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Establishment of a quantitative detection method for magnetic microparticle chemiluminescence of anti-SSA-60 antibody

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ABSTRACT

Objective: To produce 60-kDa recombinant Sjőren's syndrome antigen A (SSA-60) by gene engineering and establish and evaluate the performance of a magnetic microparticle chemiluminescence quantitative method for detecting anti-SSA-60 antibody in sera.

Methods: Recombinant antigen was prepared by gene recombination technology and purified by high affinity Ni^{2+} resin. The immunogenicity of the recombinant antigen was verified in immunized BALB/c female mice, and the immune reactivity of our recombinant antigen was assessed using the enzyme-linked immunosorbent assay (ELISA) method. With this recombinant antigen, a specific magnetic microparticles chemiluminescence assay (MMC assay) was developed and performed using various parameters.

Results: The inner-group difference among high, medium and low density sera mixtures was 7.65%, 2.24%, and 2.47%, respectively, and the inter-group precision rate was 8.25%, 6.26%, and 4.87%, respectively, using the MMC assay. The low detection limit was 1.36 relative unit per milliliter (Ru/mL), and the quantitative limit was 4.48 Ru/mL. The linear range of this method was 2–400 Ru/mL, which is wider than that of ELISA. The standard error (SE) of the differences was 31.3 between the two methods. Good correlation ($y = 1.04x - 7.86$, $R^2 = 0.979$, $P < 0.05$) and high agreement (Kappa = 0.95) were noted between the two methods.

Conclusions: These data show that the MMC assay provides high sensitivity and specificity and a wider linear range in anti-SSA-60 aab detection and fairly good agreement and correlation between the MMC assay and ELISA. The MMC assay has potential in rapid, high-throughput, quantitative and automated autoimmune antibody testing.

1. Introduction

Anti-SSA-60 autoantibody (anti-SSA-60 aab) is one of the most common autoantibodies and is associated with many autoimmune diseases, such as Sjőren's syndrome (SS), systemic lupus erythematosus (SLE), cutaneous lupus erythematosus (CLE), and neonatal lupus [1–4]. At present, anti-SSA-60 aab is an important serological diagnostic marker for SS and has been included in the diagnostic guidelines of SS [5]. In addition, anti-SSA-60 aab could be present, on average, 3.4 years before the diagnosis of SLE [6]. Anti-SSA-60 aab was detected by indirect immunofluorescence (IIF) in human laryngeal cancer epithelial (HEp-2) cells, and its presence can be confirmed by ELISA, immunodiffusion (ID) or immunoblot (IB) in most laboratories both at home and in abroad. Most of these detection methods have some limitations, e.g., they are time-consuming, manually performed, and subjective and qualitative. Some studies have demonstrated that SSA-60 antigen is underrepresented in HEp-2 cells on certain IIF slides and has a high false negative rate in IIF [7]. A 2012 College of American Pathologists (CAP) feedback analysis demonstrated that in some countries, ELISA (49.53%) and immune microspheres (38.84%) were mainly adopted using antibodies to detect extractable nuclear antigens (ENAs) [8]. According to 2012 and 2015 Chinese National Quality Control survey, the proportion of line immunoassays (LIA, simultaneously detecting multiple specific ANA in a test strip) was 90.53% to 98.50%. In

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addition, ELISAs are used in < 3% of assays for anti-ENA detection in China, and imported reagents are mostly employed [9,10]. There is a significant gap in the detection of autoantibodies. Compared with the other quantitative detection method of the single antigen envelope, which is easy to automate and standardize, most laboratories in China have mainly adopted semi-automatic and qualitative detection methods. With the development of science and technology and with the increasing demand of clinicians, the automatic quantitative detection of autoantibodies has become a necessary trend [11].

