Neg	3	1	1	2	2	6	1	8	0	2	P-ANCA	16	7
	Neg	Pos	0	4	1	5	3	2	0	1	1	1	C-ANCA	6	0
	Pos	Pos	2	1	0	0	0	0	0	0	1	0	P-ANCA + C-ANCA	0	0
													A-ANCA	3	0
GPA	Neg	Neg	4	8	2	9	10	4	1	5	9	6	Neg	16	23
$n = 56$	Pos	Neg	4	4	2	3	4	4	2	4	2	4	P-ANCA	3	5
	Neg	Pos	44	44	22	44	41	47	23	47	44	46	C-ANCA	37	28
	Pos	Pos	0	0	0	0	1	1	0	0	1	0	P-ANCA + C-ANCA	0	0
													A-ANCA	0	0
MPA	Neg	Neg	17	20	14	24	30	3	8	13	48	53	Neg	32	27
$n = 153$	Pos	Neg	125	127	33	126	118	143	39	135	99	94	P-ANCA	107	123
	Neg	Pos	3	5	2	3	4	4	2	4	5	4	C-ANCA	10	3
	Pos	Pos	3	1	1	0	1	3	1	0	1	2	P-ANCA + C-ANCA	1	0
													A-ANCA	3	0
Accuracy			0.94	0.925	0.867	0.912	0.9	0.967	0.926	0.94	0.869	0.862		0.838	0.874
Specificity			0.979	0.975	0.966	0.971	0.979	0.967	0.983	0.963	0.992	0.988		0.897	0.971
Sensitivity GPA			0.923	0.857	0.92	0.839	0.821	0.929	0.961	0.911	0.839	0.893		0.714	0.589
Sensitivity MPA			0.885	0.869	0.72	0.843	0.804	0.98	0.84	0.914	0.687	0.654		0.791	0.824
Sensitivity AAV			0.895	0.866	0.79	0.842	0.809	0.967	0.882	0.913	0.727	0.718		0.770	0.761
LR(+)AAV			42.6	34.6	23.2	29	38.5	29.3	51.8	24.67	90.87	59.83		7.476	26.24
LR(−)AAV			0.11	0.14	0.217	0.16	0.19	0.03	0.12	0.052	0.275	0.273		0.256	0.246
AUC AAV			0.966	0.951	0.961	0.95	0.971	0.979	0.981	0.966	0.846	0.892		0.891	0.886
95% CI			0.938–0.994	0.910–0.991	0.932–0.989	0.910–0.990	0.943–0.998	0.956–1	0.959–1	0.937–0.995	0.779–0.913	0.834–0.950		0.831–0.951	0.826–0.946

ANCA, antineutrophil cytoplasmic antibodies; *AAV*, ANCA-associated vasculitis; *C-ANCAs*, cytoplasmic pattern ANCAs; *GPA*, granulomatosis with polyangiitis; *MPA*, microscopic polyangiitis; *MPO*, myeloperoxidase; *Neg*, negative; *Pos*, positive; *P-ANCAs*, perinuclear pattern ANCAs; *PR3*, proteinase 3; *A-ANCAs*, atypical ANCAs; *ELISA*, enzyme-linked immunosorbent assay; *CLIA*, chemiluminescent immunoassay; *MBA*, multiplexed bead assay; *IIF*, indirect immunofluorescence; *LIA*, line immunoassay; *AUC*, the area under the curve; *LR(+) AAV*, the likelihood ratio for AAV with a positive test result; *LR(−) AAV*, the likelihood ratio for AAV with a negative test result. Euro-Diagnostica AB MPA: 148 cases; GPA: 52 cases; disease controls: 234 cases; INOVA Diagnostics ELISA, CLIA MPA: 50 cases; GPA: 26 cases; disease controls: 59 cases; Zeus Scientific MPA: 152 cases; accuracy = [number of controls that tested negative + number of GPA patients that tested positive + number of MPA patients that tested positive] / [number of controls + number of GPA+ number of MPA] [8]. 1. Euro-Diagnostica ELISA; 2. ORGENTEC ELISA; 3. INOVA ELISA; 4. AESKU ELISA; 5. YHLO ELISA; 6. YHLO CLIA; 7. INOVA CLIA; 8. Zeus MBA; 9. YHLO LIA; 10. AESKU LIA; 11. AESKU IIF; 12. INOVA IIF

