RESEARCH ARTICLE

Time-resolved fluorescent immunoassay-based combined detection of procalcitonin, C-reactive protein, heparin binding protein, and serum amyloid A1 to improve the diagnostic accuracy of early infection

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Background: Serological tests are indispensable in the diagnosis of early infection. At present, only procalcitonin (PCT) and C-reactive protein (CRP) are commonly used in clinical practice. Recently, serum amyloid A1 (SAA1) and heparin binding protein (HBP) have been shown to be new biomarkers, because SAA1 is highly sensitive and specific for viral infections, and HBP is predictive for septic shock. In this study, PCT, CRP, HBP, and SAA1 were detected in different combinations to improve the diagnostic accuracy of early infection using the biotin-avidin amplifying system-based time-resolved fluorescent immunoassay (BA-TRFIA).

Methods: A time-resolved fluorescent immunoassay for PCT, CRP, HBP, and SAA1 was developed and then tested in a clinical setting. All experiments were carried out using the DR6608 time-resolved fluorescent immunoassay analyzer.

Results: The cutoff values of PCT, CRP, HBP, and SAA1 were 0.05 µg/L, 5.59 mg/L, 3.83 µg/L, and 1.56 mg/L, respectively. The area under the ROC curve (AUC) showed that PCT > SAA1 > CRP > HBP > 0.8. A methodological comparison of the results showed that a combination of the four biomarkers had the highest accuracy for the diagnosis of infectious diseases.

Conclusion: The time-resolved fluorescent immunoassay-based combined detection of PCT, CRP, HBP, and SAA1 was shown to significantly improve the diagnostic accuracy of early infection. Thus, our results indicate that combined detection based on BA-TRFIA may represent a promising strategy in the clinical diagnosis of infection.

KEYWORDS

C-reactive protein, heparin binding protein, procalcitonin, serum amyloid A1, time-resolved fluorescent immunoassay

1 | INTRODUCTION

With the rapid development of diagnostic technologies in vitro, more rapid and accurate serological tests have played an increasingly

important role in the early diagnosis of infectious diseases. C-reactive protein (CRP), the most important acute phase protein, is considered to be an early indicator of infection and inflammation.¹⁻³ On the other hand, procalcitonin (PCT) plays a pivotal role in the diagnosis of sepsis as well as in the prescription of effective medications. However, the mechanism of sepsis is complex, which renders PCT as a poor independent indicator for the differential diagnosis of infections.⁴⁻⁶ In

Abbreviations: BA-TRFIA, biotin-avidin amplifying system time-resolved fluorescent immunoassay; CLIA, chemiluminescent immunoassay; CRP, C-reactive protein; HBP, heparin binding protein; PCT, procalcitonin; SAA1, serum amyloid A1.

recent years, studies have shown that heparin binding protein (HBP) can also be used as an indirect biomarker for the early diagnosis of sepsis, because it positively correlated with the PCT level.⁷ The combined detection of PCT and HBP serum levels can greatly improve the early diagnosis of sepsis.⁸ Unfortunately, for viral infections, the clinical significance of CRP and PCT, as well as routine blood tests, is not obvious. Thus, clinical findings are likely to be misinterpreted and patients are likely to be misdiagnosed.⁹⁻¹⁴ However, serum amyloid A1 (SAA1) has superior sensitivity and specificity in the diagnosis of viral infection.¹⁵ The introduction of SAA1 on the basis of CRP and PCT can greatly compensate for the shortcomings in the currently available viral infection detection methods and provide more valuable reference information for the identification of viral and bacterial infections as well as suitable treatment options.^{16,17} Therefore, the combined detection of PCT, CRP, HBP, and SAA1 can effectively improve the accuracy of the early diagnosis of infectious diseases.

Currently, CRP and PCT are widely used in clinical settings, whereas the use of SAA1 and HBP has not yet been popularized. Time-resolved fluorescent immunoassay (TRFIA) is an ultra-micro labeling methodology that can greatly improve the sensitivity and specificity of detection when combined with the biotin-avidin system (BA-TRFIA).¹⁸ In this article, the serum levels of PCT, CRP, HBP, and SAA1 were detected by BA-TRFIA. Based on currently available clinical diagnostic information, an analysis was performed to illustrate the clinical application value of PCT, CRP, HBP, and SAA1 in different combinations using BA-TRFIA.