SSA protein can be classified as 52 kDa (SSA-52; also called Ro52) or 60 kDa (SSA-60; also called Ro60) based on molecular weight. Ro52 and SSA-60 protein consists of two different proteins coded by different complementary DNAs (cDNAs). [12] Thus, anti-SSA-60 and anti-Ro52 represent two distinct autoantibodies systems. Traditionally, anti-SSA-60 aab detection methods, such as indirect immunofluorescence (IIF) in HEp-2 cells, immunodiffusion (ID), immunoblot or ELISA, use a mixture of both Ro52 and SSA-60 as the antigens. Some researchers have suggested that anti-Ro52 and anti- SSA-60 aab could mask each other's reactivity [13]. With advances in the expression and purification of recombinant protein, immunoassays allowed separate detection of anti-Ro52 and anti- SSA-60 aab. During the last decade, significant improvements have been made in recombinant protein technology, and recombinant antigen manufacturing is more consistent and less dependent on the biological variations of the source material [14]. In this study, we produced a recombinant SSA-60 antigen and established a magnetic microparticle chemiluminescence reagent for automatic quantitative detection of anti-SSA-60 aab using domestic instruments and evaluated its performance by a comparative method.

2. Materials and methods

2.1. Samples

This study was approved by the Institutional Review Board (IRB) of the Affiliated Hospital of North Sichuan Medical College, China. In total, 449 sera samples (male/female ratio 107:342, mean age 48 years, range 14–83 years) were obtained from patients the clinical laboratory and stored in aliquots at −20 °C until use.

2.2. Preparation and purification of recombinant antigen

Preparation and purification of recombinant antigen. The human SSA-60 protein gene sequence was amplified by polymerase chain reaction (PCR) from cDNA using primer 1 (ATGCTGGGATCCGAGGAAT CTGTAAACCAAATG) and primer 2 (ATTGGCCTCGAGAATCATATCTA ATGTGAAAT). The human SSA-60 protein gene sequence was digested with BamH I and *Xho* I restriction enzymes and subcloned into the expression vector pET41a (Novagen, USA) to construct the glutathione-S-transferase -tagged-SSA60-6*histidine-tag (GST-SSA60-6*His) recombinant plasmid.

The recombinant plasmid was transfected into the *E. coli* strain Rosetta (DE3) and incubated overnight at 37 °C. Selected monoclonal colonies were cultured in Luria-Bertani (LB) liquid medium containing 50 μg/mL kanamycin. The recombinant samples were induced with isopropyl-β-D-thiogalactoside (IPTG) when the optical density (OD) of the culture was 0.6 and incubated for 6 h at 25 °C. The cells were harvested by centrifugation at 8,000 × *g* for one minute at 4 °C. The cell pellets were resuspended in 1 mL of extraction buffer and ruptured in an ice bath by sonication. After centrifugation at 12000 × *g* for one minute at 4 °C, the supernatant and precipitate were collected to identify the protein expression by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Inclusion bodies were resuspended in extraction buffer containing 4 mol/L urea. After sonication and centrifugation, a high-affinity Ni^{2+} resin column (Millipore, USA) was employed to purify the target protein. Dissolved histidine-tag-SSA-60 protein was bound to the Ni^{2+} resin column. The column was washed extensively with elution buffer until the OD_{280} value of the flow-through solution was < 0.100. Different concentrations of imidazole elution buffer were used to elute the target protein.

2.3. Antigen identification

The immunogenicity of the fusion protein was verified in immunized BALB/c female mice. BALB/c female mice were immunized by intraperitoneal injection of 60 μg of purified fusion protein. The first immunization used Freund's complete adjuvant, and the second and third immunizations were performed with Freund's incomplete adjuvant. Mouse serum samples were collected on the seventh day after the third immunization. Different concentrations of recombinant SSA-60 antigen (Diarect, Germany) were detected by western blotting (WB). Mouse sera served as the first antibody, and sheep anti-mouse horseradish peroxidase (HRP) served as the second antibody.

We employed the ELISA method to evaluate immune reactivity and consistency between our recombinant fusion protein and the recombinant SSA-60 antigen (Diarect, Germany). Two different recombinant antigens were separately coated onto microwell plates, and serum samples were simultaneously analyzed.

