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Detection of autoantibodies to the p200-epitope of SSA/Ro52 antigen. A comparison of two laboratory assays

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Abstract

Background: Anti-p200 antibodies have been receiving growing interest in view of findings associating their presence to risk of fetal autoimmune congenital heart block (CHB). The study compares and evaluates the performance of two assays currently being used for their detection.
Methods: One hundred and sixteen pregnant women positive for anti-SSA/Ro52 antibodies were considered as the study population. Fifty women negative for anti-SSA/Ro52 antibodies were considered as the control population. Anti-p200 antibodies were analyzed using two home-made ELISA assays: one with biotinylated antigen and the other with free antigen.
Results: The specificity of the p200-free assay was significantly higher with respect to that of the p200-biotin assay ($p = 0.023$). Both methods showed a high area under curve (AUC), thus, a good accuracy. There was a significant prevalence of anti-p200 antibodies when the p200-free assay was used to analyze the sera of the pregnant women with CHB fetuses ($p = 0.007$). Cohen's κ and Spearman's ρ coefficients showed a good concordance (0.71) and a high correlation (0.93), respectively.
Conclusions: The p200-free assay with respect to the biotin-based method was more specific in detecting p200 antibodies in women positive for anti-SSA/Ro52 antibodies. In addition, only the p200-free method significantly found p200 antibodies in patients with fetal CHB.

Keywords: anti-p200 antibodies; anti-SSA/Ro52 antibodies; congenital heart block; enzyme-linked immunosorbent assay.

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Introduction

Anti-SSA/Ro and anti-SSB/La antibodies are in the majority of cases associated with Sjögren syndrome (50%–80%) and to a minor extent to systemic lupus erythematosus (20%–40%), undifferentiated connective tissue disease (20%–30%), other autoimmune diseases (5%–10%) and also in asymptomatic carriers [1, 2]. Autoimmune congenital heart block (CHB), a rare, passively acquired disease caused by the transplacental transfer of maternal anti-SSA/Ro and anti-SSB/La antibodies [3, 4], is characterized by inflammation, fibrosis and calcification of fetal cardiac conduction tissues leading to atrioventricular block (AVB) in an otherwise normal heart [4]. CHB can progress from a first-degree to an irreversible, potentially lethal third-degree AVB within a week [5, 6]. The prevalence of fetal CHB, which according to prospective studies, is 1%–5%, rises to 6%–25% for antibody-positive women with a previously affected pregnancy [5]. According to recent reports, maternal antibodies specific for a 52 kDa subunit of SSA/Ro protein, termed p200 peptide, are associated with CHB and appear to have a critical pathogenic role in its onset [7–9]. More specifically, maternal anti-SSA/Ro52 antibodies targeting the amino acids 200–239, p200 antigen, have been receiving growing interest because they have been associated to fetal risk of CHB [7, 10]. Data on the relationship between anti-p200 antibodies and CHB are nevertheless still considered controversial [10, 11].

Indeed, *in vitro* and *in vivo* studies [12–16] have demonstrated that anti-p200 antibodies correlate with fetal atrioventricular time prolongation, bind to the cell surface of cardiomyocytes, and dysregulate Ca^{2+} homeostasis causing apoptosis in affected cells. High levels of anti-p200 antibodies have, moreover, recently been found to be linked to a high risk of developing CHB [17].

These findings have led to the hypothesis that anti-p200 antibody detection can identify pregnant women whose fetuses are at risk for developing CHB, thus these antibodies could lead to a prompt diagnosis and early treatment finalized to prevent progression to irreversible third-degree AVB.

Two in-house enzyme linked immunosorbent assay (ELISA) techniques for anti-p200 antibody analysis, each using a different type of antigen and blocking agent, have been described in the literature [7, 10]. While the first method [7] detects anti-p200 antibodies using p200-free as its antigen coated directly to the plate and using a lyophilized vaccine milk as its blocking agent, the second [10] utilizes p200 conjugated with biotin at the N-terminal coated to a streptavidin-treated plate and bovine serum albumin as the blocking agent. The current study was designed to evaluate and compare the efficiency and clinical performance of the two methods in a cohort of pregnant women who were positive to anti-SSA/Ro52 antibodies and in a control group composed by women negative to anti-SSA/Ro52 antibodies.

