A novel assay for the rapid and sensitive detection of precipitating SSA autoantibodies exhibits improved diagnostic specificity

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Abstract

Background: The detection of autoantibodies to SSA antigen is considered critical for the diagnosis of primary and secondary Sjögren's syndrome as well as for a number of other rheumatological disorders including subacute cutaneous lupus and neonatal lupus erythematosus/congenital heart block. While current methods (such as ELISA) for the detection of SSA antibodies are highly sensitive, they also exhibit a significant "grey zone" leading to a high incidence of "equivocal" results, many of which constitute genuine false positives that lead to significant diagnostic confusion amongst requesting clinicians. Although the detection of precipitating autoantibodies to SSA is considered by many to have superior diagnostic specificity, traditional methods (such as immunodiffusion and immunoelectrophoresis) are considered to be cumbersome and time consuming. We describe here CIEDia, a rapid, sensitive assay for the detection of precipitating SSA autoantibodies and compare the sensitivity and specificity of this assay with a commercial ELISA assay.

Methods: Fluorescent SSA antigen was synthesised using DyLight 488 or AlexaFluor 488 reactive derivatives and commercially available purified native SSA antigen. In the CIEDia assay precipitating SSA autoantibodies are detected by immunoelectrophoresis on thin agarose gels where the precipitin formed by the interaction of patient autoantibodies with fluorescent SSA antigen can be viewed in real time using a bench top blue light transilluminator with an amber filter. Using this procedure precipitating SSA autoantibodies could be detected in less than 30 minutes with a sensitivity approaching that of ELISA.

Results: To investigate the diagnostic specificity of the new assay SSA autoantibodies in 228 patient samples that were found to be ANA positive as determined by Hep2-IF during routine laboratory testing were further analysed using a commercial ELISA assay (Phadia) and the CIEDia assay. Using the ELISA assay the results revealed 154 samples to be negative, 70 to be "equivocally" positive and 4 to be "unequivocally" positive for SSA autoantibodies. The CIEDia assay also found all of the ELISA-negative samples to be negative for precipitating SSA antibodies whereas all of the ELISA "unequivocally" positive samples were also found to be positive in the CIEDia assay. In contrast the 70 patient samples that gave an "equivocally"positive result in ELISA, only 38 were found to have precipitating SSA antibodies as determined by CIEDia. Two clinical immunologists blinded to the serological results independently reviewed patient records for all 228 samples to determine clinical presentation of Sjögren's syndrome and/or other anti-SSA related diseases using internationally accepted diagnostic criteria. The results indicated that the CIEDia assay for precipitating SSA antibodies has a diagnostic specificity of 91.6%, significantly higher than that of the commercial ELISA assay which was found to be 75.8%.

Conclusion: The CIEDia assay is a rapid, easy to perform method for the detection of precipitating autoantibodies. While the sensitivity of the method for anti-SSA antibodies was found to approach that of ELISA, the diagnostic specificity was found to be superior. It is envisaged that this novel assay could play a useful role in routine clinical laboratories to further elucidate samples yielding "equivocal" results with alternative autoantibody assays.

Introduction

The availability and technical simplicity of commercial ELISA assays for the detection of SSA antibodies have led to them being readily adopted by clinical laboratories. While ELISA techniques are more likely to detect low titre and low affinity antibodies than more traditional gel-based assays, the clinical relevance of this is poorly understood. ELISA assays do not clearly segregate patients as being either positive or negative for SSA antibodies; instead they give rise to a significant grey zone where patient samples yield low positive results and are considered as being "equivocal" for SSA antibodies (Figure 1). By contrast assays that detect precipitating SSA antibodies require a polyclonal immune response (Figure 2) and can therefore be considered as being more stringent with regard to yielding a positive result. This study proposes to resolve the dilemma of equivocal SSA patient samples by using a new, rapid and sensitive assay (CIEDia) for the detection of precipitating SSA antibodies. This alternative assay was found to have a significantly improved positive likelihood ratio for SSA-associated disease relative to a commercial ELISA assay.