2.4. MMC assay

Anti-SSA-60 aab was detected by an indirect immune method (Fig. 1). Based on the biotin-streptavidin system, biotinylated SSA-60 antigen was coated on streptavidin magnetic microparticles. During the first incubation, anti-SSA-60 aab bound to magnetic microparticles was present in serum samples. During the second incubation, HRP-labeled anti-human immunoglobulin conjugate reacted with anti-SSA-60 aab bound to the magnetic microparticles. A wash cycle removed unbound material after each incubation. Subsequently, substrate was added, and a chemiluminescence reaction was produced. The light signal was measured by a photomultiplier as relative light units, and the concentration of anti-SSA antibody in sera was calculated using a standard curve based on a calibration concentration and relative light units.

2.5. Statistical analysis

For each assay, samples were tested in duplicate, and experiments were repeated at least thrice. For comparison, we used a commercial ELISA kit according to the instructions obtained by the manufacturers. The agreement between the ELISA and the MMC assay was calculated using kappa and McNemar's tests. The Passing-Bablok test and Pearson's correlation were performed to demonstrate the linearity and correlations between the two methods. Calibration curves were obtained by plotting the logarithm of the relative light units (Y) against the analyte concentration (X) and fitting the 4-parameter logistic using ELISACalc

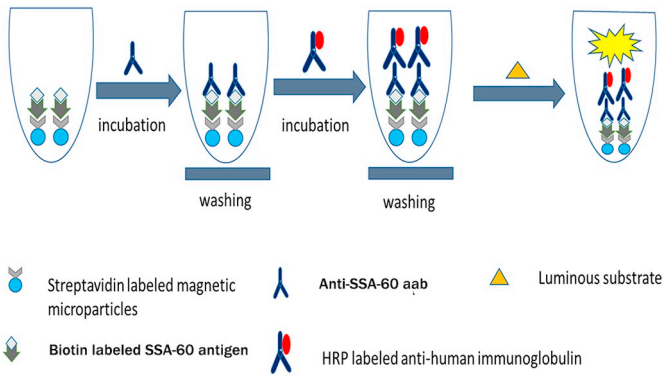

Fig. 1. Schematic diagram of the MMC assay for detecting anti-SSA-60 aab.

