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Development of a fluorescence immunochromatography method for quantitative measurement of matrix metalloproteinase-9

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ABSTRACT

Objective: Abnormal serum matrix metalloproteinase-9 (MMP-9) levels are closely related to the occurrence and development of many diseases. This study aimed to establish a fluorescence immunochromatography (FIC) method using the lanthanide fluorescent element europium(III) (Eu^{3+}) for the quantitative measurement of MMP-9 in serum.
Design & Methods: The FIC method for quantifying MMP-9 was optimized and established, and the FIC test strips (FICTS) were assembled and subsequently evaluated for sensitivity, specificity and precision. Furthermore, the reference interval and clinical sensitivity/specificity were estimated using clinical healthy/positive serum samples, and a commercial ELISA was used for comparison.
Results: We successfully established an FIC method and prepared FICTS. The analytical sensitivity of the FICTS was 0.92 ng/mL, with a linearity range of 0–1000 ng/mL. The cross-reactivity of the 7 common serum interferents was less than 1.56%. All recoveries of the intra-array and inter-array samples ranged from 102.50% to 110.99%, and all *CVs* were less than 5%. The reference interval of the FICTS was >161.15 ng/mL. The clinical sensitivity was 96.00%, and the specificity was 97.5%. The results of 270 clinical serum samples were highly coincident with the clinical diagnostic results. Pearson correlation analysis and Bland–Altman plots indicated that the FICTS and commercial ELISA results were consistent with the quantitative MMP-9 concentration.
Conclusions: The designed FIC method and test strips may be suitable for point-of-care quantitative measurement of MMP-9, which provides a new method for screening for atherosclerosis, xerophthalmia, etc.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are involved in the degradation of various proteins in the extracellular matrix (ECM) [1]. MMPs play a role in tissue remodeling (wound repair, morphogenesis, and

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angiogenesis), pathological conditions (myocardial infarction, fibrotic disorders, atherosclerosis and cancer), and most tissue inflammation [2,3]. MMPs have been proposed as biomarkers for numerous pathological conditions. As one of the most widely investigated MMPs, MMP-9 is a significant protease that is elevated in multiple pathological conditions [4,5]. A large number of studies have shown that the expression level of MMP-9 increases in atherosclerotic lesions, and the serum MMP-9 concentration is consistently associated with the expression of markers of carotid atherosclerosis and lesion vulnerability, including lipid profiles, inflammatory markers, and bone turnover markers [6,7]. Additionally, numerous studies have confirmed that excessive MMP-9 expression can enhance extracellular matrix degradation and promote plaque instability, and several human studies have demonstrated that MMP-9 may be a predictor of atherosclerotic plaque instability [8,9]. Additionally, MMP-9 is involved in the pathology of numerous inflammatory retinal degenerations, including xerophthalmia, meibomian gland dysfunction, and seasonal allergic conjunctivitis [10–12]. Therefore, the quantitative detection of serum MMP-9 has significant clinical value for the treatment of various inflammatory diseases. Unfortunately, there are few methods for detecting MMP-9.

Recently, immunochromatography test strips (ICTS) have been fully developed for point-of-care testing (POCT) [13,14]. However, the intrinsic limitations of these methods, including the nonquantitative detection of colloidal gold ICTS and low sensitivity of fluorescence ICTS (FICTS), significantly restrict their further application in clinical diagnosis. Taking advantage of rapid colorimetric qualitative detection and fluorescence quantitation, we designed a sensitive FICTS based on the addition of the lanthanide fluorescent element europium(III) (Eu^{3+}) to the sample well after reacting with the sample in advance. Furthermore, we evaluated the sensitivity, specificity and precision of the MABT using the MMP-9 antigen and evaluated the reference interval, clinical sensitivity/specificity, and comparison between healthy and seropositive serum samples. Our ultimate goal was to develop a new method for quantifying MMP-9 levels to monitor physical condition.

