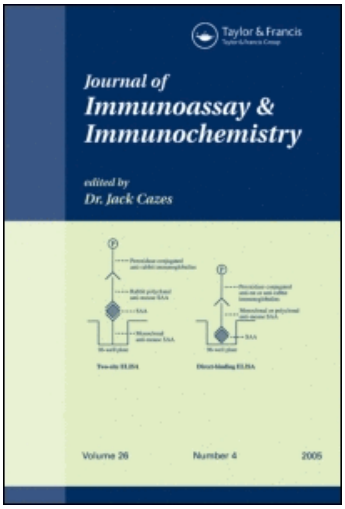

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Comparison between Chicken and Rabbit Antibody Based Particle Enhanced Cystatin C Reagents for Immunoturbidimetry

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Abstract: We have compared three commercial particle enhanced cystatin C reagents. One of the reagents utilizes chicken antibodies and the other two reagents are rabbit antibody based. We show that the chicken antibody based reagent yields a higher delta absorbance when reacting with the antigen. IgY coupled to latex particles show a strong scatter response even at high antigen concentrations in contrast to the steep decline in scatter previously reported for IgY antibodies in solution. The reagent also showed a low CV for duplicate samples. Laying hens thus seems as an interesting source of antibodies for particle-enhanced immunoassays.

Keywords: Chicken antibodies, IgY, Rabbit antibodies, Latex particles, Particle-enhanced immunoassays, Cystatin C

INTRODUCTION

There is an increasing interest in egg yolk antibodies (IgY). The use of chickens for antibody production instead of mammals avoids the second painful step, i.e., the blood collection, which can be replaced by antibody extraction from the eggs thus reducing animal suffering.[1,2] Antibodies

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derived from egg yolk have properties that differ from mammalian antibodies in several aspects and may sometimes be a more suitable choice in designing solid-phase immunometric assays than mammalian antibodies. Chicken antibodies do not activate the human complement system[3] which is a well known source of interference in sandwich immunoassays.[4] Capture antibodies bound to a solid surface are potent complement activators and the activated complement components will react with the assay antibodies thus partly blocking the antigen binding sites.[5]

Rheumatoid factor (RF) is a major source of interference in many immunoassays, reacting with the Fc portion of mammalian IgG.[6] The disease usually associated with RF is rheumatoid arthritis, but RF is also present in blood samples from patients with many other diseases and also healthy individuals.[7,8] Most immunoassays use mammalian polyclonal or monoclonal antibodies, which are subjected to RF binding, thus giving false positive results. As RF is not able to bind to IgY, chicken antibodies can be useful in assays (e.g., nephelometry, turbidimetry or ELISA) were RF could interfere.[9,10] Another interfering factor is human anti-mouse IgG antibody (HAMA). An increasing number of patients are in vivo treated with monoclonal mouse antibodies and this often provokes an antibody response in the patient resulting in HAMA production. Chicken antibodies do not react with either HAMA so they can also be used to eliminate interference due to these factors.[11]

Thus, chicken antibodies should theoretically have advantages to mammalian antibodies in immunoassays but there are reports stating that IgY are less suited for turbidimetric and nephelometric assays as immune complexes between IgY and the antigen scatter less than a corresponding complex between a rabbit antibody and the same antigen.[12] There is also a steeper decline in the absorbance when reaching antigen excess situations. These findings were based on IgY and immune complexes in solution, but there are considerable differences between a traditional turbidimetric reagent and a particle enhanced reagent. An antibody bound to a surface may have quite different properties to an unbound antibody. Recently there has been an IgY based particle enhanced turbidimetric immunoassay (PETIA) reagent for cystatin C measurements introduced on the market.[13,14] The purpose of this study was to compare the properties of this IgY based reagent with a rabbit IgG based reagents on a high throughput instrument intended for clinical use.

EXPERIMENTAL

Plasma Samples and Cystatin C Reagents

A comparison was performed with consecutive routine requests for Cystatin C. The blood samples were collected in vacutainer tubes containing Li-heparin (367376, Becton-Dickinson, Franklin Lakes, NJ, USA).

