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Simultaneous detection of *Mycoplasma pneumoniae* IgG and IgM using dual-label time resolved fluoroimmunoassay

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ABSTRACT

Anti-*Mycoplasma pneumoniae* (MP) IgM and IgG are useful serological markers for detection of MP infection. In this study, a simultaneous quantification of MP IgM and IgG was performed by time-resolved fluoroimmunoassay (TRFIA). The europium-labeled anti-human IgM and samarium-labeled anti-human IgG were used as tracers, and MP IgM and IgG were recognized in serum samples. After dissociating europium and samarium ions from the immune complex, their fluorescence intensity was recorded and used to calculate the concentrations. The linear range and sensitivity of detection were 2–5500 BU/mL and 0.5 BU/mL for IgM, and 1.5–1500 BU/mL and 0.2 BU/mL for IgG, respectively. The intra- and inter-assay coefficients of variation were 5.14% and 8.41% for IgM, and 5.44% and 8.76% for IgG, respectively. The recovery rate was 94.9–106.8% for IgM and 96.1–109.4% for IgG. The correlation rates of serum detection for 38 respiratory infected patients between dual-label TRFIA and ELISA were 0.9294 and 0.9366 for IgM and IgG, respectively. The coincidence rate between passive particle agglutination and TRFIA is 93.3%. Dual-label TRFIA is a sensitive and reliable technique for measuring MP IgM and IgG levels and could be useful for the early diagnosis of MP infection.

Introduction

Mycoplasma pneumoniae (MP) is a prokaryotic bacterium without a cell wall. It causes pneumonia mainly through its binding to the neuraminic acid receptor on the respiratory tract mucosa epithelial cells of the host, followed by the release of toxic metabolites. A serum epidemiological study has shown that, in China, 20.7–38.9% of the patients with community-acquired pneumonia (CAP) were infected by MP after the first pathological instance of community-acquired pneumonia was identified in adults [1]. In addition to the respiratory symptoms, 20–25% of the cases can experience extra-pulmonary manifestations, such as meningitis, myocarditis, hemolytic anemia, and thrombocytopenic purpura [2]. Early diagnosis could effectively control the disease caused by MP infection and reduce the mortality. Therefore, the accurate diagnosis of MP has an important clinical role.

The diagnosis of MP infection is mainly dependent on microbiological and serological assays [3–5]. The serological detection is done prominently during laboratory diagnosis, widely by particle agglutination assay and enzyme-linked immunosorbent assay (ELISA). Compared with the agglutination assay, ELISA has the advantage of detecting specific IgM and IgG antibodies, which are associated with the course of MP infection [6]. MP IgM can be used for early diagnosis or as an indicator of acute infection. It could be detected in the initial phase at about 7 days after the infection, peaks at 10–30 days, and becomes undetectable in 76.5% of the patients after 2 weeks. Although a single determination of MP IgM could be done to confirm the current infection, the negative results cannot exclude the possibility of pneumonia. MP IgG often appears 2 weeks after the onset of the disease and is the most reliable marker of MP infection. When its titer increases 4-times, primary infection might be present, but if not, then a previous infection might have relapsed. Therefore, it is more significant to simultaneously detect MP IgM and IgG for evaluating the status of MP infection [7], and demands the need for clinicians to use a more accurate and reliable diagnostic technology.

ELISA is an easy-to-perform and commonly used method, whose sensitivity toward the serum antibody is equivalent to that of polymerase chain reaction in patients with normal immune function, who have developed sufficient antibodies after MP infection. ELISA is