2. Materials and methods

2.1. Antigens, antibodies and samples

The recombinant antigen of MMP-9, its labeled monoclonal antibody (mAb) and the detection mAb were obtained from Youdi Biotechnology (Guangzhou, China). Eu^{3+}-fluorescent microspheres (particle size 210 nm) were purchased from Microdetection (MD018, China). Polyvinyl chloride (PVC) sole plates, nitrocellulose (NC) membranes, absorbent paper, sample pads and binding pads were obtained from Sartorius. Dinitrophenol (DNP) antigen (DNP-BSA) (EDD0405A) and its mAb (EKY0817A) were obtained from Seebio (Shanghai, China). A total of 270 clinical serum samples from normal subjects (120 subjects, 32.5 ± 17.4 years old), stable plaque subjects (40 subjects, 36.2 ± 18.9 years old), unstable plaque subjects (60 subjects, 37.7 ± 19.6 years old), and xerophthalmia subjects (50 subjects, 42.7 ± 17.5 years old) were obtained from the Beihua University Affiliated Hospital and stored at −80 °C. These patients were clinically confirmed. The Institutional Review Board of Beihua University Affiliated Hospital approved this study, and all the subjects provided written informed consent.

2.2. Conjugation of the labeled mAb to fluorescent microspheres

The Eu^{3+} fluorescent microspheres were activated by the classical 1-(3-dimethylaminyl)-3-ethylenediamine hydrochloride/N-hydroxysuccinimide (EDC)/NHS (Sigma, USA) method and then coupled with the label mAb of MMP-9. After washing, 14 μL of 1% EDC and 132 μL of 1% NHS were added to 1 mL of the Eu3+ fluorescent microspheres, after which the mixture was shaken for 30 min at room temperature. After centrifugation, 1 mg of labeled mAb was added to the activated fluorescent microspheres, which were gently shaken for 2 h at room temperature. Then, the fluorescent microspheres were washed and blocked (5% BSA, 1 h), resuspended in buffer (25 mmol/L Tris-base, 0.05% Tween 20, 1% BSA, pH 7.5), and stored in the dark at 4 °C. The conjugation of DNP-BSA to the fluorescent microspheres was similar to the above steps.

2.3. Coating of the NC membrane

The NC membrane has a detection line (T line) and a quality control line (C line), and the detection mAb and DNP mAb were coated on the T line and C line, respectively. The coating operation was performed using the XYZ3060 3D spraying platform with the following spraying speed parameters: 0.8 μL/cm, detection mAb: 2 mg/mL and DNP mAb: 1.5 mg/mL. After coating, the NC membranes were dried in a 37 °C air drying oven for 2 h and then sealed and stored in a dry environment.

2.4. Pretreatment of conjugate pads and sample pads

Conjugate pad pretreatment: The conjugate pads were soaked in pretreatment buffer (60 mmol/L $Na_2HPO_412H_2O$, l% Triton X-100, 0.5% PVA, 0.8% BSA, pH 7.4) for 2 h. After draining, the pads were placed in a 37 °C air drying oven for 3 h and stored in a dry environment. Sample pad pretreatment: The sample pads were soaked in another pretreatment buffer (10 mmol/L sodium tetraboric acid, 1% Triton-X100, 1% polyvinyl pyrrolidone (PVP), 0.2% sodium casein, 0.05% ProClin 300, pH 7.8) for 0.5 h. After draining, the pads were placed in a 37 °C air drying oven for 3 h and stored in a dry environment.

2.5. Preparation of the FICTS

The pretreated sample pad, pretreated bonding pad, coated NC membrane, and absorbent paper were sequentially fixed on the PVC board. After fixation, the PVC board was cut into 3 mm wide strips and loaded into a plastic cartridge, after which the preparation of the FICTS was completed. Finally, the strips were sealed, dried, and stored in the dark.

