The performance of the chemiluminescent immunoassay for measuring serum myeloperoxidase and proteinase 3 antibodies

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Funding information
National Natural Science Foundation of China; grant/award number: 61771022.

Abstract

**Background:** Enzyme-linked immunosorbent assay (ELISA) has traditionally been used to detect myeloperoxidase (MPO) and proteinase 3 (PR3) antibodies, although it is time-consuming and physically demanding. As a novel and highly effective immunoassay, we compared chemiluminescent immunoassay (CIA) with ELISA to verify the application value of CIA in MPO and PR3 antibodies detection.

**Methods:** By ELISA and CIA, serum levels of anti-MPO and anti-PR3 antibodies were measured in 63 anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) patients (AAV group), including 47 microscopic polyangiitis (MPA) patients and 16 granulomatosis with polyangiitis (GPA) patients, in addition, 68 patients in interference control group (IC group), 19 healthy subjects in healthy control group (HC group). We compared MPO and PR3 antibodies levels and positive rates measured by these two methods among groups. Relationship and coincidence rate between ELISA and CIA were investigated. Diagnostic values for clinical outcomes for MPO and PR3 antibodies were assessed by receiver operator characteristic (ROC) curve.

**Results:** In AAV patients, when detecting anti-MPO ($r = .90$) and anti-PR3 ($r = .81$), CIA was highly correlated with ELISA, companying with highly total (88.89%, 92.06%, respectively) and positive coincidence rates (84.78%, 77.27%, respectively). In HC group, anti-PR3 positive rate detected by both immunoassay were 0, anti-MPO almost were 0, which without statistically significant difference ($P = .32$). In IC group, the total (76.47%, 58.82, respectively) and positive coincidence rates (48.38%, 30.00, respectively) of anti-MPO and anti-PR3 were the lowest, but the negative coincidence rates reached 100%. By CIA, similar to ELISA, the levels of anti-MPO were significantly higher both in AAV patients (56.00; [4.40-235.30]) and MPA patients (98.00; [27.90-324.70]) compared with either IC group (3.20; [3.20-18.55]) ($P < .0001$) or HC group (3.20; [3.20-3.20]) ($P < .0001$), yielded an area under curve (AUC) of 0.76 for AAV and 0.89 for MPA, the concentration of anti-PR3 in GPA group...
1 | INTRODUCTION

Antibodies to myeloperoxidase (MPO) and proteinase 3 (PR3) are important pathogenic autoantibodies as well as the disease markers for the diagnosis of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).\(^1\)\(^-\)\(^2\) Though the diagnosis of patients who present with MPO and PR3 antibodies in blood is not necessarily vasculitis, as these antibodies can also be detected in a number of other diseases, including inflammatory bowel disease, systemic sclerosis, hyperthyroidism, and so on, MPO and PR3 antibodies are usually measured when patients are suspected of vasculitis.\(^3\)\(^-\)\(^5\) Because abnormally high levels of MPO and PR3 antibodies contribute not only to the diagnosis of vasculitis, but also to classify: on the one hand, depending on the presence of MPO-ANCA or PR3-ANCA, AAV can be divided into different disease phenotype: MPO-ANCA-associated vasculitis (MPO-AAV), PR3-ANCA-associated vasculitis (PR3-AAV), and ANCA-negative vasculitis; on the other hand, granulomatosis with polyangiitis (GPA) patients are more likely display PR3-ANCA, while MPO-ANCA is more common in patients with microscopic polyangiitis (MPA).\(^6\) Sebastian Unizony et al.\(^\text{10}\) found classification of vasculitis into MPO-AAV and PR3-AAV may apply to guide immunosuppression in AAV. Therefore, efficient, accurate, and rapid detection of MPO and PR3 antibodies is crucial for AAV patients.