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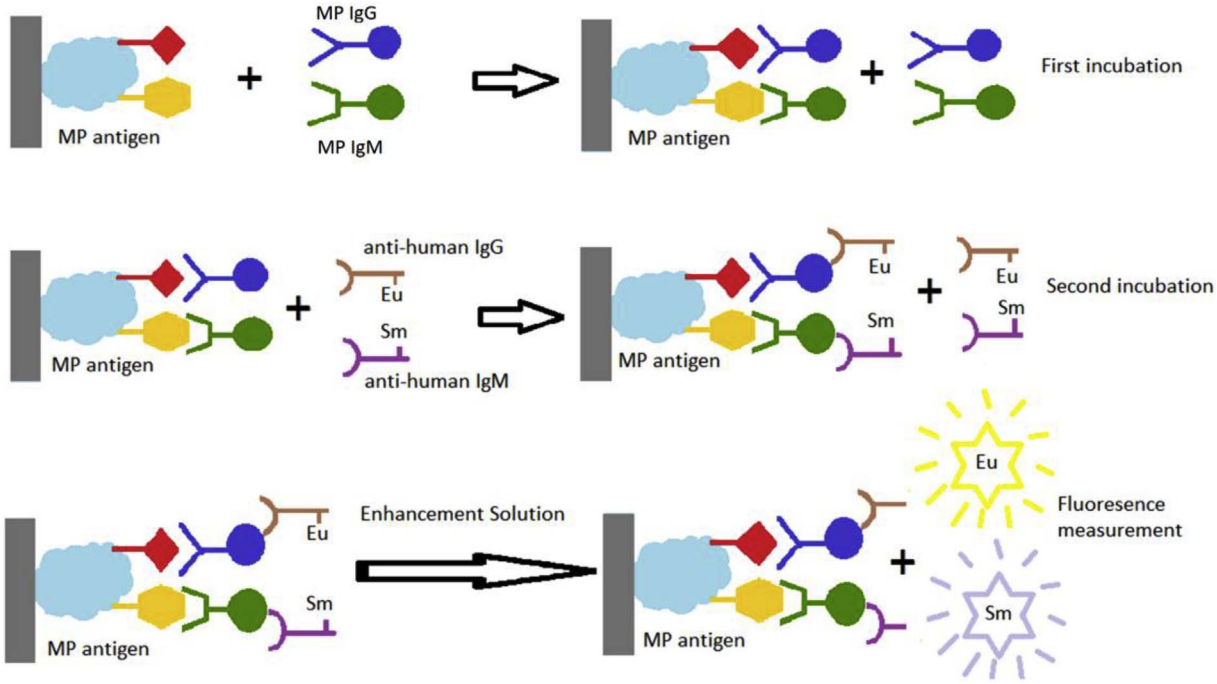

Fig. 1. Schematic illustration of dual-label TRFIA for MP IgG and IgM. The assay was performed in microtiter plates with wells coated with MP antigen. Standards or 100-fold diluted serum samples (100 μL each) were added to the wells and incubated for 1 h with continuous shaking. After removing the unbound samples by washing the plate four times, 100 μL of assay buffer containing Sm-anti-IgG and Eu-anti-IgM was added to the wells and it was incubated with continuous shaking for 1 h. After six washes with wash solution (Jiangyuan, China), 200 μL enhancement solution (Jiangyuan, China) was added to each well. The plate was shaken for 5 min and the signal of Eu and Sm was measured using a fluorometer.

affected by some factors, such as the enzyme of active macromolecules as a label, and has disadvantages of linear range and stability resulting in multiple weakly positive, threshold, and false negative cases, hence causing inaccurate clinical diagnosis. Using this method, MP IgM and IgG have to be detected separately with two kinds of kits. During the determination process, each sample was pipetted twice. Because of the difference in experimental conditions, the measurement error was large enough to affect the accuracy of IgG/IgM ratio. Therefore, simultaneous detection would be a useful approach to improve accuracy. However, common methods, including radioimmunoassay, ELISA, chemiluminescence immunoassay, and electrochemiluminescence immunoassay, could not resolve this problem.