2.6. Test procedure

The reaction mode used for the FICTS was sandwich, and the test procedure was as follows: 50 μL of serum sample, 10 μL of MMP-9 mAb-labeled fluorescent microspheres and 10 μL of DNP-BSA-labeled fluorescent microspheres were mixed thoroughly and incubated for 5 min. The solution was subsequently added to the sample well. After 15 min at room temperature, the strips were placed in a fluorescence detector (Microdetection, MD-200, China). F_T (T line fluorescence intensity), F_c (C line fluorescence intensity) and their R (F_T/F_c) ratios were recorded, and the serum MMP-9 level was analyzed using a built-in standard curve. A schematic of the method used for quantifying MMP-9 is shown in Fig. 1.

2.7. Standard curve and sensitivity assay

Standard curves for the FICTS were created using serial standard dilutions of MMP-9 (0 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, 500 ng/mL, and 1000 ng/mL diluted with normal control serum). The MMP-9 concentrations were plotted on the X-axis, and the corresponding R (F_T/F_c) was plotted on the Y-axis. A linear fit was performed, and the standard curve was drawn. The sensitivity was obtained by substituting the mean value of 20 tests of blank samples plus 2 times the standard deviation into the fitting equation (mean + 2*SD) [15].

2.8. Specificity assay

For the specificity tests, several common serum interferents, including MMP-3, high-sensitivity C-reactive protein (hs-CRP), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), hemoglobin, bilirubin, and cholesterol, were added to the normal control serum at high concentrations and measured using the FICTS. The detection procedure was repeated three times for each interferent. The specificity of the FICTS is reflected by the cross-reactivity. The lower the cross-reactivity is, the stronger the specificity of the FICTS. Crossreactivity (%) = (determined concentration)/original concentration × 100% [16].

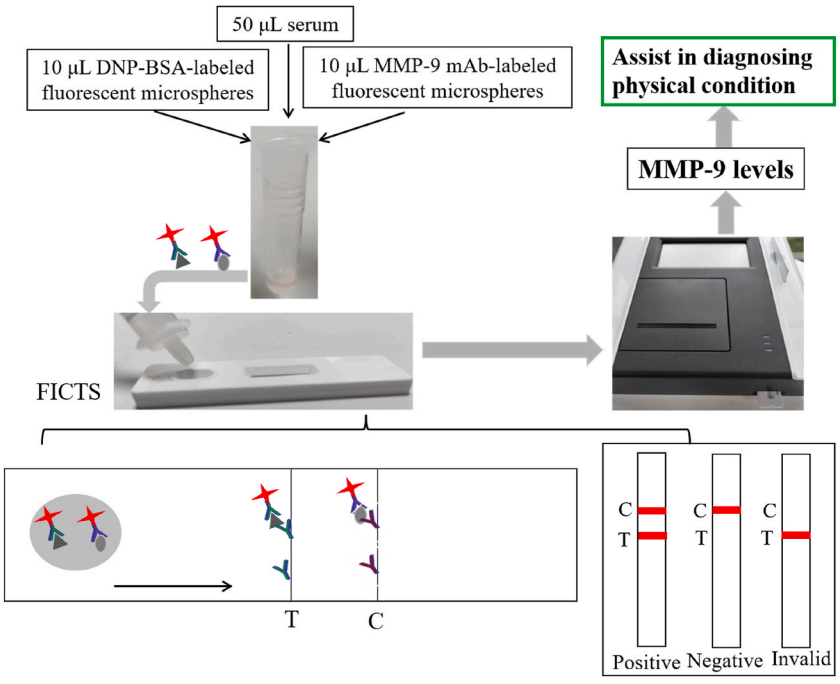

Fig. 1. Schematic of the quantitative measurement of MMP-9 levels by the FICTS.

2.9. Precision

The precision of the FICTS was assessed by measuring the recoveries and CVs. The known concentrations of the MMP-9 antigen (50 ng/mL, 200 ng/mL and 800 ng/mL) were added to the normal control serum, and the mean and SD of the MMP-9 concentration were calculated through six independent experiments. Recovery (%) = (determined concentration-basal concentration)/spiked concentration × 100. CV (%) = SD/mean × 100 [17].