Materials and methods

Study populations

One hundred and forty-one pregnant women positive for antibodies directed against 52 kDa and/or 60 kDa subunits of SSA/Ro proteins and/or against anti-SSB/La antibodies with a mean age of 33.3 years ± 5 SD were included in the study. Their mean week of gestation (wg) at enrolment was 13 wg ± 7 SD range 7–27. The patients were affected by Sjögren syndrome in 56 cases, undifferentiated connective tissue diseases in 47, systemic lupus erythematosus in five, rheumatoid arthritis in four, antiphospholipid syndrome in two, thrombocytopenia in one and discoid lupus in one; while 25 women were asymptomatic. Twenty-four women, all positive for anti-SSA/Ro52, were diagnosed with fetal CHB between the 20th and 27th wg (mean 23 wg ± 3 SD). Nine CHB were third-degree blocks and 15 a second-degree block. Six women decided to terminate their pregnancy, three were treated with steroids remaining stable, while 15 started a therapeutic protocol already described [18] following regression or no progression of the block in three and three cases, respectively.

One hundred and sixteen pregnant women (mean age 32.5 years ± 4.7 SD) whose sera were positive for antibodies directed against 52 subunits of SSA/Ro protein were considered as the study population. The control group consisted of 50 women, all were anti-SSA/Ro52 negative, matched for age with the study population; 25 of them were pregnant and positive for anti-SSA/Ro60 and/or anti-SSB/La antibodies, the other 25 were healthy blood donors. All the samples were stored at −80 °C until they could be tested. Among all the pregnant women 24 had fetuses affected with CHB, while 117 had unaffected fetuses.

The study was carried out in accordance with ethical principles outlined in the Declaration of Helsinki and all the patients gave informed consent. The Institutional Review Board for observational studies and the Audit Committee of the University of Padua's Medical Centre approved the study design.

ELISA assays for anti-p200-antibody detection

The anti-p200 antibodies were analyzed utilizing two home-made ELISAs: one used p200 alone as its antigen (p200-free) and the other used p200 with biotin (p200-biotin). Synthetic peptides representing amino acids 200–239 (i.e. p200) of SSA/Ro52 protein with and without biotin conjugated at the N-terminal end were synthesized by Thermo Biosciences (Ulm, Germany).

The p200-free ELISA assay was carried out in accordance with a home-made method outlined by Salomonsson et al. [7] with minimal variations. Briefly, high-binding 96-well plates (Nunc, Odense, Denmark) were coated (1 µg/well) with p200 diluted in carbonate buffer (pH 9.6) and incubated overnight at +4 °C. Plates were blocked with 200 µL phosphate buffered saline (PBS)/0.05% Tween/5% milk powder and incubated for 1 h at room temperature. Sera were tested at 1:500 dilution in PBS/0.05% Tween/1% milk powder and incubated for 2 h. Affinity-purified alkaline phosphatase-conjugated goat anti-human IgG antibodies (Sigma-Aldrich, St. Louis, MO, USA) were added at 1:2000 dilution and incubated at room temperature for 1 h. After incubation phosphatase substrate tablets (Sigma-Aldrich) dissolved in Mg-carbonate buffer (pH 9.8) were added. The absorbance was measured at 405 nm.

The p200-biotin ELISA assay was performed following an in-house method proposed by Strandberg et al. [10] with some variations. Briefly, high-binding 96-well plates (Nunc Odense, Denmark) were coated with 100 µL of 3 mg/mL streptavidin (Sigma-Aldrich) diluted in deionised water and incubated for 2 days at +4 °C. The plates were then dried at 37 °C (24 h) and stored at +4 °C until they were used. They were washed with wash buffer (0.15 M NaCl, 0.006 M $NaH_2PO_4 \cdot H_2O$, 0.05% Tween-20) and blocked with 200 µL/well of PBS +4% BSA. The plates were then washed once with PBS and coated with 100 µL of 3 mg/mL biotin-p200 peptide in coating buffer (0.03 M Na_2CO_3, 0.07 M $NaHCO_3$) for at least 6 h at room temperature. Sera were tested at 1:300 dilution in 0.15 M NaCl, 0.006 M $NaH_2PO_4 \cdot H_2O$, 0.05% Tween-20, 2% BSA and incubated by shaking for 2 h. After washing, affinity-purified alkaline phosphatase-conjugated goat anti-human IgG antibodies (Sigma-Aldrich) were added at 1:1000 dilution and incubated at room temperature for 1 h. After incubation phosphatase substrate tablets (Sigma-Aldrich) dissolved in Mg-carbonate buffer (pH 9.8) were added. The absorbance was measured at 405 nm.