Time resolved fluoroimmunoassay (TRFIA) is a technique that uses lanthanide chelates as tracers, gaining some advantages, such as low background, high sensitivity, broad detection range, and good stability [8]. Notably, only TRFIA could perform multiple analyses based on the difference in fluorescence waves of lanthanides. The technology has been successfully applied to detect various biological molecules [9–14]. In recent years, dual-label TRFIA has become an important tool in food safety and clinical examination, such as for the detection of clothianidin, diniconazole, cardiac troponin T, and myoglobin [15–18]. Here, simultaneous serological detection of anti-MP IgG and IgM antibodies by dual-label TRFIA method is reported.

Materials and methods

Chemicals and reagents

Auto DELFIA-1235 TRFIA analyzer was supplied by Perkin-Elmer Life and Analytical Science/Wallac Oy (Finland). Enzyme-linked immunosorbent assay (ELISA) kit for MP antibody detection, MP-IgG antibody standard and MP-IgM antibody standard were obtained from Savyon Diagnostics Company (Israel). MP antigen was purchased from Microbix Biosystems Company (Canada). Rabbit anti-human IgM and IgG were purchased from Sigma (USA). Eu^{3+}- and Sm^{3+}- labeling kits were obtained from Perkin-Elmer Company (USA). Bovine serum albumin (BSA) were supplied by the Institute of Biological Products Department of Health (China). 96-well polystyrene microtiter plates were obtained from Nunc International (Denmark). Sephadex-G25 was purchased from Pharmacia Company (USA). All additional chemicals and reagents used were of analytical grade.

Preparation of antigen-coated plate

MP antigen (Microbix Biosystems Co., Canada) was diluted using 50 mmol/L Na_2CO_3—$NaHCO_3$ buffer (pH 9.6) and 100 μL of the diluted antigen was added to each well of the 96-well microtiter plate (Nunc co., Denmark). After overnight incubation at 4 °C, the coated plates were washed twice and blocked with 200 μL 50 mmol/L carbonate buffer (pH 9.6) containing 3 g/L BSA for 2 h at room temperature. After discarding the blocking buffer, the plates were vacuumed and later stored in sealing bags at −20 °C until further use.

Preparation of labeled antibodies

Referring to the manual of the labeling kits (PE Co., USA), the rabbit anti-human IgG and IgM antibodies (Sigma-Aldrich, USA) were reacted with samarium ion (Sm^{3+}) and europium ion (Eu^{3+}) chelates, respectively. One microgram of anti-human IgG antibody was loaded on a PD-10 column, eluted with 50 mmol/L Na_2CO_3—$NaHCO_3$ buffer (pH 8.5) containing 0.155 mol/L NaCl, and collected from the first elution peak. IgG (2 g/L) was mixed with 0.2 mg Sm^{3+} chelates overnight at room temperature. The unbound reagent was removed by using a 1 × 40 cm column packed with Sepharose CL-6B (Pharmacia, USA) and the elution buffer (80 mmol/L Tris HCl, pH 7.8). The descending fraction was analyzed by measuring fluorescence using AutoDELFIA1235 fluorometer (PE co., USA). After collecting the protein peak, Sm-IgG was diluted with the elution buffer containing 2 g/L BSA and stored in a freeze-dried form. Eu-IgM was labeled using the same procedure as that used for Sm-IgG as mentioned above. As calculated from the element's photon counts and protein concentrations, the molar ratios of Sm/IgG and Eu/IgM were 7.5 and 8.1, respectively.

Assay principle and procedure

The TRFIA method was based on antibody-antigen reaction and signal amplification of the second antibody (Fig. 1). If the specimens contained MP IgM/IgG, they would bind to the solid phase of the plate coated with MP antigen. After addition of Eu^{3+}-anti-human IgM antibody and Sm^{3+}-anti-human IgG antibody conjugates, the complexes of MP-MP IgG-Sm^{3+} anti–IgG and MP-MP IgM-Eu^{3+} anti-IgM were formed, respectively. Later, enhancement solution led to dissociation of the complexes and release of Eu and Sm. The fluorescence was read using a time-resolved fluorescence spectrometer and its intensity was proportional to the concentration of MP IgM/IgG.