2.10. Reference intervals

A total of 120 healthy subjects were used for reference interval determination. The FICTS was used to detect the serum MMP-9 concentration, and the numerical values were subjected to a normality test using SPSS 22.0. The one-sided upper limit of the 95% reference interval range was applied to identify the reference intervals. The one-sided upper limit was calculated using the following formula: mean + 1.*64 SD* [17].

2.11. Clinical sensitivity/specificity

A total of 270 clinical serum samples from healthy individuals (120 subjects), stable plaque individuals (40 subjects), unstable plaque individuals (60 subjects), and xerophthalmia individuals (50 subjects) were used for clinical sensitivity/specificity evaluation. The FICTS was used to determine the serum MMP-9 concentration, and the normality of the samples was subsequently determined based on the reference interval. The clinical sensitivity/specificity were calculated for the clinical diagnostic results. Sensitivity = true positive/(true positive + false-negative) × 100%, specificity = true negative/(true negative + false-positive) × 100%. The laboratory technicians who measured MMP-9 levels were blinded to the clinical outcomes of the patients.

2.12. Comparison with the commercial ELISA kit

Moreover, 270 serum MMP-9 levels were also measured with a commercially available ELISA kit (R&D Systems, Minneapolis, MN). The level values obtained from the two methods were compared using Pearson correlation analysis and Bland–Altman plots. GraphPad Prism 5 software was used for Pearson correlation analysis and Bland–Altman plots.

2.13. Statistical analysis

The data were statistically analyzed using SPSS 22.0 and graphed using GraphPad Prism 5 (GraphPad Software, USA). All the results are presented as the means ± *SDs*.

3. Results

3.1. TRFIA standard curve

The standard curve of MMP-9 is presented in Fig. 2. The curve equation is y = 0.0372x + 1.9042 (R^2 = 0.9902). Over wide concentration ranges (0–1000 ng/mL), the curve exhibited a well-defined linear relationship. The sensitivity was 0.92 ng/mL.

3.2. Specificity results

The FICTS was used to detect 7 high-concentration interferents (MMP-3, hs-CRP, IL-6, TNF-α, hemoglobin, bilirubin and cholesterol) that were added to the control serum, and the results are shown in Table 1. There was very low cross-reactivity (all no more than

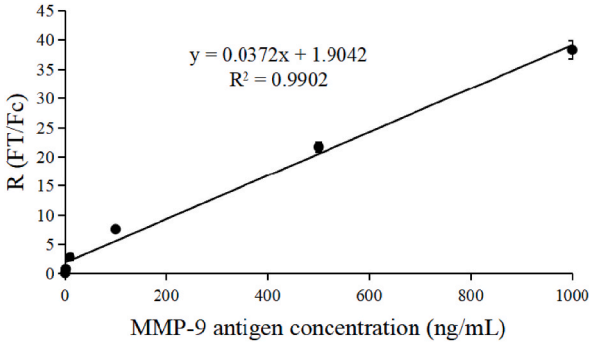

Fig. 2. The standard curve of the FICTS for quantifying MMP-9. R=F_T/F_C.

Table 1
Specificity of the FICTS.

Interferents	Original concentrations (ng/mL)	Determined concentrations (ng/mL)	Cross reactivity (%)
MMP-3	500	7.80 ± 0.91	1.56
hs-CRP	500	5.23 ± 0.40	1.05
IL-6	500	2.83 ± 0.56	0.57
TNF-α	500	6.10 ± 0.70	1.22
Hemoglobin	500	5.70 ± 0.86	1.14
Bilirubin	500	5.00 ± 0.78	1.00
Cholesterol	500	7.46 ± 0.35	1.49

1.56%), indicating that the FICTS had high specificity for quantifying MMP-9, and the common interferents in the serum did not affect the quantitative results of MMP-9.