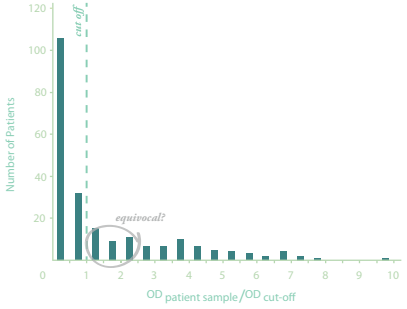

Figure 1. Current ELISA methods for the detection of SSA antibodies give rise to a significant number of "equivocal" results. 228 Patient samples that were found to ANA positive by Hep2-IF during routine laboratory testing were assayed for SSA antibodies using a commercial ELISA kit (Phadia).

Methodology

SSA antibodies were assayed using a commercial ELISA kit (Phadia, *www.phadia.com*) in 228 patient samples that were found to be positive for antinuclear antibodies by Hep2 immunofluorescence during routine laboratory testing. Fluorescent (Alexafluor 488 or DyLight 488) derivatives of native SSA antigen were provided by AROTEC Diagnostics Limited (*www.arodia.com*). Antigen derivitisation did not result in any significant loss of reactivity with SSA antibodies when assessed by ELISA and Western blot. The experimental set up for the real-time detection of precipitating SSA antibodies using fluorescent SSA antigen (CIEDia) is shown in Figure 3. Clinical assessment of patient records for the presence of Sjögren's syndrome or other SSA-associated disease was carried out according to Vitali et al.[1] by two consultant clinical immunologists blinded to the serological results. Statistical analysis was carried out according to the McNemar test[2].

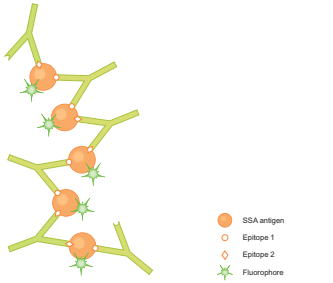

Figure 2. Detection of precipitating SSA autoantibodies using fluorescently labelled antigen. Polyclonal SSA antibodies form a multimeric aggregate that appears as a fluorescent precipitate during counterimmunoelectrophoresis.

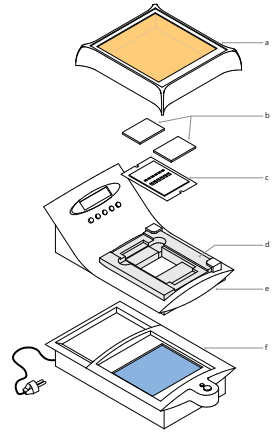

Figure 3. Apparatus for the real time detection of precipitating SSA autoantibodies. **a.** E-Gel® Safe ImagerTM transilluminator amber filter (invitrogen); **b.** CIEDia buffer strips; **c.** CIEDia agarose immunoelectophoresis gel; **d.** CIEDia adapter; **e.** E-Gel® iBaseTM power system (invitrogen); **f.** E-Gel® Safe ImagerTM transilluminator (invitrogen). The apparatus is assembled together with the agarose gel **(c)** and the buffer strips **(b)** are applied. Patient samples are added to the anode wells and fluorescent SSA to the cathode wells. The amber filter **(a)** is placed on top and current is applied through the power system **(e)**. Precipitating SSA antibodies can be detected by direct observation through the amber filter after activation of the transilluminator **(f)**.