Assay evaluation

Sample collection was approved by the medical ethics committee of Affiliated Wuxi People's Hospital of Nanjing Medical University. The dose-response linear curve was estimated by running a set of standards for ten runs. Precision of the assay was calibrated from coefficients of variation (CV). Three aliquots of serum samples with low, middle, and high concentrations of MP IgG and IgM were analyzed with 25 runs in an assay for intra-CV and in 10 runs in an assay for inter-CV by dual-label TRFIA for MP IgG and IgM. The sensitivity was determined from the dose equal to the mean fluorescence (n = 20) of blank sample ± SD. To calculate the reference range, serum specimens from 50 cases of healthy donors were collected and subjected to MP TRFIA. This range gave 99% confidence interval. The recovery rate was calculated from the difference between observed dose and known dose, which was determined from the serial 2-fold dilution of the three sera by established dual-label TRFIA. To evaluate the correlation between dual-label TRFIA and ELISA kits (SeroMP IgG and IgM kits, Savyon Diagnostics, Israel), serum samples of 38 patients were subjected to both the methods simultaneously. To reconfirm the consistency, 30 samples were determined by passive particle agglutination (MP Antibodies kit, Fujirebio Inc., Japan) and proposed TRFIA. Definition of 'positive' and 'negative' for the passive particle agglutination was based on the reaction image observed by naked eyes. The detection process was following the manual of the kit. Stability of the assay was estimated by comparing that of the kits incubated at 37 °C for 7 days and stored under normal conditions. To obtain the clinical specification, 50 cases with no respiratory infection were enrolled and their sera were determined for the presence of MP antibodies. Clinical positive rate of the assay was calculated from antibodies of MP IgG and IgM in serum samples by MP dual-label TRFIA. In this evaluation, 100 cases of pneumonia and 100 cases of upper respiratory infection (URI) were enrolled after being clinically defined. (All participants gave informed consent to take part in the study, which was approved by the Committee for Ethical Affairs of the Nanjing Medical University, protocol number 2015/31)

Statistical analyses

Software Origin 8.0 (OriginLab, USA) and SPSS 19.0 (IBM, USA) were used to analyze the data. The comparison test and correlation coefficient were determined to analyze the correlation between the results of the two methods. $P < 0.05$ was considered statistically significant.

Results

Working ranges and standard curves

The calibrators of dual-label TRFIA for MP IgM and IgG ranged from 2 to 5500 BU/mL and from 0.5 to 1500 BU/mL, respectively. The linear ranges were 2–5500 BU/mL for IgM and 1.5–1500 BU/mL for IgG without the high-dose hook effect (Fig. 2). The R^2 of the standard curve was 0.935 for IgM and 0.949 for IgG. The effective dose (ED)20, ED50, and ED80 for IgM were 7.99, 58.51, and 473.75 BU/mL, respectively, whereas those for IgG were 3.8, 19.24, and 97.75 BU/mL, respectively.

Precision

Three aliquots of serum samples in low, middle, and high concentrations were collected and determined as quality controls for MP IgM and IgG. As shown in Table 1, the intra- and inter-coefficients of variation (CVs) were 4.5–5.64% and 7.39–9.02% for IgM, and 3.57–6.84% and 8.54–9.00% for IgG, respectively. The average of intra- and inter-CV was 5.14% and 8.41% for IgM, and 5.44% and 8.76% for IgG, respectively.

Reference range

Serum specimens were collected from 50 healthy individuals, including 25 men and 25 women, aged 1–65 years, and MP was detected by established TRFIA method. The mean value of MP IgM and IgG was 7.67 ± 4.16 BU/mL and 7.58 ± 4.12 BU/mL, respectively. Therefore, the reference range for MP IgM and IgG was 0–18.4 BU/mL and 0–18.2 BU/mL, respectively, with 99% confidence interval.