3.3. Precision results

The precision of the FICTS was assessed by detecting the control serum that had been spiked with the MMP-9 antigen (50, 200 and 800 ng/mL). As shown in Table 2, all average recoveries of the intra- and interarray samples ranged from 102.50% to 110.99%, and all *CVs* were less than 5%. The precision of the FICTS was high, and the FICTS met the requirements of clinical immunoassay kits.

3.4. Reference interval

A total of 120 serum samples from healthy subjects were analyzed by the FICTS, and the concentrations of the MMP-9 antigen were determined. A normality test using SPSS 22.0 confirmed that 120 values were normally distributed, and the one-sided upper limit was 161.15 ng/mL (calculated using the mean + 1.64*SD*). This means that the reference interval of the FICTS is > 161.15 ng/mL.

3.5. Clinical sensitivity/specificity

A total of 270 clinical serum samples, including 120 healthy serum samples and 150 positive serum samples, were analyzed by the FICTS, after which the negative/positive results were judged in accordance with the reference interval. The numbers of true positives, false-negatives, true negatives and false-positives were counted, and the clinical sensitivity and specificity were subsequently calculated. The clinical sensitivity was 96.00%, and the specificity was 97.5%. These results showed that these FICTS can be used for detecting high levels of abnormal MMP-9, which indicates the occurrence and development of some diseases.

3.6. Comparison with the commercial ELISA kit

The comparison results are shown in Fig. 3. For Pearson correlation analysis, the Pearson r was 0.9782. For Bland–Altman plots, the 95% limits of agreement ranged from −32.32 ng/mL to 21.01 ng/mL, 12/270 of the points were outside this limit, and the mean of the two methods was −5.65 ng/mL. Pearson correlation analysis and Bland–Altman plots indicated that the FICTS and commercial ELISA results were consistent with the quantitative MMP-9 concentration.

4. Discussion

MMPs play a considerable role in atherosclerosis and inflammatory retinal degeneration. MMP-9 is positively correlated with common carotid artery intimal media thickness both in stroke patients and asymptomatic subjects [18]. A study of 3186 participants (767 participants (24.6%) who experienced major disability or died) showed that the addition of serum MMP-9 to conventional risk factor data improved the ability to predict the combined outcome of death or major disability [19]. A study of 558 acute ischemic stroke patients indicated that adding MMP-9 to the conventional risk factor model substantially improved the discrimination and reclassification of poststroke depression, revealing that the detection of serum MMP-9 has an important prognostic role in poststroke depression [20]. Research has shown that an increase in the MMP-9 level is responsible for ocular surface inflammation and dry eye,

Table 2
Precision results of the FICTS.

	Spiked concentration	mean ± SD	Recovery (%)	*CV* (%)
Intra-array (n = 6)	50	139.17 ± 6.48	107.19	4.66
	200	297.8 ± 9.61	106.12	3.23
	800	905.6 ± 13.11	102.50	1.45
Interarray (n = 6)	50	141.07 ± 6.84	110.99	4.85
	200	306.13 ± 14.11	110.28	4.61
	800	908.73 ± 17.54	102.89	1.93

