Determination of Anticyclic Citrullinated Peptide Based on Biotin–Streptavidin-Amplified Time-Resolved Fluoroimmunoassay

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Background: A rapid and sensitive time-resolved fluoroimmunoassay (TRFIA) based on the biotin–streptavidin amplification system was developed for the determination of anticyclic citrullinated peptide (anti-CCP). **Methods:** Europium-labeled streptavidin derivatives combined with europium and anhydride of diethylene triamine pentaacetic acid were used to label streptavidin, biotin was coupled with rabbit anti-human IgG to form a biotin–anti-human IgG bridge between streptavidin–europium and the anti-CCP antibody in the immunoassay. The anti-CCP assay was carried out by measuring the fluorescence of Eu³⁺–streptavidin at 615 nm. **Results:** The presented method produced a wide linear

range from 0.58 to 9,463 U/ml, while it was only 591.4–18.48 U/ml when using an ELISA kit, and featured a detection limit up to 0.5 U/ml for anti-CCP. The values determined by the biotin–streptavidin–TRFIA and ELISA correlated well ($R^2 = 0.8927$). The method was applied to determine anti-CCP in serum samples with satisfied recoveries of 96.45–104.63%. **Conclusion:** The assay results obtained by the present method showed that biotin–streptavidin-amplified TRFIA improve the traditional ELISA kit for anti-CCP detection. Therefore, it offers a better alternative immunoassay in rheumatoid arthritis management. J. Clin. Lab. Anal. 29:474–479, 2015. © 2014 Wiley Periodicals, Inc.

Key words: cyclic citrullinated peptide; rheumatoid arthritis; time-resolved fluoroimmunoassay

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease, with a prevalence of 0.3–1% worldwide, which leads to progressive joint erosion and substantial disability if not treated early. A reliable and specific test for a marker present early in the disease would be useful for identifying RA patients prior to the occurrence of joint damage (1).

Antibodies to citrullinated proteins (ACPA) are specific serological markers for RA (2). ACPA constitute a growing family of autoantibodies, and the major breakthrough came with the realization of the crucial role of peptide epitopes, which were citrullinated posttranslationally, and the subsequent development of the anticyclic citrullinated peptide antibodies (anti-CCP) test (3). The anti-CCP an-

tibodies can be detected in up to 80% of patients with RA, which is highly specific for the disease, and may be of value for both the diagnosis and prognosis of RA, particularly implicated in more erosive disease course (4).

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Time-resolved fluoroimmunoassay (TRFIA), a nonradioimmunoassay, offers better sensitivity than other traditional FIA methods due to the unique fluorescence properties of lanthanide chelates and the time-resolved measurement mode, which allows the specific fluorescence to be measured after the background fluorescence has already declined (5). Bioanalytical assays based on TRFIA have made a substantial impact on routine and research areas (6).

Up to now, the use of Eu^{3+} -labeled streptavidin (Eu^{3+} -streptavidin) and time-resolved fluorometry has been well suited as an indirect label for its relatively high affinity to biotin and low nonspecific binding properties. As a tetrameric structure with four binding sites for biotin, it can be used for amplifying signal (7). Moreover, a universal detection reagent, europium chelate, as a fluorescent marker has attracted much attention for high-throughput screening of large populations of biologically active samples (8,9).

In this study, the europium chelate of Eu^{3+} -streptavidin was prepared and used as a signal generation reagent after its binding to the biotin moieties of the surface immunocomplex. A novel, rapid, biotin-streptavidin-amplified TRFIA for the determination of anti-CCP was developed and is described here. This method was applied to determine anti-CCP in human serum samples with satisfactory results.

MATERIALS AND METHODS

Chemicals and Instrumentation

Auto DELFIA1235, Eu^{3+} -labeled reagent, and Eu^{3+} -labeled enhancement solution were purchased from Wallac Oy (Turku, Finland). The 96-well polystyrene microtiter plates and recombinant CCP antigen were obtained from AESKU (Dresden, Germany). Monoclonal rabbit anti-human IgM was purchased from Sigma (St. Louis, MO). N2-[*p*-isocyanate-benzyl]diethylenetriamine tetraacetic acid (DTTA) was purchased from Sigma Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Institute of Biological Products Ministry of Health (Shanghai, China). Sephadex G25 was purchased from Pharmacia (Piscataway, NJ). Concentrated wash buffer and enhancement solution were provided by Institute of Nuclear Medicine (Jiangsu, China). Other reagents used were of analytical reagent grade.