Assay sensitivity

The sensitivity of the method defined as the minimum level of determination was 0.5 BU/mL for MP IgM and 0.2 BU/mL for MP IgG, calculated from the mean fluorescence of 20 blank samples ± SD.

Recovery rate

Three clinical serum specimens with known concentration were used in this recovery study. They were diluted to 1:100–800 and then subjected to dual-label TRFIA for detection of MP IgG and IgM. The recovery rate of the assay was calculated by dividing the observed content by the expected content as shown in Table 2, and was 94.9–106.8% for MP IgM and 96.1–109.4% for MP IgG.

Correlation with ELISA

Thirty-eight serum samples were determined by established TRFIA and commercial ELISA. The coefficient of correlation was calculated from the results. The linear equation for MP IgM was $y = 0.60501x + 11.143$, with the correlation rate of 0.9294, whereas that for MP IgG was $y = 0.56203x + 9.3986$, with the correlation rate of 0.9366. The results of dual-label assay were comparative to those of commercial kits as shown in Fig. 3.

Coincidence rate with passive particle agglutination

Thirty serum samples were analyzed by established TRFIA and passive particle agglutination. As shown in Table 3, the positive rate of passive particle agglutination was 80.0% and the negative rate was 20.0%. The positive rate of MP IgG and IgM for TRFIA was 46.7% and 73.3%, and the negative rate was 53.3% and 26.7%, respectively. The coincidence rate was 93.3%.

Comparison of working range

To determine the working range of this method, an aliquot of highly positive serum for MP IgM and IgG was diluted from 5520 to 0.55 BU/mL and from 1500 to 0.55 BU/mL, respectively. The series of diluted samples were confirmed by dual-label TRFIA and commercial ELISA simultaneously, the results of which are plotted in Fig. 4. The fluorescent value of MP-IgM TRFIA, ranging linearly from 7570 to 1299316, was 171.6 times higher than that of ELISA from 0.728 to 2.475.

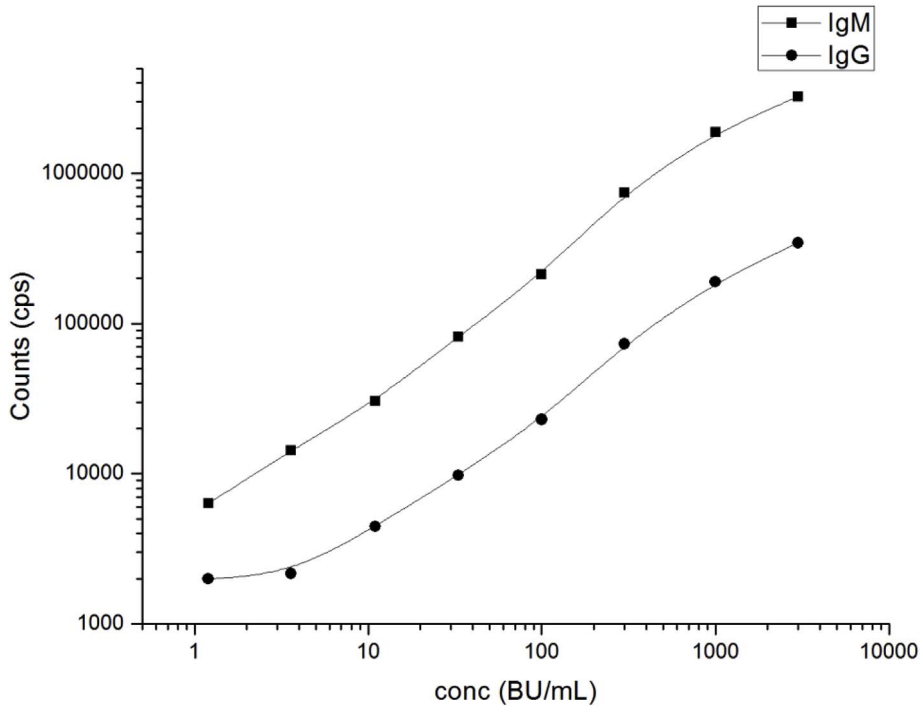

Fig. 2. Standard curves of dual-label TRFIA for MP IgM and IgG.