The seven point calibration curve (see Supplemental Material, Figures 1 and 2) were built for both assays using a high positive serum in order to turn absorbance values into arbitrary units (AU/mL). The cut-off levels were calculated as the 99th percentile of

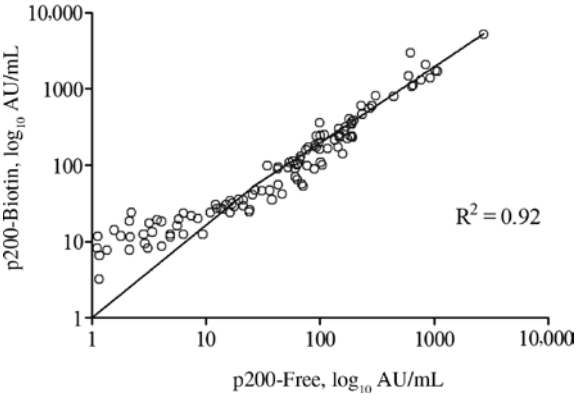

Figure 1: Graphic representation of anti-p200 antibody levels showing a significant correlation between the two ELISA assays.

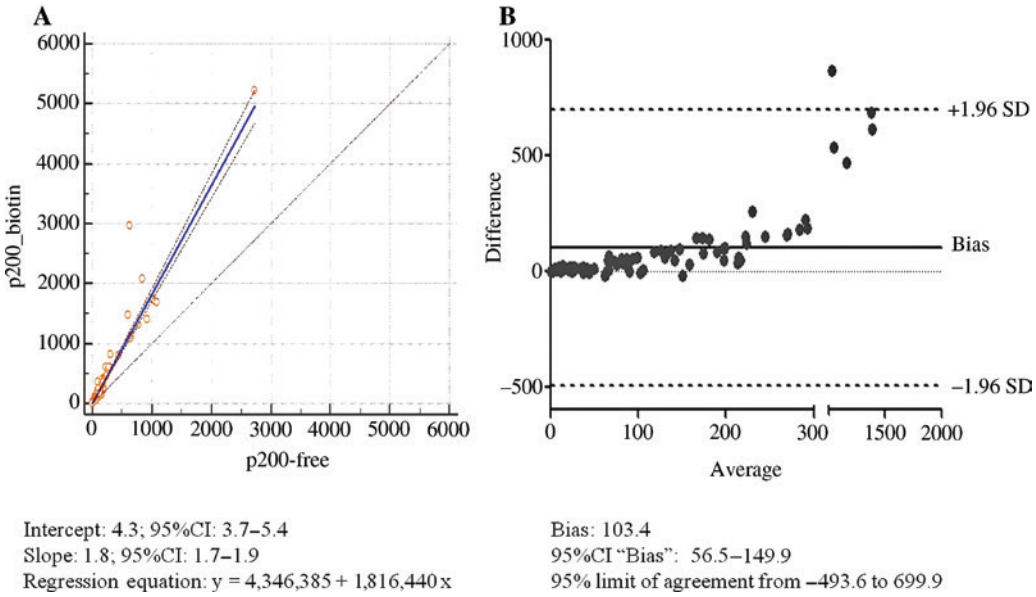

Figure 2: The analyses demonstrate the presence of a systematic overestimation error of the p200-biotin compared to p200-free one. Passing-Bablok regression (A) and Bland-Altman plots (B) show the results obtained using the p200-free method vs. those using p200-biotin one.

values obtained testing the sera of 100 healthy women who were age-matched with the study population.