Enzyme-linked immunosorbent assay (ELISA) is the traditional method for the detection of MPO and PR3 antibodies, but it has some defects, such as time-consuming, laborious, and little scale testing.\(^8\) With many advantages, including automation, good repeatability, and high testing throughput, the chemiluminescence technology has an increasingly wide utilization in a variety of detections, suggesting the chemiluminescent immunoassay (CIA) may be a promising tool for routine detecting of MPO and PR3 antibodies.\(^9\)\(^-\)\(^12\) In recent years, several studies in Australia and the United States have shown that the CIA can detect MPO and PR3 antibodies as accurately as ELISA.\(^13\)\(^-\)\(^14\) In the present study, in order to verify that whether the CIA can also detect MPO and PR3 antibodies accurately and efficiently in our population, we assessed the analytical performance of CIA for MPO and PR3 antibodies; additionally, we evaluated the diagnostic performance of CIA for GPA and MPA by comparing it with ELISA method.

2 | MATERIALS AND METHODS

2.1 | Study population

During this prospective investigation, a total of 150 subjects in the Peking University Third Hospital were enrolled from June, 2017, through May, 2018. The first group (AAV group) was collected from 63 AAV patients, aged 20-84 years old, 34.93% were male (Table S1, which demonstrated the baseline demographical characteristics of subjects). We strictly followed the international recommendations to determine AAV.\(^15\)\(^-\)\(^16\) According to its classification criteria, AAV patients were further classified into MPA group (n = 47, group aged 20-84 years old, 31.91% were male) and GPA group (n = 16, aged 29-77 years old, 43.75% were male).\(^17\)\(^-\)\(^18\) The second group (interference control group, IC group) was obtained from 68 patients with MPO and/or PR3 positive non-AAV patients, including connective tissue disease (n = 19), renal insufficiency (n = 13), interstitial pneumonia (n = 9), inflammatory bowel disease (n = 4), hyperthyroidism (n = 4), others (n = 19). These patients ranged from 17 to 91 years old, 32.35% were male. The third group (healthy control group, HC group) was gathered from 19 healthy individuals, aged 22-77 years old, 42.11% were male. In the present study, the control group was composed of IC group and HC group. The protocol was approved by the local Institutional Ethics Committee.

2.2 | Reagents and apparatus

Chemiluminescent immunoassay assay was determined by a kit (QUANTA Flash assays, INOVA Diagnostic, Inc.), according to the manufacturer’s instructions described in the assay procedure. The assay was performed on BIO-FLASH instrument (Biokit S. A.). ELISA assay was measured by commercial ELISA kits (Euroimmun).

2.3 | Precision, limit of quantitation and linearity

To evaluate the precision of CIA for MPO and PR3 antibodies, the precision analysis was performed according to the requirements listed in the Clinical and Laboratory Standards Institute (CLSI) EP15-A2 document.\(^19\) Low and high concentration serum was measured four times...
daily for five consecutive days, within-run precision and laboratory precision were calculated. Diluent assay was used to evaluate the limit of quantitation (LOQ), high level serum was diluted with sample diluent in different ratio to near the expected lower reportable limit. The prepared samples were measured twice, and then compared the expected value with the mean measured value, and the coefficient of variation (CV) and recovery were calculated. The lowest concentration at which the CV were less than or equal to the allowable error (≤ 25%) was considered the LOQ. As the requirements of the CLSI EP6-A2 document illustrated, linearity analysis was performed based on dilution linearity: serum with concentrations near the expected upper limit were selected and diluted with the recommended diluent to prepare a series of sample concentrations. By means of least-squares fit, linearity was analyzed by plotting the measured results compared with the expected results based on dilution factor.

2.4 | Statistical analysis

Statistical analysis was performed using SPSS version 22.0. Normally distributed variables were expressed as mean ± SD, while abnormal distributed variables were described as median (25-75 interquartile interval) or number (%). Test the normality distribution of data variables was performed by the Kolmogorov-Smirnov test (P > .10). To compare normally distributed variables between two groups, the Student's t-test was utilized. Otherwise, non-normal distributed one used Mann-Whitney U test. Categorical variables were presented as percentages, compared with X² test. Spearman's test was used when assessed correlations among variables. To compare the diagnosis value of antibodies, receiver operator characteristic (ROC) curves were generated, and the area under the ROC curve (AUC) were determined. GraphPad Prism 8 was used to evaluate the linearity of the assay. All statistical tests were two-tailed, and P < .05 was considered statistically significant, and P < .01 was considered as a highly statistical significance.