Cystatin C immunoparticles with chicken antibodies were obtained from Gentian (Code 1014, Gentian, Moss, Norway). Cystatin C immunoparticles with rabbit antibodies were obtained from Dade Behring (N Latex Cystatin C, Dade Behring, Deerfield, IL, USA) and Dako (Cystatin C PET, Dako, Glostrup, Denmark). The study was approved by the local ethical board at Uppsala University (01-167).

Cystatin C Measurements on Architect ci8200

Plasma cystatin C measurements with Gentian reagents on Architect ci8200 (Abbott Laboratories, Abbott Park, IL, USA) was performed using the following instrument settings: Primary wavelength 548 nm and secondary wavelength 700 nm, sample blank position 18 and spline calibration method. 220 μL reagent 1 and 3 μL sample were mixed with 45 μL reagent 2 in accordance with the manufacturer recommendations.[14]

Plasma cystatin C measurements with Dako reagents on Architect ci8200 were performed using the following instrument settings: Primary wavelength 548 nm and secondary wavelength 700 nm, sample blank at position 18 and spline calibration method. 167 μL reagent 1 was mixed with 2 μL sample and 33 μL reagent 2. This protocol followed as close as possible the protocol recommended by Dako for Modular (Roche, Mannheim, Germany).[15]

Plasma cystatin C measurements with Dade-Behring reagents on Architect ci8200 was performed using the following instrument settings: Primary wavelength 572 nm, sample blank and spline calibration method. 145 μL reagent 1 (3 mL supplement reagent (Dade Behring) and 42 mL diluent (Dade Behring)) was mixed with 20 μL reagent 2 (undiluted N Latex Cystatin C, Dade Behring) and 15 μL sample diluted 1:50 with Dade Behring diluent. The sample dilution was performed automatically by the instrument.[16]

Delta Absorbance at 548 nm Measured by Spectrophotometry

Buffer, latex particles, and patient samples were mixed in the same proportion as used on the Architect ci8200 except that the volumes were increased to give a total volume of 0.9–1.0 mL. After mixing buffer and latex particles the absorbance at 548 nm were measured with a spectrofotometer (Shimadzu 1601PC, Kyoto, Japan). The patient sample was added and the content of the cyvette was mixed. A second absorbance was measured after an additional five minutes and a delta absorbance was calculated.

Antigen Excess

Antigen excess effects on the Gentian cystatin C immunoassay was determined by spiking a serum sample up to 50 mg/L and analyzing the samples

in serial dilutions on a Modular P (Roche Diagnostics, Mannheim, Germany) at Gentian AS. A serum/plasma sample of 3 μL and 230 μL of assay buffer was mixed. After 16.5 cycles (each cycle is 17.9 seconds), 40 μL immunoparticles and 20 μL water were added. After 18 cycles the first absorbance measurement was made, and after 34 cycles the last measurement was made. The difference in these two absorbance measurements was converted to mg/L in accordance with the regression fit of the calibration curve. In total the analysis time was 10 min and 50 seconds, in which the agglutination reaction lasts for 5 minutes and 25 seconds. Primary/secondary wavelength is 546/700 nm.

Analysis of Latex Particles

The cystatin C immunoparticles with chicken antibodies and cystatin C immunoparticles with rabbit antibodies were investigated by Dynamic Light Scattering using a Nicomp 380 Submicron Particle Size Analyzer from Particle Sizing Systems (Santa Barbara, USA) to obtain the particle size distribution before and after addition of cystatin C to the particle suspension. The particles were first measured before addition of cystatin C. Then 9 μL of cystatin C (9 mg/L) was added to 100 μL of each particle suspension and incubated for five minutes before the next runs were started. The measurements were taken at room temperature and the run time was set to five minutes for each measurement. All reported diameters are the mean of three repetitive measurements.

RESULTS

Cystatin C Analysis with the IgY Reagent (Gentian) on Architect ci8200

The instrument adds patient sample and buffer. After mixing, the absorbance is measured every eighteenth sec. Each reading point is presented as a dot in Fig. 1. After point 16, the antibody reagent is added and the samples are mixed. The last measurement is point 33. The delta absorbance for 548 and is calculated. Figure 1 shows the kinetics of three samples with different cystatin C concentrations. The sample with the highest cystatin C concentration gives the highest delta absorbance.