Analytical sensitivity was evaluated by comparing the results obtained on stepwise dilution of the same anti-p200 positive sera with the two assays. Analytical specificity of anti-p200 antibody binding was evaluated by testing as potential interference anti-SSA/Ro60 and anti-SSB/La positive sera.

ELISA assays for anti-SSA/Ro52, anti-SSA/Ro60 and anti-SSB/La antibody testing

The antibodies were tested using a home-made ELISA assay following the method outlined by Klauninger et al. [19] with minor variations. Briefly, high-binding 96-well plates (Nunc) were coated overnight (0.1 mg/well) with recombinant Ro52 and La antigens and with native Ro60 protein (Arotec Diagnostics Limited, Wellington, New Zealand) diluted in carbonate buffer (pH 9.6). Plates were blocked with 200 µL/well of phosphate-buffered saline PBS, 0.05% Tween-5% milk powder and sera were tested at 1:1000 dilution in PBS-0.05% Tween-1% milk powder. Bound antibodies were detected by affinity-purified alkaline phosphatase-conjugated goat anti-human IgG antibodies (Sigma-Aldrich) at 1:1000 dilution. Phosphatase substrate tablets (Sigma-Aldrich) dissolved in Mg-carbonate buffer (pH 9.8) were used as substrate. The absorbance was measured using a microplate reader at 405 nm, and the optical density was converted into arbitrary units using a high positive serum to build the three calibration curves at seven dilution points. The cut-off values were calculated as the 99th percentile of values obtained by testing the sera of 100 healthy women who were age-matched with the patients. All sera were also evaluated for anti-SSA/Ro52, /Ro60 and anti-SSB/La with an ELISA kit (Orgentec Diagnostika, Germany). The relevant results were compared with those obtained by the home-made ELISA.

Statistical analysis

Statistical analysis was performed using the 22.0 version of the Statistical Package for the Social Sciences software (Chicago, IL, USA). The positive and negative predictive values were calculated considering "positive" those patients with positive anti-SSA/Ro52 results and "negative" those negative for these antibodies. For the same reason the sensitivity, specificity and results of receiving operating characteristics (ROC) curves were analyzed on anti-SSA/Ro52 positive and negative patients.

The association between categorical variables was analyzed using Fisher's exact test; odds ratios with 95% confidence intervals were calculated. To compare the median antibody levels of women with CHB with those of women without, the Mann-Whitney test was employed. Cohen's κ coefficient was calculated to assess the agreement between the two assays. The correlation coefficient between the antibody levels attained using the two methods, was calculated using Spearman's test. Correlation, linear association and agreement between the two assays were evaluated by Passing-Bablok analyses and Bland-Altman plots. The overall accuracy of the two assays was expressed as the area under the curve (AUC) calculated using ROC curve analysis. A p-value <0.05 was considered statistically significant.

Results

Analytical sensitivity revealed that at equal dilutions, the method employing p200 free revealed OD higher than p200-biotin one, i.e. at a dilution of 1:1000 the corresponding OD was 0.8 for p200 free and 0.4 for p200-biotin, thus revealing larger analytical sensitivity of p200 free than p200 biotin assay. While, analytical specificity was 94%

Table 1: The prevalence of anti-p200 antibodies in women positive and negative to anti-SSA/Ro52 according to the two different ELISA assays used here.

	Anti-SSA/Ro52+ n=116	Anti-SSA/Ro52– n=50	p-Value	OR [95% CI]
p200-Free, n (%)	95 (81.9)	3 (6)[a]	<0.0001	70.8 [20.1–249.7]
p200-Biotin, n (%)	106 (91.4)	12 (22)[a]	<0.0001	33.6 [13.4–84.0]

ELISA, enzyme linked immunosorbent assay; OR, odd ratio; CI, confidence interval. [a]These unspecific positivities may depend on the lack of absolute purity of both p200 free and p200 biotin antigens.

using p200 free and 76% using p200 biotin with a significant difference (p=0.023). Overall, intra- and inter-assay coefficients of variation (CV) were <10% for all the tests. They were obtained using 16 replicates both for intra-and inter-assay CVs. More specifically, the intra-assay CVs of p200-free and p200-biotin assays were 3.0% and 2.2%, respectively, while the inter-assay CVs were of p200-free and p200-biotin tests were 9.0% and 4.4%, respectively.