Table 1
Precision of dual-label TRFIA for MP IgM and IgG in serum controls.

Sample	MP IgM (BU/mL)		MP IgG (BU/mL)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
Low				
Intrabatch (n = 25)	11.53 ± 0.65	5.64	8.92 ± 0.61	6.84
Interbatch (n = 10)	11.37 ± 0.84	7.39	8.33 ± 0.75	9.00
Middle				
Intrabatch (n = 25)	49.52 ± 2.23	4.50	52.68 ± 1.88	3.57
Interbatch (n = 10)	52.82 ± 4.66	8.82	56.27 ± 4.92	8.74
High				
Intrabatch (n = 25)	95.68 ± 5.04	5.27	89.63 ± 5.29	5.90
Interbatch (n = 10)	90.80 ± 8.19	9.02	92.11 ± 7.87	8.54

Correspondingly, the linear working ranges of MP IgM were found to be 2.07–5520 BU/mL and 6.21–100 BU/mL by TRFIA and ELISA, respectively. Therefore, the detectable range of TRFIA was 50-times greater than that of ELISA. For MP IgG detection, the optical density (OD) of ELISA was noted to be three times difference between 0.759 and 2.280 and the fluorescence counts of TRFIA were 36.4 times difference from 1634 to 59433. Moreover, a good linearity range was revealed from the dilution curve from 1.65 to 100 BU/mL by commercial ELISA and from 0.55 to 1500 BU/mL by established TRFIA. The minimum level of ELISA was three times higher than that of TRFIA, and the maximum level of ELISA was 1/15th lower than that of TRFIA. This indicated that the working range of ELISA was limited for MP IgG detection. Moreover, the detectable range of MP IgM and IgG by the developed TRFIA was more than ten-times that of ELISA.

Specification and positive rate

Fifty cases with no respiratory infection were used for estimation of the levels of MP IgG and IgM in serum by dual-label TRFIA. According to the cut-off value, 2 cases were false-positive and the remaining 48 cases were true-negative, so the clinical specificity of the method was 96%. One hundred patients with pneumonia and 100 with URI were confirmed by clinical diagnosis and enrolled. Their serum samples were collected and subjected to established dual-label TRFIA for detection of MP IgM and IgG. In the pneumonia group, the positive rate of MP IgG

Table 2
Recoveries of dual-label TRFIA for MP IgG and IgM.

		MP IgM			MP IgG		
Sample	Dilution	Observed (BU/mL)	Expected (BU/mL)	O/E (%)	Observed (BU/mL)	Expected (BU/mL)	O/E (%)
1	1:100	78.8			83.8		
	1:200	40.4	39.4	102.5	42.5	41.9	101.4
	1:400	18.7	19.7	94.9	20.5	20.95	97.8
	1:800	9.6	9.85	97.5	10.5	10.48	100.2
2	1:100	51.7			36.8		
	1:200	25.2	25.85	97.5	17.8	18.4	96.7
	1:400	13.8	12.925	106.8	9.83	9.2	106.8
	1:800	6.8	6.465	105.2	4.42	4.6	96.1
3	1:100	37.8			47.6		
	1:200	19.4	18.9	102.6	23.2	23.8	97.5
	1:400	9.2	9.45	97.4	12.5	11.9	105
	1:800	4.85	4.73	102.5	6.51	5.95	109.4