2 | MATERIALS AND METHODS

2.1 | Materials

PCT monoclonal antibodies 16B5 and 42, and SAA1 monoclonal antibodies VSA31 and VSA25 were purchased from HyTest, Finland. CRP monoclonal antibodies 005M and 239 were purchased from Meridian Life Science, Inc, Memphis, TN, USA. HBP monoclonal antibodies 2H6 and 1E2 were purchased from Zhuhai Bomei Biotechnology Co., Ltd., Zhuhai, Guangdong, China. Streptavidin was purchased from Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, and the Eu-Labeling kit was purchased from Guangzhou Darui Biotechnology Co., Ltd., Guangzhou, Guangdong, China. The DR6608 time-resolved fluorescent immunoassay analyzer was purchased from Guangzhou Foshan Daan Medical Equipment Co., Ltd., Foshan, Guangdong, China.

2.2 | Serum collection

Blood samples from 497 patients supplied by the Department of Clinical Laboratory of the Second Affiliated Hospital of Wenzhou Medical University were collected from October 2017 to February 2018. The initial diagnosis of all patients was suspected to be a bacterial or viral infection. The age of 240 males and 257 females was from 1 to 93 years old, and the average age was 52.13 ± 26.15 years old. Among the 497 cases, 182 patients were clinically tested for

PCT, 200 patients were tested for CRP, and the remaining 115 patients were tested for both PCT and CRP. No patient was clinically tested for HBP and SAA1. In addition, blood samples were obtained from 150 healthy people (controls) enrolled at the Wenzhou Medical University in 2017. The age of 60 males and 90 females was from 21 to 27 years old, and the average age was 24 ± 3 years old. All samples were collected by venipuncture in anticoagulant tubes. The total volume of each specimen was approximately 2-5 mL. Centrifugation was performed at a speed of 5000 rpm for 10 min at 4°C. The plasma was collected, numbered, and stored at -80°C.

2.3 | Preparation of EU³⁺-SA

We combined 13.84 μ L of the Eu-labeling reagent (20 mg/mL) with 236.16 μ L of the SA reagent (2 mg/mL), followed by gentle mixing and overnight incubation at 4°C. The separation of EU³⁺SA from unreacted EU³⁺ chelate was performed using an EMD Millipore Amicon Ultra-0.5 centrifugal filter unit. The stability of EU³⁺SA was increased by adding purified BSA to a final concentration of 0.1%, followed by storage at -20°C.

2.4 | Principle of the assay

This experiment combined the advantages of both the biotin-avidin immune amplification and the time-resolved fluoro-immunoassay, which is based on the double-antibody sandwich method. Specifically, the capture antibody and the biotinylated detection antibody were conjugated to two antigenic determinants on the antigenic molecule, followed by the formation of solid-phase capture antibody-antigen-biotinylated detection antibody immune complexes. Subsequently, EU³⁺-SA and the fluorescent enhancement solution were added to the reaction mixture. Because the amount of the capture antibody and the biotinylated detection antibody in the reaction mixture were much higher than that of the antigen to be detected, the amount of the formed immune complex positively correlated with the amount of the antigen to be detected in the sample within a certain linear detection range. In this way, the amount of the antigen to be detected in the sample could be determined by measuring the fluorescent intensity of the label within the immune complex.