ELISA Kit and Reference Standard of the Anti-CCP Preparation

Commercial ELISA kits of the anti-CCP IgG were obtained from AESKU, Dr. Fenning (Kirchzarten, Germany), and ORGENTEC (Mainz, Germany), respectively.

Reference standard of the anti-CCP was obtained from AESKU.

Preparation of Solid Antigen

Each well of polystyrene microtiter plates was coated with 100 μl of recombinant CCP antigen diluted with coating buffer (pH 9.6, 50 mmol/l Na_2CO_3 - NaHCO_3) by incubating at 4°C overnight. The coating buffer was discarded. After three times wash, some binding sites not occupied by the coating buffer were then blocked by 250 μl 3 g/l BSA (Institute of Biological Products Department of Health, Shanghai, China) in phosphate buffer solution (PBS; pH 7.4) and the plates were incubated at 4°C overnight. After the blocking buffer was discarded, the plates were vacuum-dried and stored at -20°C until analysis.

Biotin Labeling of Anti-Human IgG

One milligram of *N*-hydroxysuccinimidobiotin (Promega, Madison, Wisconsin) was added to 0.5 mg of a 1 mg/ml solution of rabbit anti-human IgG (St. Louis, MO) in pH 7.2, 0.05 M PBS. The reaction mixture was incubated at 37°C for 1 h and then dialyzed overnight at 4°C with PBS. The biotin-labeled antibody was stored at -20 °C until use.

Eu^{3+} -Labeled Streptavidin Preparation

Eu^{3+} labeling of streptavidin (Sigma) was performed using a Eu^{3+} -labeling Kit (EG&G-Wallac, Turku, Finland) strictly according to the manufacture's instructions. One milligram of streptavidin was loaded on a PD10 column and eluted using carbonate buffer (pH 8.5, 50 mmol/l Na_2CO_3 - NaHCO_3) containing 0.155 mol/l NaCl. Then streptavidin was mixed with 0.2 mg Eu^{3+} -DTPAA (diethylenetriamine pentaacetic acid; Sigma Aldrich) followed by vigorous stirring at 30°C for 20 h. The resulting mixture was then fractionated on a Sepharose CL-6B (1 × 40 cm; Pharmacia) with pH 7.8, 80 mmol/l Tris-HCl buffer. The absorbance values of the elution were measured at 280 nm to obtain protein concentrations. After purification, equal amount of AR glycerin was added before subpackaging and stored at -20°C until use.

Anti-CCP Antibody Assay

The procedures for BAS-TRFIA of serum anti-CCP antibody were performed using an indirect ELISA technique. Hundred microliters of serum diluted with assay buffer was pipetted into each coated microtiter well, and incubated for 30 min at 25°C. The plates were washed four

times with wash buffer, then 100 μ l of rabbit anti-human IgG was added to each well, and incubated with shaking for 30 min at 25°C. The plates were washed again four times, then pipetted with 100 μ l buffer, diluted with Eu^{3+} -labeled streptavidin to each well, and incubated with shaking for 15 min. After washing six times with wash buffer, 100 μ L of enhancement solution was pipetted into each well. The plates were shaken for 5 min. Eu^{3+} fluorescence intensity (cps) was measured with auto DELFIA1235. The concentrations of anti-CCP antibody in respective samples were determined based on the calibration curves using Multicalc software program.