179 routine patient samples (mean, range) were analysed in duplicate with the IgY reagent to evaluated imprecision. The coefficient of variation for duplicate samples was 0.58%. 118 of these samples were in the 0.9–2.0 mg/L range which is the clinically most important range. The CV in this range was 0.73%.

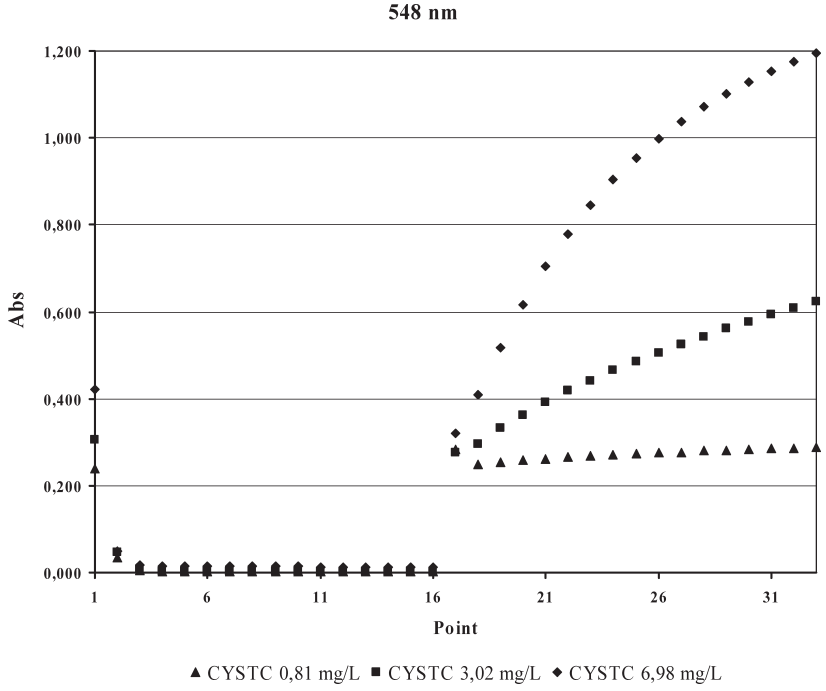

Figure 1. Kinetics of the turbidimetric reactions for three samples with different cystatin C concentrations on Architect ci8200. The instrument measures the absorbance every eighteenth sec. Each dot in the figure represents a separate reading. Patient sample and buffer is added and mixed prior to the first reading. After point 16, the antibody reagent is added and the samples are mixed. The last measurement is point 33. The delta absorbance for 548 is calculated automatically by the instrument.

Delta Absorbance at 548 nm Measured by Spectrophotometry

The delta absorbance at 548 nm for the IgY reagent was 1.189, while the delta absorbances for the Dade reagent was 0.500 and for the Dako reagent 0.216 with the same patient sample (7.0 mg cystatin C/L). The IgY reagent thus yielded more than two-fold higher delta absorbance than the Dade-Behring reagent and more than five-fold higher delta absorbance than the Dako reagent.

Antigen Excess

The effect of antigen excess was studied by plotting the measured cystatin C concentration (measured in duplicates) vs theoretical cystatin C concentration (Fig. 2). Patients' samples do not exceed 12 mg/L even in patients with very severe kidney disease. In this study a theoretical concentration of 50 mg/L

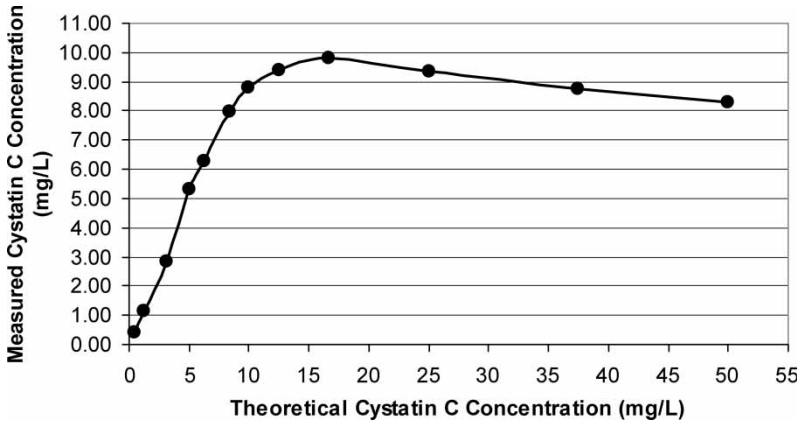

Figure 2. Antigen excess curve for the Gentian cystatin C immunoassay. Measured cystatin C concentration (mg/L) vs theoretical cystatin C concentration. The plot shows that antigen excess is not present below 50 mg/L cystatin C.

cystatin C was reported as 9 mg/L cystatin C. Thus, even a six-fold excess of antigen in comparison with severe kidney disease did not result in an antigen excess situation that could be interpreted as a test result within the patient range.