2.5 | BA-TRFIA assay

The capture antibody-coated 96-well plate was removed and maintained at room temperature for 15 min. The reference standard or diluted serum sample was added according to our laboratory's instructions, and the plate was incubated at room temperature by shaking at 600 rpm (the incubation times for the assay of PCT, CRP, HBP, and SAA1 were 30, 45, 30, and 45 min, respectively). At the end of the first incubation, the plate was washed 5 or 6 times, and then air-dried. The biotinylated detection antibody was diluted (B-42 and B-239, 1:400; B-2H6 and B-VSA31, 1:100) with assay buffer, added to the 96-well plate, and incubated (the incubation

times for the assay of PCT, CRP, HBP, and SAA1 were 60, 45, 60, and 60 min, respectively). The plate was washed six times after the end of the second incubation. Thereafter, EU³⁺-SA was diluted (PCT, 1:2000; CRP, 1:3200; HBP, 1:700 and SAA1, 1:1600) with assay buffer, added to the 96-well plate, and incubated (the incubation times for the assay of PCT, CRP, HBP, and SAA1 were 60, 30, 45, and 60 min, respectively). At the end of the third incubation, the plate was washed 5 to 6 times and then air-dried. Finally, the fluorescent enhancement liquid was added, and the plate was incubated in the dark by shaking for 3 min. After warming up the instrument for at least 30 min, the plate was read by placing it in the detection tank of the DR6608 time-resolved fluorescent immunoassay analyzer.

2.6 | Clinical evaluation

The serum levels of PCT, CRP, HBP, and SAA1 in 150 healthy subjects were individually measured to calculate the cutoff values for the BA-TRFIA. A total of 497 serum samples were used to measure the levels of PCT, CRP, HBP, and SAA1. After analyzing the ROC curves, we used the currently recognized chemiluminescent value as the reference point. The PCT and CRP clinical test results were obtained from the Siemens automatic chemiluminescence immunoassay analyzer and the Roche Cobas e601 fully automatic chemiluminescence immunoassay analyzer, respectively. Because the clinical use of HBP and SAA1 is not widespread, their results were not available. The four biomarkers were combined in different combinations to explore the diagnostic value of each combination in the detection of infections. A combined detection was judged as positive when one of the

biomarkers in the combination was higher than the critical value. A combination was judged as negative when all biomarkers in the combination were lower than the critical value. Statistical analysis was performed using IBM SPSS Statistics 20.0, Armonk, New York, USA.

3 | RESULTS

3.1 | Identification of recombinant proteins

PCT, CRP, HBP, and SAA1 recombinant proteins were prepared in our laboratory, and their authenticity was verified by Western blotting or dot blotting. Figures S1A,C,E,G show that the four recombinant proteins were successfully prepared. Furthermore, each recombinant protein reacted with its specific monoclonal antibody. The antigenicity of each protein was identified by the BA-TRFIA. Figures S1B,D,F,H show that the four recombinant proteins reacted with their specific monoclonal antibodies in double-antibody sandwich reactions.

3.2 | ROC curve analysis

The reliability of the BA-TRFIA is shown in Figure 1 and Table 1. The AUC values of PCT, CRP, HBP, and SAA1 were 0.978 (95% CI, 0.949-1.000), 0.841 (95% CI, 0.787-0.895), 0.808 (95% CI, 0.593-1.000), and 0.920 (95% CI, 0.877-0.963), respectively. A comparison of the AUC values of the biomarkers showed the reliability to be PCT > SAA1 > CRP > HBP. Furthermore, the AUC value for CRP was lower than that for CLIA, but it was higher for PCT than that for CLIA by the BA-TRFIA.

FIGURE 1 The ROC curves for PCT, CRP, HBP, and SAA1. The graph was prepared using IBM SPSS statistics 20.0

Area under the curve					
Test result variate	Area	Standard error ^a	Progressive Sig. ^b	Asymptotic 95% Confidence Interval	
				Lower limit	Upper limit
PCT (CLIA)	0.925	0.029	0.000	0.867	0.982
PCT (BA-TRFIA)	0.978	0.014	0.000	0.949	1.000
CRP (CLIA)	0.864	0.034	0.001	0.797	0.931
CRP (BA-TRFIA)	0.841	0.028	0.002	0.787	0.895
HBP (BA-TRFIA)	0.808	0.109	0.005	0.593	1.000
SAA1 (BA-TRFIA)	0.920	0.022	0.000	0.877	0.963

^aUnder nonparametric assumptions.