Evaluation on the Kits

- (1) *Precision testing.* Three pools of mixed serum specimens with high, intermediate, and low concentration of anti-CCP antibody were subpackaged and stored at -20°C . Mixed with the serum specimens and evaluating their concentrations with samples, the coefficient of variations (CVs) was calculated.
- (2) *Linear range testing.* Serum from patient with the highest anti-CCP antibody concentration was diluted in a twofold serial dilution manner, and the anti-CCP antibody concentrations of diluted specimens were detected using the established BAS-TRFIA kit and a commercial ELISA one, respectively. The linear curve of dilution concentration was obtained with dilution multiple as the horizontal abscissa and fluorescent intensity as the vertical ordinate.
- (3) *Sensitivity testing.* The sensitivity of the assay was back-calculated by the obtained mean fluorescent counts ($n = 20$) of the zero standard plus 2SD in the calibration curve.
- (4) *Clinical applications.* The specificity of anti-CCP antibody detection was evaluated by testing serum from 52 healthy blood donors, 32 systemic lupus erythematosus (SLE), 27 Sjogren's syndrome (SS), 10 scleroderma, 20 mixed connective tissue disease (MCTD), 23 multiple sclerosis (MS) patients.
- (5) *Correlation test.* The anti-CCP antibody concentrations of 32 serum specimens from patients with RA were detected using the established BAS-TRFIA kit and a commercial ELISA kit, respectively, and then the values obtained were analyzed.
- (6) *Stability testing.* New BAS-TRFIA kit and three ELISA kits were put into the 37°C water box for 7 days and then compared with the one stored in routine conditions.
- (7) *Coefficient of recovery.* Three sera with high antibody concentrations were diluted with sample buffer and assayed in the new TRFIA kit.

Fig. 1. Serial dilutions of a specimen with the highest anti-CCP IgG concentration were tested with TRFIA and ELISA, respectively. Double Y-axis curve was determined according to dilution strength (X-axis) as well as BAS-TRFIA fluorescence intensity and ELISA absorbance (the two Y-axes).

Statistical Analyses

Data were analyzed using the SPSS 13.0 software. Regression analysis and analysis of variance were used to calculate correlation and test linearity, respectively. Interassay and intra-assay variation was calculated using the CV. The statistical significance level was set at 0.05.

RESULTS

Linearity

A strong positive specimen from 9,081 to 0.55 U/ml was diluted and the serial dilutions with the established TRFIA as well as ELISA were detected. The curve of detectable range of the two methods is shown in Figure 1, in which we observe that for the established TRFIA kit there was a good linear range within 9,463–0.58 U/ml, whereas it was within 591.4–18.48 U/ml when using an ELISA kit, indicating that the method of TRFIA we established has a wider detectable range than the commercial ELISA.

Calibration Curve for Determination of Anti-CCP IgG

As shown in Figure 2, the time-resolved fluorescence intensities are directly proportional to the concentrations of anti-CCP IgG. The concentrations of anti-CCP IgG were 2,365.8, 591.44, 147.86, 36.96, 9.24, 0.58 U/ml. The line equation for the calibration curve of anti-CCP IgG was $Y = 3.79071 + 0.79505X + 0.01094X^2$, where X is the concentration of anti-CCP IgG (GPL U/ml) and Y is fluorescence intensity. The sensitivity of the assay, defined as the mean signal of the zero standard plus 2SD, was 0.5 U/ml.

Fig. 2. Standard curves for anti-CCP IgG by TRFIA at different concentrations (2,365.8, 591.44, 147.86, 36.96, 9.24, 0.58 U/ml).

TABLE 1. Intra-batch and Inter-batch Precision Analysis

	BAS-TRFIA (U/ml)		ELISA (U/ml)	
	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)
High				
Intrabatch ($n = 20$)	241.5 $\pm$ 7.63	3.16	242.8 $\pm$ 14.86	6.12
Interbatch ($n = 10$)	240.9 $\pm$ 10.33	4.29	243.1 $\pm$ 17.64	7.26
Intermediate				
Intrabatch ($n = 20$)	72.7 $\pm$ 2.78	3.82	72.6 $\pm$ 5.94	8.18
Interbatch ($n = 10$)	72.3 $\pm$ 3.38	4.68	73.1 $\pm$ 7.26	9.93
Low				
Intrabatch ($n = 20$)	14.15 $\pm$ 0.60	4.24	14.32 $\pm$ 1.38	9.61
Interbatch ($n = 10$)	14.23 $\pm$ 0.71	4.97	14.37 $\pm$ 1.54	10.74