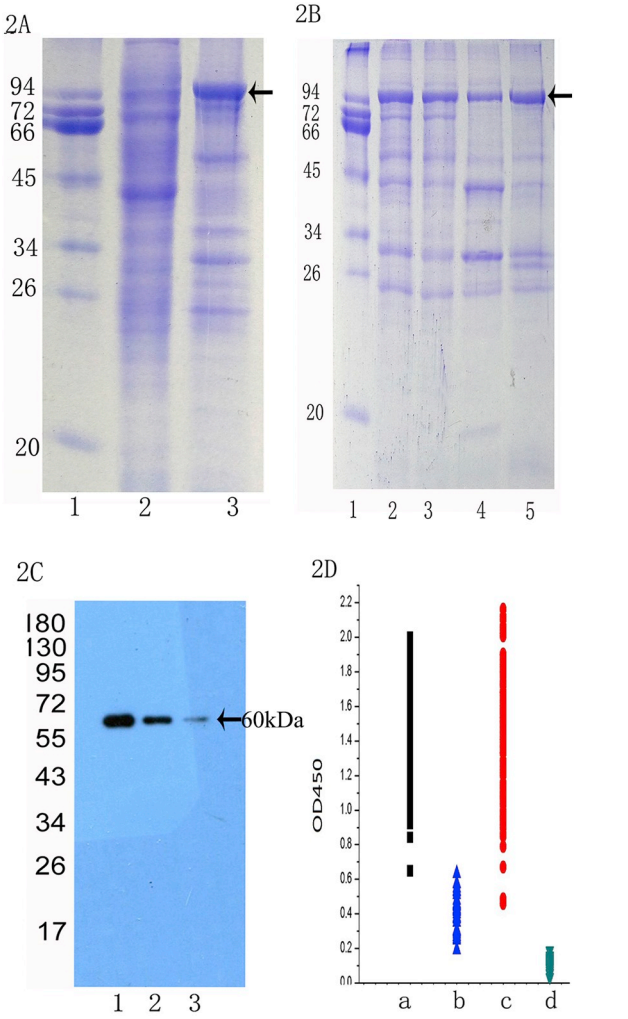

Fig. 2. A. The expression of GST-SSA60–6*His protein as assessed by SDS-PAGE. **Lane 1**, molecular mass marker; **Lane 2**, supernatant; **Lane 3**, cell pellet. **B.** Purification and characterization of the fusion protein. **Lane 1**, molecular mass marker; **Lane 2**, starting material before loading onto the column; **Lane 3**, flow-through; **Lanes 4–5**, eluate obtained after applying 20 mM and 60 mM imidazole, respectively, to the column. **C.** Western blot analysis of recombinant SSA-60 antigen with fusion protein antiserum. **Lanes 1–3**, 20 ng, 10 ng and 5 ng of recombinant SSA-60 antigen reacted with fusion protein antiserum, respectively. **D.** Comparison study of our fusion protein and the recombinant SSA-60 antigen. **a.** SSA-60 (contrast) + anti-SSA-60 aab positive serum samples; **b.** SSA-60 (contrast) + anti-SSA-60 aab negative serum samples; **c.** SSA-60 (our) + anti-SSA-60 aab positive serum samples; **d.** SSA-60 (our) + anti-SSA-60 aab negative serum samples.

data program. Statistical analysis was performed using McNemar's and Passing-Bablok tests in SPSS 22.0, MedCalc statistical software version 15.2.2, and Microsoft Excel 16.0.

3. Results

3.1. Characterization of the recombinant antigen

According to SDS-PAGE analysis results (Fig. 2A), the induced fusion protein was found predominantly in the cell pellets, suggesting that the fusion protein was insoluble in the form of inclusion bodies. The molecular weight of the fusion protein was 96 kDa (indicated by arrow above), which is consistent with the expected size.

Purification of the fusion protein was performed using Ni^{2+} resin columns. SDS-PAGE analysis showed that the concentration of the target protein in 60 mM imidazole elution buffer was higher, and the total protein purity was > 90% (Fig. 2B).

The antiserum could specifically recognize the recombinant SSA-60 antigen (Diarect, Germany) by WB (Fig. 2C), indicating that the fusion protein had sufficient immunogenicity and produced a high titer of antibody in immunized animals.

Based on a comparable ELISA study, we found that our fusion protein exhibited good immune reactivity and agreement with the recombinant SSA-60 antigen (Diarect, Germany). In addition, the distinction between negative and positive was obvious in clinical serum samples. [Fig. 2D].

3.2. Performance of the MMC assay

To estimate the reliability of the MMC assay for the detection of anti-SSA-60 aab, we evaluated various parameters. According to IUPAC criteria, the detection limit of this proposed method was 1.36 Ru/mL, and the quantitative limit was 4.48 Ru/mL.

The specificity or anti-interference ability was quantified in pooled sera containing other similar autoantibodies. The results for anti-SSA-60 aab were negative, suggesting that there were no significant cross-reactions with other autoantibodies. [Table 1].

The within-assay precision was calculated by measuring analytes under conditions of high, medium and low of substrate concentrates repeated at least 20 times in one run, and the between-assay precision was calculated. The results were obtained on seven separate days. The intra-assay precision and inter-assay precision were < 8% and < 10%, respectively. [Table 2].

The concentration of mixed high value anti-SSA-60 aab serum was calibrated thrice with ELISA, and the average was 1492.5 Ru/mL. Serial dilutions (1:200 to 1:204800) of mixed serum with sample buffer were assessed in parallel tested to determine the linear range for ELISA and the MMC assay (Fig. 3A). The curve for anti-SSA-60 aab obtained from ELISA was linear in the range of dilution concentrations of 1:1600 to 1:102400 (2–200 Ru/mL) and conformed to manufacturer's specifications. The correlation coefficient (R) was 0.948 and indicated a higher linear correlation with the range of dilution concentrations. However, in the MMC assay, the curve was linear in the range of 1:800 to 1:102400 (2–400 Ru/mL), and R was 0.989.