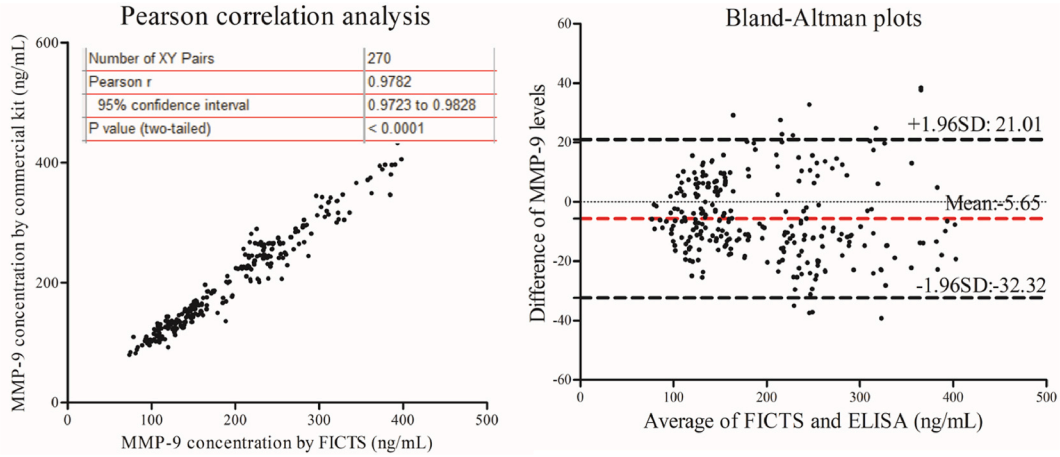

Fig. 3. Pearson correlation analysis and Bland–Altman plots of MMP-9 levels measured by the FICTS and ELISA.

which is a reliable quantitative index of ocular surface inflammation and tends to increase proportionally with the severity of dry eye [21,22]. An increase in the MMP-9 level on the ocular surface in turn escalates the production of other inflammatory cytokines and mediators, exacerbating the chronic inflammatory cycle [23]. Therefore, the detection of serum MMP-9 is highly valuable for monitoring the occurrence and development of atherosclerosis and inflammatory retinal degeneration.

In clinical practice, the main detection method for detecting serum MMPs is the enzyme-linked immunosorbent assay (ELISA) [24,25]. Currently, the most commonly used detection method for detecting serum MMP-9 is ELISA [19,20], in addition to the time-resolved fluorescence immunoassay (TRFIA) we have established [26]. Considering the time-consuming nature and low specificity of ELISA, FIC has recently been developed to make testing more convenient and efficient [13]. In this study, we attempted to establish a rapid, sensitive FIC method for quantifying MMP-9 and determining the FICTS. The prepared FICTS showed high sensitivity (0.92 ng/mL) and high specificity (all cross-reactivity values were less than 1.56%). All average recoveries of the intraarray and interarray ranged from 102.50% to 110.99%, and all CVs were less than 5%. For clinical sample validation, we selected 120 healthy serum samples and 150 positive serum samples, and the MMP-9 test results were highly consistent with the clinical diagnostic results, with 96.00% clinical sensitivity and 97.5% specificity. Compared with the commercial ELISA kit used in Section 2.12 (comparison with the commercial ELISA kit) (test time: 3.5 h, sensitivity: 0.156 ng/mL, linear range 0–20 ng/mL, intra-assay precision: 2–2.9%, interassay precision: 6.9–7.9%, recovery: 91–99%), the FICTS exhibited comparable or superior detection performance. Additionally, the test results of 120 normal individuals inferred a preliminary reference interval; that is, when MMP-9 levels >161.15 ng/mL, the subjects may suffer some disease combined with clinical symptoms, which will help clarify the patient's physical condition. Additionally, compared with the sensitivity, specificity, and precision of the reported TRFIA method (sensitivity of 0.85 mg/mL, cross-reactivity of less than 2.65%, recovery of 94.58%–109.82%, and CV of less than 5.46%) [26], the FICTS exhibited comparable or superior detection performance with the additional advantage of detection time (20 min).

In conclusion, a rapid, sensitive FIC method based on the lanthanide fluorescent element Eu^{3+} was first established and validated for the quantitative detection of MMP-9 in serum. The prepared FICTS has high sensitivity, specificity, precision, and high clinical sensitivity/specificity. Based on the physiological effects of MMP-9 and its relationship with various diseases in the body, we speculate that the detection of MMP-9 levels combined with clinical symptoms can be used for screening various diseases; therefore, the FICTS may provide an efficient and useful technique for the rapid quantitative detection of MMP-9 to monitor physical conditions.

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CRediT authorship contribution statement

Jingyan Huang: Writing – original draft, Validation, Project administration. **Cuicui Chen:** Writing – original draft, Methodology. **Huankun Liang:** Validation, Supervision, Software. **Wenqi Dong:** Writing – review & editing, Data curation. **Laiqing Li:** Conceptualization. **Hongyan Ma:** Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analysed during this study are included in this published article.

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