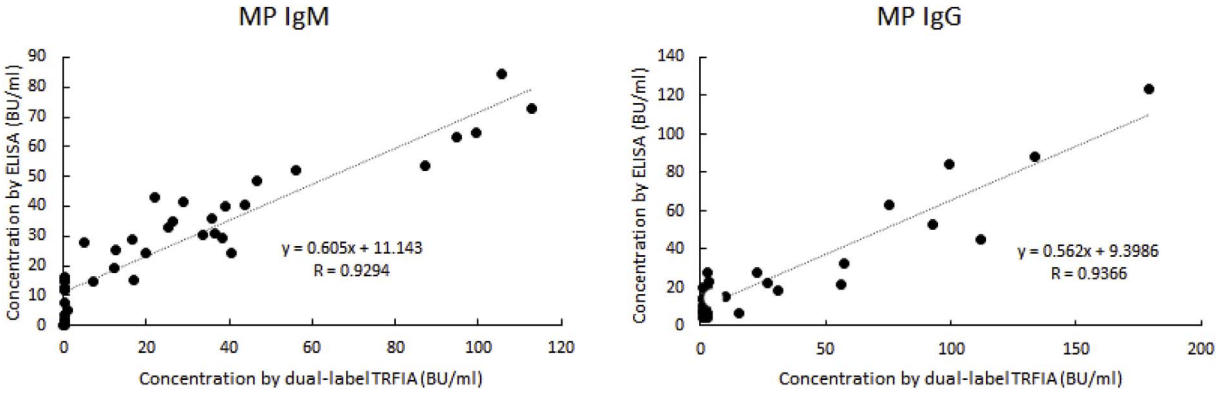

Fig. 3. Correlation between dual-label TRFIA and ELISA for MP IgM and IgG in 38 serum samples.

Table 3
Concordance between TRFIA and passive particle agglutination.

	Passive Particle Agglutination	TRFIA	
		MP IgG	MP IgM
Positive rate (%)	80.0	46.7	73.3
Negative rate (%)	20.0	53.3	26.7
Concordance rate (%)	93.3		

and IgM was 22% and 53%, respectively. In patients with URI, the positive rate of MP IgG and IgM was 9% and 67%, respectively.

Stability

The stability test was performed using established TRFIA and ELISA kits by keeping them at 37 °C for 7 days. The performance of stressed and normal kits were compared simultaneously. Using these kits, 10 serum specimens with high concentration of MP IgG and IgM were analyzed. In case of the kits stored under normal conditions, the signals of TRFIA and ELISA for MP IgM decreased by 7.2% and 16.6%, whereas those for MP IgG decreased by 9.4% and 15.1%, respectively. After storing at 37 °C for 1 day, the kit could be reserved at 2–8 °C for 2 months. According to the good properties, the dual-label kits could remain stable at 2–8 °C for more than 1 year. Furthermore, a slight decrease in fluorescence signal of the plates was observed after overnight placement without discarding the enhancement solution. Therefore, the dual-label TRFIA for MP IgM and IgG was stable and reliable for long-term storage.

Discussion

TRFIA method using rare earth ions as tracers is one of the most sensitive methods. It has significant advantages of high sensitivity, wide detection range, easy performance and no radioactive pollution. [19–21] Furthermore, it particularly provides dual-label analytical method detecting two analytes in a single test. Since its unique properties of lanthanide chelates, TRFIA provides multiple labelling technique with Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+}. Among them, Eu and Sm were mostly used in pairs, because of same excitation spectrum and same enhancement solution for signal amplification. Moreover, they are different in emission wavelengths with 30 nm interval and fluorescence lifetime, which of Eu is 730 us and of Sm is 50 us. Dual-label TRFIA with Eu and Sm had been developed for analysis different compounds and the corresponding instruments are commercially available. [15–17,22,23]