^bZero hypothesis: real area = 0.5.

TABLE 1 ROC curve-related information

3.3 | Methodological comparison

The authenticity of each biomarker is shown in Table 2. The sensitivity of PCT was the highest (99.65%), but its specificity was the lowest (66.67%) and its Youden's index was also low (0.66). The specificity of SAA1 was the highest (85.71%), but its negative predictive value (NPV) was low (28.13%). Although the NPV (23.94%) and the Youden's index (0.67) of CRP were low, the sensitivity, specificity, accuracy, and positive predictive value (PPV) were greater than 80%. Lastly, the sensitivity, accuracy, and PPV of HBP were approximately 98%, but the specificity, Youden's index, and NPV were low.

When two biomarkers were detected, the sensitivity and NPV of PCT+CRP, PCT+HBP, and PCT+SAA1 were all 100%. Furthermore,

the accuracy and PPV were all greater than 97%, but the specificity was less than 50% and the Youden's index was less than 0.5. In addition, the Youden's index and NPV of CRP+HBP, CRP+SAA1, and HBP+SAA1 were unsatisfactory.

When three biomarkers were detected, the authenticity of PCT+CRP+HBP and PCT+HBP+SAA1 yielded similar results. The sensitivity and NPV were all 100%, but the specificity (33.33%) and the Youden's index (0.33) were poor. PCT+CRP+SAA1 and CRP+HBP+SAA1 also yielded similar results. The sensitivity and PPV were as high as 99.12%, but the specificity (50%) and the Youden's index (0.49) were higher than PCT+CRP+HBP and PCT+HBP+SAA1, but still lower.

When four biomarkers were detected, the rate of missed diagnosis and misdiagnosis was greatly reduced. Based on the results of 115 clinical specimens, each evaluation index was optimal.

TABLE 2 Methodological comparison

Grouping	Sensitivity, %	Specificity, %	Accuracy, %	Youden's index	PPV, %	NPV, %	Total
PCT	99.65	66.67	98.65	0.66	98.97	85.71	297
CRP	81.69	85	81.9	0.67	98.77	23.94	315
HBP	98.96	55.56	97.64	0.55	98.62	62.5	297
SAA1	84.35	85.71	84.44	0.70	98.8	28.13	315
PCT+CRP	100	25	97.39	0.25	97.37	100	115
PCT+HBP	100	44.44	98.32	0.44	98.29	100	297
PCT+SAA1	100	25	97.39	0.25	97.37	100	115
CRP+HBP	97.35	50	96.52	0.47	99.1	25	115
CRP+SAA1	90.14	80.95	89.52	0.71	98.51	36.96	315
HBP+SAA1	99.12	50	98.26	0.49	99.12	50	115
PCT+CRP+HBP	100	33.33	98.26	0.33	98.25	100	115
PCT+CRP+SAA1	99.12	50	98.26	0.49	99.12	50	115
PCT+HBP+SAA1	100	33.33	98.26	0.33	98.25	100	115
CRP+HBP+SAA1	99.12	50	98.26	0.49	99.12	50	115
PCT+CRP+HBP+SAA1	100	100	100	1	100	100	115

3.4 | Clinical verification

Analysis of the levels of PCT, CRP, HBP, and SAA1 in 115 clinical specimens is shown in Table 3. The differential diagnosis of bacterial and viral infections can also be achieved by examining the SAA1/CRP ratio. The occurrence of sepsis can be better predicted, and the severity of the condition can be better determined by the combined detection of PCT and HBP. Among 25 patients screened by differential detection, 20 cases were negative for CLIA, but positive for BA-TRFIA. The final clinical diagnosis was as follows: 1 case of pulmonary infection with Guillain-Barre syndrome, 8 cases of pneumonia, 1 case of respiratory infection, 1 case of chest pain within the lung, 1 case of intestinal perforation, 1 case of left soft tissue infection, 2 cases of vasculitis, 1 case of acute pancreatitis, 1 case of tenosynovitis, and 1 case of pulmonary nodules with a mediastinal cyst. There were six cases negative for CRP and positive for SAA1 (two cases were also included in the previous 20 cases). The final clinical diagnosis was as follows: cough, left rotator cuff tear, lung infection, left osteomyelitis, chest pain within the lung, peptic ulcer with hypertension, and coronary heart disease. The remaining 1 case was positive for PCT and negative for HBP, and the final clinical diagnosis was colon cancer.