3.3. Comparison

To further demonstrate the reliability of the MMC assay for the determination of anti-SSA-60 aab, a total of 449 clinical serum samples were tested in parallel with the proposed method and the commercially available ELISA kit (EUROIMMUN, Germany). First, the serum samples were diluted 200-fold with sample buffer as an initial result. If the sample diluted 800-fold was out of the range, the result was multiplied

Table 1
Cross-reactivity results for other autoantibodies.

Analyte concentration category	LIA	MMC assay (Ru/mL)	ELISA (Ru/mL)
Rib-p	3+	7.24	6.51
NUC	3+	7.76	5.8
U1-nRNP	3+	< 2	3.6
Ro52	3+	3.89	< 2
PCNA	3+	3.49	2.78
Jo-1	3+	3.89	3.46
CENP-B	3+	5.31	7.89
SS-B	3+	< 2	2.34
Scl-70	3+	< 2	< 2
SmD1	3+	< 2	< 2
PM-SCL	2+	< 2	< 2

LIA: line immunoassay (EUROLine, Germany).
MMC assay: proposed method.
ELISA: (EUROIMMUN, Germany).

Table 2
MMC assay precision.

Intra-assay precision				Inter-assay precision		
Analyte concentration	Mean value	SD	CV	Mean value	SD	CV
Low	8.88	0.68	7.65%	9.61	1.28	8.25%
Medium	64.10	1.44	2.24%	60.36	3.61	6.26%
High	135.82	3.35	2.47%	131.33	6.41	4.87%

by four to obtain a final result. The initial results < 2 Ru/mL and final results > 800 Ru/mL were removed. In total, 111 samples for which the concentration were within the detection range (2–800 Ru/mL) were selected to demonstrate the correlation between the two methods using different statistical analysis. Good linear relativity was noted between the methods ($y = 1.04x - 7.86$, $R^2 = 0.979$) in Fig 3B. The least squares analysis ($P < 0.05$) and Passing-Bablok ($P > 0.05$) further demonstrated a good correlation between the two methods [Table 3]. The standard error (SE) of the differences was 31.3 between two methods. Differences in testing results between the two methods were evaluated with McNemar's test and the Kappa statistic for correlated proportions and agreement. The Kappa value was 0.95, showing high agreement between tests [Table 4].

4. Discussion

Antinuclear antibodies (ANAs) are fundamental for the diagnosis of autoimmune diseases. In recent decades, with antigen specificity determined with a second assay [15,16], the demands for more specific ANA or ENA and different specificities have increased significantly, resulting in a technical evolution and the development of novel diagnostic platforms, including ELISA, bead immunoassays, fluorescent multiplex flow immunoassays (MFIs), and others. [17-20].

The MMC assay employs a two-step chemiluminescence assay as a measurement technique using antigen-coated solid phase magnetic particles. Compared with conventional liquid-solid phase assays, magnetic microparticles suspended in solution with a liquid-liquid phase system have demonstrated superiority in their ability to provide a large contact area, thus increasing the reaction rate, increasing the reaction speed, and expanding the linear range [21]. In this study, the method was automated on a Chinese Chemical immuno-luminescence analyzer, needing only 48 min for a result, compared to ELISA at 90 min. Compared to ELISA, the method is automated, faster, easier to standardize, and has better sensitivity and a wider linear range. In addition, the method allows for a continuous workload.