Assay Precision

The precision of the assay was also analyzed by measuring three pools of mixed serum specimens with high, intermediate, and low concentration of anti-CCP IgG 20 times in one series (intra-assay) and in duplicate in eight different series, and data were compared with those obtained by the ELISA kit. As shown in Table 1, the intra-assay CVs detected by TRFIA at mean anti-CCP IgG concentrations of 241.5, 72.7, 14.15 U/ml were 3.16%, 3.82%, and 4.24%, respectively, and the interassay CVs at mean concentrations of 240.9, 72.7, 14.23 U/ml were 4.29%, 4.68%, and 4.97%, respectively. In comparison, the intra-assay CVs detected by ELISA at mean concentrations of 242.8, 72.6, 14.32 U/ml were 6.12%, 8.18%, and 9.61%, respectively, and the interassay CVs at mean concentrations of 243.1, 73.1, 14.37 U/ml were 7.268%, 9.93%, and 10.74%, respectively. Obviously, the TRFIA kit we established for anti-CCP IgG detection was better than the commercial ELISA kit in intra- and interassay precision with either rank of concentration.

TABLE 2. Specificity of Anti-CCP Antibody in Different Types of Patients

Case and control group	n	Negative 15 U/ml	Positive $\geq$ U/ml	Specificity (%)
Healthy blood donors	52	51	1	98.1
SLE	32	31	1	96.9
SS	27	26	1	96.3
Scleroderma	10	10	0	100
MCTD	20	19	1	95.0
MS	23	22	1	95.7

Clinical Application

Each well of polystyrene microtiter plates was coated with recombinant CCP antigen. The specificity of anti-CCP antibody detection was evaluated by testing serum from 52 healthy blood donors, 32 SLE, 27 SS, 10 scleroderma, 20 MCTD, and 23 MS patients. Cross-reactivities are shown in Table 2.

Correlation With ELISA

Concentration results of 32 serum samples determined by TRFIA were compared with those obtained by an ELISA kit. The scatter plots showed that the two methods correlated well (Fig. 3) and the correlation coefficient of the results obtained from the two assays was 0.8927.

Stability of the TRFIA Kits

The assay kits were stored at 4°C and water bathed at 37°C for 7 days. Then, the difference of fluorescence intensity or absorbance—that is decline rate of

Fig. 3. Correlation between the BAS-TRFIA and ELISA.

TABLE 3. Stability of the BAS-TRFIA and ELISA

Groups	4°C preserve 7 days	37°C water bath 7 days	The declined rate of combination (%)
ELISA 1(abs)	2.56	1.63	36.3
ELISA 2(abs)	2.39	1.69	29.3
ELISA 3(abs)	2.71	2.23	17.7
BAS-TRFIA (cps)	2,451,236	2,153,415	12.1

combination—was measured both by TRFIA and ELISA. We observed that it exhibited no visible change except for a decline by 12.1% for TRFIA (17.7%, 29.3%, 36.3% for the commercial ELISA) in the rate of combination when compared with the one stored in routine conditions, suggesting that the established assay kit had good stability and could be stored at 4°C for 1 year (Table 3). Moreover, the accuracy decreased as time was extended by ELISA, but after enhancement solution was added the detection of fluorescent results was not affected up to the next day. Thus, it can be seen that TRFIA is more stable.

Coefficient of Recovery

The recoveries of the first sample were 100.98%, 102.25%, and 96.45%, respectively. The recoveries of the second sample were 100.46%, 98.18%, and 101.59%, respectively. The recoveries of the third sample were 104.63%, 97.18%, and 98.17%, respectively. The average recovery rate calculated by the average of nine individual values by the formula of observed concentrations × 100/expected concentration in Table 4) was 99.98%.

TABLE 4. Coefficient of Recovery: Three Sera With High Antibody Concentrations Were Diluted With Sample Buffer and Assayed in the New BAS-TRFIA Kit

Sample	Dilution	Observed (U/ml)	Expected (U/ml)	O/E (%)
1	1:100	266.4		
	1:200	134.5	133.2	100.98
	1:400	68.1	66.6	102.25
	1:800	32.1	33.3	96.40
2	1:100	175.6		
	1:200	88.2	87.8	100.46
	1:400	43.1	43.9	98.18
	1:800	22.3	21.95	101.59
3	1:100	88.5		
	1:200	46.3	44.25	104.63
	1:400	21.5	22.125	97.18
	1:800	10.86	11.0625	98.17

DISCUSSION

To facilitate early diagnosis, a serological marker of RA that is detectable very early in the disease is needed. As mentioned above, several recent studies have shown that anti-CCP can be detected very early in the course of RA and may be helpful in early diagnosis.