3 | RESULTS

3.1 | Analytical performance of CIA for MPO and PR3 antibodies

As illustrated in Table 1, precision studies of CIA for MPO and PR3 antibodies revealed both within-run and laboratory precision CVs of <5%. The concentration of anti-MPO and anti-PR3 could be detected as low as 5.8 CU and 9.4 CU, respectively. Recovery of anti-MPO (96.67%-109.08%) and anti-PR3 (98.40%-110.59%) included in predefined acceptable limits (85%-115%). The analysis of anti-MPO and anti-PR3 demonstrated the linearity in the range of 3.20-603.30CU (R² = .9948) and 2.30-1657.40 (R² = .9985), respectively, was within the linear range given by kit instructions. Additionally, the linearity of MPO and PR3 antibodies was satisfactory, as the slopes showed a value of nearly 1 (1.016 and 0.9976 for anti-MPO and anti-PR3, respectively). By testing a series of standard substances with known concentrations, test results were compared with the known concentrations, and the equations for anti-MPO and anti-PR3 were obtained by least-squares fit: Y = −1.366 + 1.229 X − 0.0003535X² and Y = −2.916 + 0.8921 X + (6.366×10⁻³)X², respectively. The assays of MPO and PR3 antibodies were reliable (R² = .9985 and R² = .9994, respectively) (Figure 1).

3.2 | Quantitative performance of CIA for MPO and PR3 antibodies

By ELISA, as showed in Figure 2A and B, the levels of anti-MPO were significantly higher in AAV patients (68.60; [18.70-158.80])

| Table 1 Analytical performance of CIA for anti-MPO and anti-PR3 |
|---------------------------------------------------------------|
| **Within run precision**                                      |
| L                                                             |
| Accuracy (recovery rate)                                      |
| Reference interval                                           |
| **Laboratory precision**                                      |
| L                                                             |
| H                                                             |
| **Limit of Quantitation**                                     |
| **Linear range**                                              |
| R²                                                            |
| Fit regression equation                                       |
| Reference interval                                           |

Abbreviations: CIA, chemiluminescent immunoassay; CU, chemiluminescent unit; MPO, myeloperoxidase; PR3, proteinase 3.
and MPA patients (105.40; [47.94-204.3]) compared with either IC group (12.57; [0.66-75.47]) \( (P < .0001) \) or HC group (0.17; [0.13-0.67]) \( (P < .0001) \). By CIA, similarly, the levels of anti-MPO were significantly higher both in AAV patients (56.00; [4.40-235.30]) compared with either IC group (3.20; [3.20-18.55]) \( (P < .0001) \) or HC group (3.20; [3.20-3.20]) \( (P < .0001) \) (Figure 2C, D), IC group was significantly higher vs HC group \( (P < .01) \).

No matter by ELISA method or CIA method, in GPA group, the concentrations of anti-PR3 (102.60; [48.13-226.00], 66.65; [24.43-150.00], respectively) were significantly higher than that in IC group (34.06; [2.08-66.66], 2.3; [2.3-10.95], respectively) \( (P < .0001) \) and HC group (0.58; [0.20-1.83], 2.3; [2.3-2.3], respectively) \( (P < .0001) \) (Figure 3A, B). Although both methods demonstrated higher levels of PR3 antibody in AAV patients (2.87; [1.32-47.24], 2.3; [2.3-2.3], respectively) than in HC group (0.58; [0.20-1.83], 2.3; [2.3-2.3], respectively) \( (P < .05) \), the concentrations of anti-PR3 in AAV patients (2.87; [1.32-47.24]) was lower than in IC group (34.06; [2.08-66.66]) \( (P < .05) \) by ELISA, meanwhile there was no statistical difference between AAV patients (2.3; [2.3-2.3]) and IC group (2.3; [2.3-10.95]) \( (P = .51) \) by CIA (Figure 3C, D). Both methods demonstrated significantly higher levels of anti-PR3 in IC group vs HC group \( (P < .01) \).