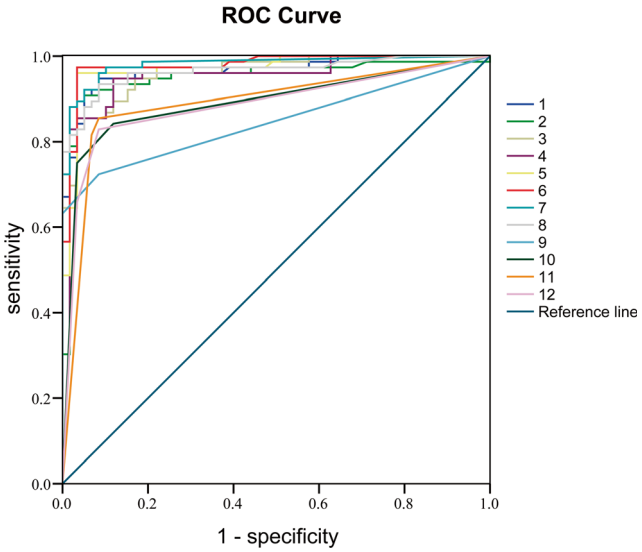

Fig. 2 ROC curve of 12 methods for ANCA detection. Receiver operating characteristic curve for eight quantitative antigen-specific immunoassays, two line immunoassays (LIAs), and two indirect immunofluorescence (IIF) methods for antineutrophil cytoplasmic antibody (ANCA) detection. ROC curve analysis showed significant differences between IIF, LIA, and antigen-specific immunoassays results. The results of eight quantitative antigen-specific immunoassays were better than those of IIF and LIA. (1) Euro-Diagnostica ELISA. (2) ORGENTEC ELISA. (3) INOVA ELISA. (4) AESKU ELISA. (5) YHLO ELISA. (6) YHLO CLIA. (7) INOVA CLIA. (8) Zeus MBA. (9) YHLO LIA;. (10) AESKU LIA. (11) AESKU IIF. (12) INOVA IIF

cannot be completely abandoned at present. The problem might be resolved gradually with further development in the field.

In our study, MPA and MPO-ANCAs accounted for the majority of the case group, which is consistent with the majority of MPO-ANCA-positive results in the 2004 study by Xin et al. [12]. A limitation of this study was that more samples should be enrolled for further investigation, and the ANCA detection strategies should be further evaluated in clinical practice.

In conclusion, both the first-generation ELISA and high-quality antigen-specific immunoassays for MPO-ANCAs and PR3-ANCAs could be used for screening and confirmation in Chinese patients suspected of having GPA and MPA, without combining with IIF to have good diagnostic performance. For LIA, combination with IIF is still needed to prevent missed diagnosis. Negative detection results inconsistent with clinical manifestation still require confirmation by the IIF methods or other high-quality antigen-specific immunoassays. CLIA seems to be a method worth recommending for the detection of MPO-ANCA and PR3-ANCA. Histopathology is an important basis for diagnosing ANCA-negative AAV.

Acknowledgments This study was supported by grants from the National Nature Science Foundation Key Research Project of China (2017YFC0909002) and the National Basic Research Program of China (No.2105CB553704).

The authors would like to express their gratitude toward Tianjin Super Biotechnology Development Co., Ltd.; Guangzhou Kangrun Biotech Co., Ltd.; Autoimmune BU., Werfen China; Shenzhen YHLO Biotech Co., Ltd.; Beijing H&J Novomed Co., Ltd.; Zeus Scientific Inc.; Shanghai Kexin Biotech Co., Ltd.; and Beijing Dacheng Biotech Co., Ltd, for providing reagents for the study.

Compliance with ethical standards

Statement The study was performed according to the 1997 Declaration of Helsinki of the World Medical Association and has been approved by the ethics committee of each participating center, all the participants gave written informed consent.

Disclosures None.

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