Herein, we developed a new sandwich TRFIA with biotin–streptavidin system as a quantitative and rapid immunological assay for measuring anti-CCP in clinical serum samples, since the new method combined the advantages of both high sensitivity of traditional TRFIA and high affinity of biotin to streptavidin tetrameric bond. Moreover, the detectable signal can be amplified because of the tetrameric structure with four binding sites of streptavidin for biotin. The experimental results indicated that the new TRFIA is a promising immunofluorometric method with highly sensitive and specific, meanwhile, the assay provided wider dynamic working ranges and better reproducibility. In Figure 1, TRFIA demonstrated a good linear range within 9,463–0.58 U/ml, by contrast, within 591.4–18.48 U/ml of an ELISA kit. The expanded detection range will improve earlier detection and higher sensitivity in RA diagnosis (10, 11). Furthermore, the serologic-positive patients will be treated timely and effectively, subsequently, preventing irreversible joint damage (12).

By comparison, the new assay has satisfied correlation with general commercial ELISA kit with the R^2 value 0.8927 (Fig. 3), moreover, its stability performance is better than the commercial ones, which will guarantee the more stable yield (Table 3). In sum, the new TRFIA with biotin–streptavidin system as a quantitative and rapid immunological assay for measuring anti-CCP is a promising alternative assay for RA diagnosis and monitor.

CONFLICT OF INTEREST

All authors declare that they have no competing interests.

REFERENCES

1. Raptopoulou A, Sidiropoulos P, Katsouraki M, Boumpas DT. Anti-citrulline antibodies in the diagnosis and prognosis of rheumatoid arthritis: Evolving concepts. *Crit Rev Clin Lab Sci* 2007;44(4):339–363.
2. van Gaalen FA, Linn-Rasker SP, van Venrooij WJ, et al. Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: A prospective cohort study. *Arthritis Rheum* 2004;50(3):709–715.
3. van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP antibodies: The past, the present and the future. *Nat Rev Rheumatol* 2011;7(7):391–398.
4. Forslind K, Ahlmén M, Eberhardt K, Hafström I, Svensson B; BARFOT Study Group. Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: Role of antibodies to citrullinated peptides (anti-CCP). *Ann Rheum Dis* 2004;63(9):1090–1095.
5. Du L, Cheng S, Wang S. Determination of diethylstilbestrol based on biotin-streptavidin-amplified time-resolved fluoroimmunoassay. *Luminescence* 2012;27(1):28–33.
6. Hu Z, Liu J, Ye Y, Zhou Y, Yu L. Detection of anticardiolipin antibody igm by sm(3+)-labeled time-resolved fluoroimmunoassay. *J Immunoassay Immunochem* 2013;34(3):255–265.
7. Suonpaa M, Markela E, Ståhlberg T, Hemmilä I. Europium-labelled streptavidin as a highly sensitive universal label. Indirect time-resolved immunofluorometry of FSH and TSH. *J Immunol Methods* 1992;149(2):247–253.
8. Qin QP, Lovgren T, Pettersson K. Development of highly fluorescent detection reagents for the construction of ultrasensitive immunoassays. *Anal Chem* 2001;73(7):1521–1529.
9. Huhtinen P, Soukka T, Lövgren T, Härmä H. Immunoassay of total prostate-specific antigen using europium(III) nanoparticle labels and streptavidin-biotin technology. *J Immunol Methods* 2004;294(1–2):111–122.
10. van Genderen FT, Gorus FK, Pipeleers DG, van Schravendijk CF. Sensitive and specific time-resolved fluorescence immunoassay of rat C-peptide for measuring hormone secretory and storage capacity of beta-cells in vivo and in vitro. *Endocrinology* 1993;134(5):1934–1939.
11. Farid S, Azizi G, Mirshafiey A. Anti-citrullinated protein antibodies and their clinical utility in rheumatoid arthritis. *Int J Rheum Dis* 2013;16(4):379–386.
12. Landewe RB, Boers M, Verhoeven AC, et al. COBRA combination therapy in patients with early rheumatoid arthritis: Long-term structural benefits of a brief intervention. *Arthritis Rheum* 2002;46(2):347–356.