### 3.3 The associations between CIA and ELISA

As described in Table 2, in AAV group, when detecting anti-MPO \( (r = .90, P < .0001) \) and anti-PR3 \( (r = .81, P < .0001) \), CIA was highly correlated with ELISA. In IC group, CIA was moderately correlated with ELISA to measure anti-MPO \( (r = .67, P < .0001) \) and anti-PR3 \( (r = .71, P < .0001) \), while there was no relationship between CIA and ELISA when measuring MPO \( (P = .48) \) and PR3 \( (P = .73) \) antibodies in HC group.

From the analysis of all cases, CIA was highly positively correlated with ELISA in the detection of anti-MPO \( (r = .82, P < .0001) \) and anti-PR3 \( (r = .75, P < .0001) \).

### 3.4 Qualitative performance of CIA for MPO and PR3 antibodies

The positivity for anti-MPO and anti-PR3 measuring by both ELISA and CIA was characterized as values more than 20. Figure 4...
HOU et al. illustrated the prevalence of anti-MPO and anti-PR3 in AAV group, IC group and HC group detected by ELISA and CIA. In AAV group, there were no statistically difference in the positive rates of anti-MPO (\(P = .18\)) detected by both immunoassays, nor in the positive rates of anti-PR3 (\(P = .34\)). In IC group, by CIA, the positive rates of anti-MPO (\(P < .01\)) and anti-PR3 (\(P < .01\)) were lower compared with by ELISA. In HC group, the positive rates of anti-PR3 were 0 by both immunoassays, similarly, without statistically significant difference (\(P = .32\)), the positive rates of anti-MPO were almost 0.

3.5 | The coincidence rate between CIA and ELISA

As described in Table 3, in AAV group, the total coincidence rates of anti-MPO and anti-PR3 reached to 88.89% and 92.06%, respectively, with the highly positive coincidence reached to 84.78% and 77.27%, respectively. The lowest total coincidence rates of anti-MPO (76.47%) and anti-PR3 (58.82%) were in IC group, with the positive coincidence reached to 48.38% and 30.00%, respectively. In HC group, the total coincidence rates of anti-MPO and anti-PR3 reached to 94.74% and 100.00%, respectively. The negative coincidence rate of each group was above 90%.

3.6 | The diagnosis performance of CIA compared with ELISA

Receiver operating characteristic curves were used to evaluate the diagnosis performance of anti-MPO for MPA and anti-PR3 for GPA. As demonstrated in Figure 5A and B, both by ELISA and CIA, anti-MPO had good discriminatory performance with an area under curve (AUC) of 0.86 and 0.89, respectively, (95% CI = [0.79; 0.92], [0.83; 0.95], respectively, \(P < .0001\)) with a cutoff value of 40.91 (Se = 85.11%, Sp = 70.11%) and 21.3 (Se = 80.85%, Sp = 82.76%), respectively. Similarly, either through ELISA or CIA, anti-PR3 had good discriminatory performance with an AUC of 0.85 and 0.92, respectively, (95% CI = [0.76; 0.94], [0.87; 0.98], respectively, \(P < .0001\)) with a cutoff value of 46.63 (Se = 81.25%, Sp = 68.97%) and 22.8 (Se = 81.25%, Sp = 87.36%), respectively. ROC curves were used to evaluate the diagnosis performance of
anti-MPO and anti-PR3 for AAV. Both by ELISA and CIA, anti-MPO had good discriminatory performance with an AUC of 0.74 and 0.76, respectively, (95% CI = [0.66; 0.82], [0.68; 0.84], respectively, \(P < .0001\)) with a cutoff value of 17.18 (Se = 76.19%, Sp = 63.22%) and 13.6 (Se = 68.25%, Sp = 77.01%), respectively, while the ROC of anti-PR3 for AAV yielded an AUC value of 0.53 and 0.51, respectively, (95% CI = [0.43; 0.62], [0.41; 0.61], respectively, \(P = .58\) and \(P = .85\), respectively) (Figure 5C,D).