We evaluated the proposed method for anti SSA 60 aab by comparing to a classical IgG ELISA method We also found that the MMC assay demonstrated good Kappa agreement with the commercial ELISA for anti-SSA-60 aab detection if we used the cut-off recommended by the manufacturer. A total of seven cases were discrepant, but two of them corresponded to weak positives. However, the other five cases had obvious differences. In the five discrepant results that did not correspond with clinical findings that corroborated the results, it was noteworthy that two of the results were positive by ELISA and confirmed by blotting. Furthermore, other results were negative both by the MMC assay and blotting.

One putative reason for the significantly different results for anti-SSA-60 aab detection is the antigen source. It is important to note that differences in antibody binding to antigens can also result from variability in the source and composition of antigens used for autoantibody detection [22]. The ELISA antigen was extracted from bovine thymus, and the relevant binding epitopes of anti-SSA-60 aab slightly differed compared with the recombinant antigen.

Our study had some limitations. We did not study our own optimal cut-off values for the anti-SSA-60 aab test. Healthy people have significantly lower titers and avidity of circulating autoantibodies [23,24]. Furthermore, the prevalence of autoantibodies is influenced by sex, age, race and other factors [25]. This highlights the importance of determining our own cut-off values. Another limitation is that we did not analyze the performance of the two methods in specific connective tissue disease. Some diseases were represented by only a small number of patients or not included, such as systemic sclerosis (SSc) and myositis, etc. And most of the patients were undergoing treatment or had inactive diseases.

5. Conclusion

We successfully produced recombinant SSA-60 antigen and established MMC assay methods to detect anti-SSA-60 aab. With the utilization of magnetic microparticles and recombinant antigen, the MMC assay was established for domestic instruments, demonstrating high sensitivity and specificity and a wider linear range. It is our belief that

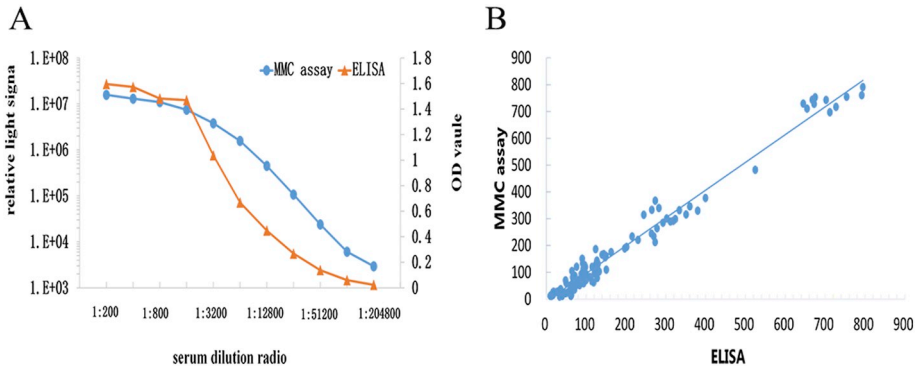

Fig. 3. A. Comparison of the detectable ranges for anti-SSA-60 aab between the MMC assay and ELISA. **B.** Plot of the results obtained with the proposed method versus ELISA for 111 samples.

Table 3
Correlation analysis results for ELISA and the MMC assay.

Statistical analysis	Sample, no	Slope (95%CI)	Intercept (95% CI)	P	Correlation coefficient
Least squares	111	1.0366 (1.0075–1.0658)	−7.8610 (−15.8358–0.1138)	$P < 0.05$	0.9892
Passing-Bablok	111	1.0323 (0.9945–1.0820)	−11.9819 (−13.4976 - -1.0871)	$P = 0.09$	

Table 4
McNemar's test between the proposed method and ELISA results for 449 samples.

		Proposed method		Total
		−	+	
ELISA	−	324	2	326
	+	5	118	123
Total		329	120	449

Positive/Negative: ± .
The cutoff value is 20 for both methods.

the novel technology platform combined with the high sensitivity and wider linear range observed in this study has potential in rapid, high-throughput, quantitative and automated autoimmune antibody testing. This study has laid a foundation for further application of other autoantibodies in magnetic microparticle chemiluminescence assays.

Conflict of interest statement

The authors declare that no competing interests exist.

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