Anti-p200 antibodies significantly prevailed in anti-SSA/Ro52 positive women with respect the anti-SSA/Ro52 negative ones by using both the p200-free and p200-biotin assays (Table 1). The predictive positive and negative values of the p200-free method were, respectively, 96.9% and 69.1%, while those of the p200-biotin method were, respectively, 89.8% and 79.2%.

Table 2: Comparison of performance of p200-free and p200-biotin.

	p200-Free	p200-Biotin	p-Value
Sensitivity, %[a]	81.9	91.4	ns
Specificity, %[b]	94.0	76.0	0.023
Cohen's κ	0.71		<0.0001
Spearman's ρ	0.93		<0.0001

ns, not significant. [a]Evaluated in anti-SSA/Ro52 positive subjects; [b]evaluated in anti-SSA/Ro52 negative subjects.

Data concerning the comparison between the performance of p200-free and p200-biotin assays are outlined in Table 2. There was no significant difference in the sensitivity of the two assays, but there was a significant difference in their specificity for anti-SSA/Ro52 positive sera. It was significantly higher for the p200-free with respect to the p200-biotin assay. According to Cohen's κ coefficient, there was a significant, good agreement between the two assays. Spearman's analysis uncovered a strong significant correlation between the antibody levels detected by the two methods. As illustrated in Figure 1, there was a significant linear relationship between the anti-p200 antibody titers detected using the two assays. Passing-Bablok analyses and Bland-Altman plots were agreed in demonstrating the presence of a systematic overestimation error of the p200-biotin method compared to p200-free one (Figure 2A and B). Both p200-free and p200-biotin assays were found to have a high level of accuracy with an AUC of 0.96 and of 0.93, respectively, (Figure 3A and B). When the 24 women with fetuses affected with CHB were tested for anti-p200 antibodies, only the p200-free technique showed a significantly higher antibody prevalence with respect to the women with unaffected fetuses (Table 3). There was also a significant difference between the antibody levels of women with CHB and women without CHB,

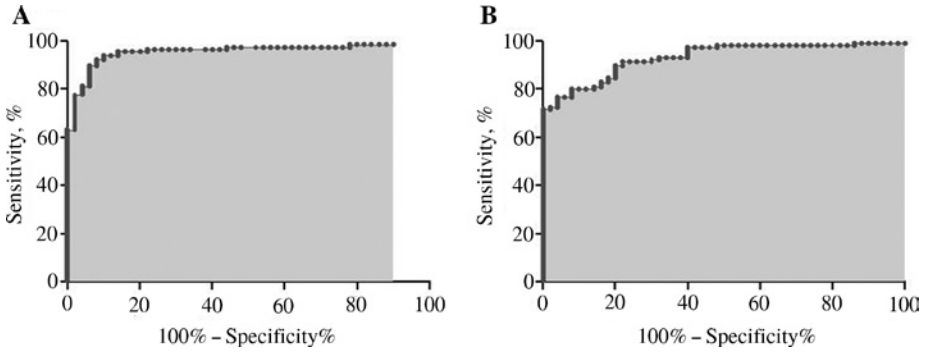

Figure 3: The receiving operating characteristics (ROC) curves are built using anti-SSA/Ro52 positive patients vs. anti-SSA/Ro52 negative ones.
They show a high area under curve for both p200-free (A) and p200-biotin (B) assays, so revealing a very good accuracy of both tests in detecting anti-p200 antibodies.

Table 3: Prevalence of p200 antibodies according the two assays in CHB+ and CHB– pregnant women.

	CHB+ n=24	CHB– n=117	p-Value	OR [95% CI]
p200-Free, n (%)	22 (91.7)	75 (64.1)	0.007	6.2 [1.4–27.5]
p200-Biotin, n (%)	23 (95.8)	94 (80.3)	ns	–

CHB+, pregnant women with fetuses affected by congenital heart block; CHB–, pregnant women with unaffected fetuses; ns, not significant.