4 | DISCUSSION

Enzyme-linked immunosorbent assay, a conventional immunoassay for anti-MPO and anti-PR3 detecting, performed as preferred screening and diagnostic tool for anti-MPO and anti-PR3 because of its ease of operation and low cost.\(^2\)\(^1\) In our present study, by ELISA, MPO and PR3 antibodies were used to distinguish AAV group from IC group and HC group. Firstly, in MPA patients, the levels of anti-MPO were significantly higher compared with either IC group (\(P < .0001\)) or HC group (\(P < .0001\)). Meanwhile, in GPA group, the concentrations of anti-PR3 were significantly higher than that in IC group (\(P < .0001\)) and HC group (\(P < .0001\)). Lastly, the AUC of anti-MPO (0.86) and anti-PR3 (0.85) in the diagnosis of MPA and GPA was statistically significant, suggesting ELISA was an effective detection method for MPO and PR3 antibodies in the diagnosis of MPA and GPA. However, the ELISA method was not perfect, ELISA detection needed batch detection, detection speed was moderately with assay times between 1.5 and 3 hours, it was necessary to explore new rapid and effective anti-MPO and anti-PR3 detection methods. The CIA can automate the detection process simply in a short time (30 minutes).\(^2\)\(^2\) In addition, previous studies have found that the CIA can quickly and sensitively detect multiple substances.\(^2\)\(^3\)-\(^2\)\(^4\) Could it compensate for ELISA deficiency while also detecting MPO and PR3 antibodies as effectively as ELISA?

Through CIA performance analysis, with good precision, accuracy, and reliable range, CIA showed good application potential in anti-MPO and anti-PR3 analysis. In addition, by CIA, MPO antibodies and PR3 antibodies had the ability to assist the diagnosis of MPA and GPA, respectively. Firstly, compared with IC group and HC group, MPA group and GPA group had higher concentrations of anti-MPO and anti-PR3 respectively. Secondly, the AUC of anti-MPO (0.89) and anti-PR3 (0.92) in the diagnosis of MPA and GPA was statistically significant. Finally, both methods confirmed higher levels of anti-MPO in the AAV group compared to the HC and IC groups (\(P < .0001\)), with an AUC of 0.74 and 0.76, respectively, while anti-PR3 failed to diagnose AAV effectively, yielded an AUC value of 0.53 and 0.51, respectively. The low proportion of GPA patients (25.40%) in the selected AAV patients might account for the failure of anti-PR3 to diagnose AAV.

In the AAV group, neither the positive rate of anti-MPO nor anti-PR3 by CIA was statistically different from by ELISA. Additionally, when detecting anti-MPO (\(r = .90\)) and anti-PR3 (\(r = .81\)), CIA was
highly correlated with ELISA, companying with highly total coincidence rates (88.89%, 92.06%, respectively) and positive coincidence (84.78%, 77.27%, respectively) of anti-MPO and anti-PR3, which was similar to previous study.\textsuperscript{14} It suggested that CIA could accurately and effectively detect MPO and PR3 antibodies in AAV patients.

In HC group, when measuring anti-MPO and anti-PR3, though there was no relationship between CIA and ELISA ($P = .48$, .73, respectively), the total coincidence rates of these two methods reached to 94.74% for anti-MPO and 100.00% for anti-PR3, in addition, anti-PR3 positive rate detected by both immunoassay were 0, without statistically significant difference ($P = .32$), anti-MPO almost were 0. Commonly, MPO and PR3 antibodies in healthy people are also mostly negative. It indicated that the CIA could be used to measure MPO and PR3 antibodies in healthy population.

In IC group, by CIA, the positive rate of anti-MPO and anti-PR3 was lower than by ELISA. The total (76.47%, 58.82, respectively) and positive (48.38%, 30.00%, respectively) coincidence rates of anti-MPO and anti-PR3 were the lowest, but the negative coincidence rates reached 100%. This suggested that although the CIA failed to detect all positive samples as sensitive as ELISA, the CIA did detect