Results

Performance data of the CIEDia assay for the detection of precipitating SSA antibodies is shown in Figure 4. CIEDia is a countercurrent immunoelectrophoresis technique operated at a pH where the antigen is negatively charged while antibodies are positively charged. Fluorescent labelling of the SSA antigen allows the operator to observe its migration through the gel matrix in real time when an electric current is applied. Precipitating antibodies in SSA positive patient samples will migrate towards the labelled antigen, halt its migration and thereby give rise to a readily observable precipitin band. Antibodies in SSA negative patient samples will fail to impede the migration of the labelled antigen and no precipitin will be formed. The assay is rapid, simple to perform and detects precipitating antibodies with a sensitivity that is far superior to traditional gel-based methods. The assay was used to test for the presence of precipitating SSA antibodies in the 228 patient samples that had previously been tested by ELISA by an operator blinded to these results. The results (Figure 5) show that, although there was a perfect correlation for the two assays for patient samples found to be negative for SSA antibodies in ELISA, precipitating SSA antibodies could also not be detected in a significant number of patient samples found to be above the cut-off for the ELISA assay. The incidence of ELISA positive / CIEDia negative samples was particularly significant for samples with an ELISA result immediately above the cut-off (i.e. for grey-zone "equivocal" samples). Examination of patient records revealed that for 122 patients sufficient information was available to undertake a clinical assessment. Statistical analysis of the results is presented in Table 1. The results indicate that, although the CIEDia assay is less sensitive than the ELISA assay, it is significantly more specific with a superior positive likelihood ratio (LR+).

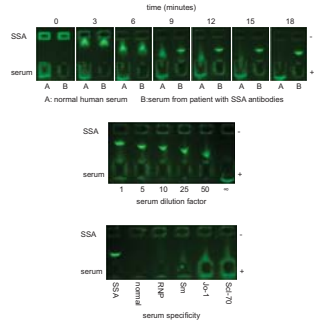

Figure 4. Rapid and sensitive detection of precipitating SSA autoantibodies. **Top panel:** time course of the formation of fluorescent immunoprecipitate during immunoelectrophoresis. **Middle panel:** detection of precipitating SSA autoantibodies at different serum dilutions. **Bottom panel:** specificity of the CIEDia assay; only patient samples containing SSA antibodies are able to form an immunoprecipitate.

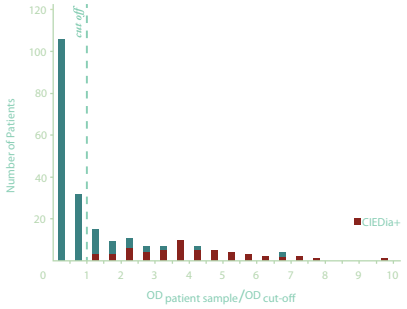

Figure 5. Detection of precipitating SSA autoantibodies - comparison with a commercial ELISA assay. The patient samples tested in Figure 1 were reassayed using the CIEDia assay. It can be observed that there are a significant number of ELISA SSA positive samples that negative for SSA precipitating antibodies.

	Disease positive		Disease negative	
	ELISA	CIEDia	ELISA	CIEDia
Result positive	23	19	23	8
Result negative	4	8	72	87

	Sensitivity	Specificity	LR+	LR-
CIEDia	70.4%	91.6%	8.4	0.3
ELISA	85.2%	75.8%	3.5	0.2

Table 1. Sensitivity and specificity of the CIEDia assay for detecting precipitating antibodies; comparison with a commercial ELISA assay. **Top panel:** assay results of 122 patients for which sufficient data was available to make a clinical assessment (positive or negative) for SSA-related disease. **Bottom panel:** calculation of sensitivity, specificity and likelihood ratios for the commercial ELISA and CIEDia assays (LR+, likelihood ratio positive; LR-, likelihood ratio negative).

Discussion

ELISA methods for the detection of SSA antibodies are known to be very sensitive and as such have a very good negative predictive value (NPV). A trade-off to this high sensitivity is the fact that such assays give rise to a significant number of low positive "equivocal" results, many of which are likely to be false positives where SSA associated disease is absent. It is our proposal that clinical samples found to be positive for SSA antibodies with a sensitive entry diagnostic procedure should be re-examined with an alternative more stringent procedure that exhibits a significantly higher positive predictive value (PPV). We believe that the CIEDia assay described here constitutes such a confirmatory procedure that will help resolve the diagnostic confusion caused by low positive results inherent to current SSA antibody assays.

References

1. Vitali, C. et al. (2002) Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis;61:554-558
2. Altmann, DG (1991) Practical statistics for medical research. Chapman & Hall, London, UK

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