Previously, using ELISA detection, a large number of cases were found near the threshold or were shown to be weakly positive. In these cases, the poor accuracy and repeatability also affected diagnosis of the infection status. According to the results of our comparison study, this may be because ELISA provided a limited linear range for both MP IgM and IgG detection. However, these limitations could be overcome by the developed TRFIA, which has higher sensitivity of < 1 BU/mL, a much lower cut-off value of 18 BU/mL, and a much wider working range. TRFIA used for detection of MP IgG and IgM in serum samples also showed good sensitivity and high specificity. Importantly, the good correlation (> 0.9) between the dual-label TRFIA and commercial ELISA meant that there was no significant difference between the two assays in addition to there being high consistency. In our study, the positive coincidence rate of 93.3% between TRFIA and passive particle agglutination methods proved its high reliability. However, for the

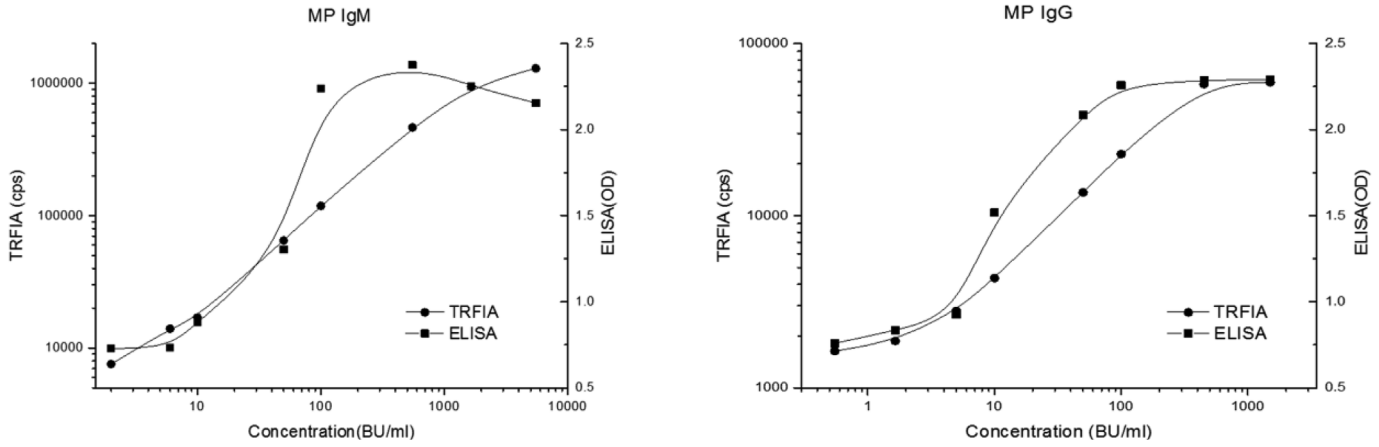

Fig. 4. The linear range of ELISA and TRFIA.

further use, kit stability was one of key factors that we were concerned about. The results of proposed kits showed good long-term storage and less thermal sensitivity with a decline in fluorescence of less than 10%, which was lower than that of ELISA. In order to compare the signal stability in the last reaction, ELISA strips must be assayed using a colorimeter within half an hour, while TRFIA could be stored effectively for 1 day. Considering the better thermal and signal stability, TRFIA shows superiority and application value.

Using lanthanide atoms with powerful fluorescence, instead of radioactive isotopes, as tracers, this newly developed immunoassay does not cause radioactive pollution, and has improved sensitivity and detection range. The novel method was also time-saving and could complete assay procedures within 2–3 h. The commercial MP kits separate the procedure of IgG and IgM measurement that is one test one result. In this study, dual-label TRFIA could simultaneously analyze both MP IgG and IgM, thus improving the measurement accuracy and even reducing the clinical workload and cost. It is a helpful tool for the early diagnosis and effective treatment of MP infection.

Author contributions statement

Z.G. Hu. and J. Liu. conceived the study and designed the experiments.Y.Zhang., X. Yang. and J. Qian. conducted the experiments and wrote the manuscript. X.H. Gu. and J. Zhang. analyzed the results.

Competing financial interests

Yi Zhang, Xue Yang, Jun Qian, Xiaohong Gu, Jue Zhang, Jie Liu, and Zhigang Hu declare no competing financial interests.

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