|                | CIA       |         |         |         |
|----------------|-----------|---------|---------|---------|
|                | Positive  | Negative| Total   | Coincidence rate |
| **AAV**        |           |         |         |         |
| Anti-MPO       |           |         |         |         |
| ELISA          |           |         |         |         |
| Positive       | 39        | 7       | 46      | 84.78%(39/46)  |
| Negative       | 0         | 17      | 17      | 100.00%(17/17) |
| Total          | 39        | 24      | 63      | 88.89%(56/63)  |
| Anti-PR3       |           |         |         |         |
| ELISA          |           |         |         |         |
| Positive       | 17        | 5       | 22      | 77.27%(17/22)  |
| Negative       | 0         | 41      | 41      | 100.00%(41/41) |
| Total          | 17        | 46      | 63      | 92.06%(58/63)  |
| **IC**         |           |         |         |         |
| Anti-MPO       |           |         |         |         |
| ELISA          |           |         |         |         |
| Positive       | 15        | 16      | 31      | 48.38%(15/31)  |
| Negative       | 0         | 37      | 37      | 100.00%(37/37) |
| Total          | 15        | 53      | 68      | 76.47%(52/68)  |
| Anti-PR3       |           |         |         |         |
| ELISA          |           |         |         |         |
| Positive       | 12        | 28      | 40      | 30.00%(12/40)  |
| Negative       | 0         | 28      | 28      | 100.00%(28/28) |
| Total          | 12        | 56      | 68      | 58.82%(40/68)  |
| **HC**         |           |         |         |         |
| Anti-MPO       |           |         |         |         |
| ELISA          |           |         |         |         |
| Positive       | 0         | 0       | 0       | (0/0)      |
| Negative       | 1         | 18      | 19      | 94.74%(18/19) |
| Total          | 1         | 18      | 19      | 94.74%(18/19) |
| Anti-PR3       |           |         |         |         |
| ELISA          |           |         |         |         |
| Positive       | 0         | 0       | 0       | (0/0)      |
| Negative       | 0         | 19      | 19      | 100.00%(19/19) |
| Total          | 0         | 19      | 19      | 100.00%(19/19) |

Abbreviations: AAV, Anti-neutrophil cytoplasmic antibody-associated vasculitis; CIA, chemiluminescent immunoassay; ELISA, Enzyme-linked Immune Sorbent Assay; HC, Healthy control; IC, Interference control; MPO, Myeloperoxidase; PR3, Proteinase 3.
HOU et al. | all negative ELISA samples. Additionally, the clinical characteristics of non-vasculitis patients with serologies positive MPO and PR3 antibodies were not significantly different from those without MPO and PR3 antibodies. The serologies positive for MPO-ANCA and/or PR3-ANCA alone frequently failed to diagnosis as systemic vasculitis, might even cause diagnostic dilemma. Furthermore, CIA was highly correlated with ELISA. It indicated that CIA was generally competent to detect MPO antibody and PR3 antibody in IC group.

5 | CONCLUSION

Performed well in measuring MPO and PR3 antibody, CIA could be applied to the detection of MPO and PR3 antibodies in AAV patients and healthy population, though not all the positive samples were sensitively detected in IC group. As ELISA, CIA was competent to detect MPO and PR3 antibodies in these subjects, thus distinguish AAV patients from IC group and HC group and effectively diagnose MPA and GPA.

5.1 | Limitation of the study

The limitation of this study was failing to investigate sufficient number of inflammatory bowel disease and hyperthyroidism patients in IC group due to the small sample size. Because ANCA contributed to the classification of inflammatory bowel disease, besides, after propylthiouracil treatment, patients with hyperthyroidism were more likely to develop AAV. CIA may also contribute to monitor MPO and PR3 antibody in these patients. In this way, similar studies in large population are warranted.

AUTHOR CONTRIBUTIONS

Study concept and design: Xiu-zhu Hou, Li-yan Cui; data curation: Xiu-zhu Hou, Jing Liu, Jiansuo Zhou; analysis and interpretation of data: Xiu-zhu Hou, Tiancheng Wang; drafting of the manuscript: Xiu-zhu Hou; funding acquisition: Li-yan Cui; statistical analysis: Xiu-zhu Hou, Jing Liu, Li-yan Cui; study supervision: Li-yan Cui; writing – review and editing: Xiu-zhu Hou, Li-yan Cui.

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