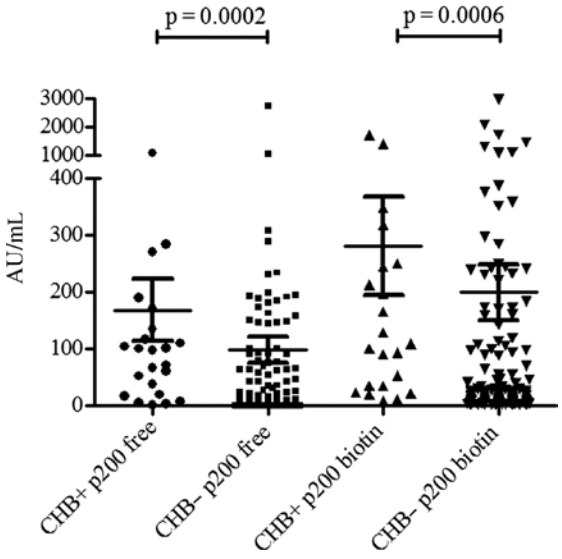

Figure 4: Serum titers of anti-p200 antibodies detected by p200-free and p200-biotin assays in women with or without fetuses affected by congenital heart block.

using raw results both in the p200-free and p200-biotin assay (Figure 4).

The agreement of home-made ELISAs and commercial kits for detection of anti-SSA/Ro52, /Ro60 and anti SSB/La antibodies showed the following Cohen's κ values 0.53/$p < 0.0001$, 0.48/$p < 0.0001$ and 0.51/$p < 0.0001$, respectively.

Discussion

The aim of the current study was to compare, for the first time, the performance of two ELISA assays used to determine anti-p200 antibodies, a promising tool for CHB management. Both methods were performed in our laboratory by the same technician following indications outlined in the literature [7, 10] with minor variations. The stepwise dilutions of the same positive samples with both methods revealed that for equal dilutions, the method employing p200 free as the antigen demonstrated an OD higher than the p200-biotin method. So, the choice to use different serum working dilutions, in particular the highest for the p200 free technique was appropriate.

The intra- and inter-assay coefficients of variation were indeed found to be good for the two techniques. The large number of sera that were examined ensured the validity of these results. A high proportion of CHB cases was detected in the patient cohort, but the high incidence of CHB found in our patient population may have been linked to the fact that the majority of the pregnant women with CHB (17%) were referred to our center by rheumatologic centers throughout Italy. Thus, it is important to remember that the study cannot in any way be considered epidemiological.

The most relevant finding uncovered by our study was the specificity of anti-p200 antibodies for anti-SSA/Ro52 positive sera detected by the p200-free ELISA assay, which was significantly higher than that linked to the p200-biotin method. Another noteworthy finding was the significant prevalence of anti-p200 antibodies in the pregnant women with CHB affected fetuses that was found when the p200-free assay was used; the difference in prevalence in the CHB unaffected and affected groups was not significant when the p200-biotin based assay was used.

There was a good level of agreement between the two methods according to other analyses. In fact, the prevalence of anti-p200 antibodies in the anti-SSA/Ro52 positive women as well as the predictive positive value produced by the two assays were very similar. Moreover, Cohen's κ and Spearman's ρ coefficients showed, respectively, a good concordance and a high correlation. Although Passing-Bablok analyses and Bland-Altman plots revealed a systematic overestimation error of the p200-biotin method compared to the p200-free one.

The low number of pregnant women negative for anti-SSA/Ro52 antibodies can be considered a study limitation, but it is admittedly difficult to find anti-SSA/Ro52 antibody negative subjects in an anti-SSA/SSB positive population.

The high specificity in detecting anti-p200 antibodies in anti-SSA/Ro52 positive patients along with the significant association between CHB and anti-p200 antibodies found using the p200-free ELISA assay can be considered study strengths. In fact, these results could also be taken into account by manufacturers to develop new commercial kits aimed to the spread of the determination of anti-p200 antibodies. There are also some practical advantages that should be considered linked to the p200-free method, which is quicker and more cost-effective.

In conclusion, the p200-free assay with respect to the biotin-based method was more specific in detecting p200 antibodies in women positive for anti-SSA/Ro52 antibodies. In addition, only the p200-free method significantly found p200 antibodies in patients with fetal CHB.

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