4 | DISCUSSION

In this study, we found that none of the biomarkers were absolutely sensitive or specific, especially for infectious diseases with complex etiologies and multiple manifestations. Individually, PCT, CRP, HBP, and SAA1 have indispensable roles in the diagnosis and tracking of different infectious diseases. However, the combination of the four biomarkers renders the clinical diagnosis of infectious diseases more convenient and accurate. Therefore, the combined detection of PCT, CRP, HBP, and SAA1 can provide clinicians with a more reliable early diagnosis.

To improve the diagnostic accuracy, it was critical to develop a more sensitive approach. Based on the respective advantages of the time-resolved fluorescent immunoassay and the biotin-avidin system, this study combined the two approaches to develop the BA-TRFIA, which detected PCT, CRP, HBP, and SAA1. Due to the inherent shortcomings of this approach, the method could not simultaneously detect multiple biomarkers. Instead, the biomarkers were separately detected. However, with the popularity of automated instruments, this shortcoming can be largely ignored.

In this study, we evaluated the ROC curves and the authenticity-related indicators of PCT, CRP, HBP, and SAA1. By contrast, the ROC curve indicated that the order of the AUC values was $PCT > SAA1 > CRP > HBP > 0.8$, and each biomarker had a certain accuracy. The BA-TRFIA yielded a higher AUC value for PCT than for CLIA; that is, PCT had a higher clinical diagnostic value than CLIA. However, the BA-TRFIA was less useful when detecting CRP. Unfortunately, we found that there were several deficiencies when individually detecting the biomarkers, including low sensitivity or specificity. Presently, the combined detection of the four biomarkers has been used clinically to improve the diagnostic rate of bacterial and viral infections. After comparing various combinations, we found that a combination of PCT, CRP, HBP, and SAA1 had the greatest clinical value. The clinical verification once again highlighted the importance and necessity of the combined detection of the four biomarkers. Therefore, it is our opinion that the combined detection of PCT, CRP, HBP, and SAA1 in evaluating patients for potential bacterial and viral infections contributes to the more timely and accurate diagnosis of infectious diseases, especially in the early stages of sepsis.

5 | CONCLUSIONS

In summary, the individual detection of PCT, CRP, HBP, and SAA1 had some value in the diagnosis of infectious diseases, but there was evidence of misdiagnoses and missed diagnoses. However, the combined detection of the four biomarkers improved the sensitivity and accuracy of the diagnosis in a timely and effective manner, especially for the early prediction of sepsis. In general, the combined detection of the four biomarkers improved the detection rate of infectious diseases, ensured the accuracy of the diagnosis, and enhanced the clinical application of the BA-TRFIA.

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TABLE 3 Clinical verification

Groups	Number	PCT ($\mu\text{g/L}$)	HBP ($\mu\text{g/L}$)	SAA1 (mg/L)	CRP (mg/L)	SAA1/CRP
Virus	5	0.6 ± 0.3	9.0 ± 3.5	24.3 ± 12.8	2.1 ± 1.9	78.7 ± 108.3
Bacteria	98	3.2 ± 6.2	62.1 ± 57.2	40.0 ± 29.9	122.5 ± 81.6	0.5 ± 0.7
Sepsis	9	7.5 ± 15.8	92.8 ± 37.6	51.6 ± 41.2	156.6 ± 72.4	0.37 ± 0.24
Cardiovascular diseases	8	1.0 ± 1.2	27.0 ± 35.6	39.3 ± 35.8	76.4 ± 61.6	1.0 ± 1.4
Tumor	2	0.3 ± 0	13.9 ± 10.6	26.5 ± 17.2	37.8 ± 20.2	1.3 ± 1.2

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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