Analysis of Latex Particles

The Dynamic Light Scattering analysis resulted in a mean diameter of 125.5 ± 0.35 nm for the cystatin C immunoparticles with chicken antibodies (Gentian) and 126.5 ± 1.1 nm for the cystatin C immunoparticles with rabbit antibodies (Dako). After incubation with cystatin C, the mean diameter of the immunoparticles with chicken antibodies had increased to 142.6 ± 0.75 nm, while the immunoparticles with rabbit antibodies had a mean diameter of 127.9 ± 2.2 nm.

DISCUSSION

IgY is found in birds, reptiles and amphibian. It is the functional equivalent of mammalian IgG, has a molecular weight of approximately180 kDa and consists of two heavy and two light chains.[12] IgY possesses two antigen-binding sites and should, in principle, precipitate and agglutinate multivalent antigens. This does not always occur in solution even if the antibodies bind strongly to the antigen. Some studies report that IgY mainly precipitates the antigen at raised salt concentrations with an optimal precipitation at NaCl

concentration tenfold over the physiological concentration (e.g., 1.5 M NaCl).[17] The problems with poor precipitation are not related to the Fc region or to valency. Chicken IgY has the expected valency of 2.0 both in the presence or absence of high salt.[18,19] It seems more likely that the lower precipitation capacity is due to the conformation differences that exist between mammalian IgG and chicken IgY.[20] IgY does not possess the hinge region that gives the IgG molecule much of its flexibility. These studies were performed with IgY in solution. Antibodies bound to a solid surface may have different properties than free antibodies. We have thus compared the turbidimetric activity of IgY based immunoparticles with rabbit IgG based immunoparticles against the same antigen. A second comparison is performed based on measurements of dynamic light scattering of the antibody coated particles and their antigen complexes, respectively. This study clearly indicates a more extensive aggregate formation in case of the Gentian particles, to judge from the larger increase in average diameter following complex formation. The Dako and Gentian reagents are intended for turbidimetric assays while the Dade-Behring is intended for nephelometric use. We have previously reported that all three methods yielded low CV on the Architect ci8200 instrument.[14–16] We found that the kinetics of the reaction between the antigen and the three immunoparticles tested were similar, but the IgY based immunoparticles yielded a higher delta absorbance on the Architect instrument. This may be related to the evolutionary differences between birds and mammals.[21] The higher delta absorbance was confirmed by the spectrophotometric test. The IgY based reagent yielded a very low CV for duplicate analyses of patient samples. In our routine work we have performed more than 30,000 cystatin C assays. In none of these tests the antigen content has exceeded approximately 11 mg/L and thus within the measuring range of all three methods. The antigen excess curve shows that there is a very broad safety range to antigen excess conditions with the IgY particles. Colloidal dispersions of IgY-covered latex particles have also been shown to be more stable than those sensitized by IgG, which may improve reagent stability.[22,23]

CONCLUSIONS

IgY seems well suited for the use in particle enhanced turbidimetric immunoassay (PETIA) reagent. In contrast to previous reports on IgY antibodies in solution, IgY bound to particles gave higher delta absorbance and a lower method CV than the corresponding rabbit antibodies. Thus, the IgY based cystatin C PETIA shows that chicken IgY can provide a high analytical quality. This in combination with the other advantages of chicken antibodies (e.g. lack of complement RF and HAMA interference) makes IgY an interesting alternative to mammalian antibodies for this type of immunoassay.

ABBREVIATIONS

IgY, immunoglobulin Y; IgG, immunoglobulin G; HAMA, human anti-mouse IgG antibody; RF, rheumatoid factor; PETIA, particle enhanced turbidimetric immunoassay.

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