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Comparison of Anti-D Titration Testing by Automated Column Agglutination Technology on Glass-Beads and Standard Tube Method

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Abstract*— *Introduction: *Antibody titration is important in the follow-up of alloimmunised pregnant women and anti-D is still the most relevant specificity for hemolytic feto-neonatal disease risk. Column agglutination technology is an attractive alternative to traditional tube method for antibody titration because of its technical ease, operator independence, and automation possibility. This study's objective was to compare automated column agglutination technology on glass-beads and standard tube method in performing anti-D titrations.* ***Methods:*** *Serial plasma dilutions from 67 samples with detectable anti-D (IP, passive or AI, immune) were prepared with Ortho Vision platform and tested against R_0r 3% reagent red cell using Anti-IgG Ortho BioVue cassettes. The reaction grades read by the system were reported and Titre Score (TS) was also calculated. Titration results were compared with those obtained by parallel serial dilution with the tube technique against the same single-dose phenotype 3% red cells, following AABB standard method.* ***Results:*** *Anti-D titer range was 1-512 both in column and in tube. Column titer was identical to tube titer in 68.6% of samples, differed of 1 dilution in 22.5% and of 2 dilutions in only 9% of cases. The grade of agreement between the two methods was assessed and the bias in the mean differences was computed. In the IP antibody group (n=37) anti-D column titration results were 0.27 (95% CI = -0.73 to 1.27) additional dilutions greater than in tube. The median TS values in tube test were 3 (range 3-23) in prophylactic category and 29.5 (range 3-90) in the immune group. In BioVue titration, the median TS values were 6 (range 3-33) and 40 (range 3-103), respectively. At a tube titer of 16, the sensitivity of BioVue titrations in immune patients was maximal (100%) at the same column titer of 16, whereas specificity was maximal (100%) at a column titer of 32.* ***Conclusions:*** *Our data show a good correlation in anti-D titration results against single-dose reagent cells between automated column agglutination technology on glass-beads and standard technique in tube. A maximum difference of 2 dilutions was observed with column data showing an overall higher sensitivity, as expected, but with a high percentage of concordance. The grade of agreement between the two methods was particularly good in patients with low titer anti-D with supposed passive nature. TS results were also evaluated and confirmed as a good tool to predict the nature, passive or immune, of detected anti-D. Further data are needed to compare titration results in immune anti-D and to determine clinically significant range for referral to a high-risk obstetrician.*

Keywords— *Anti-D, HDFN, antibody titration, standard tube method, automated column agglutination technology on glass-beads.*

I. INTRODUCTION

Anti-RhD antibodies still remain an important cause of haemolytic disease of the fetus and the newborn (HDFN), though its incidence has been reduced by the introduction of ante- and peri-natal anti-D prophylaxis. For a correct management of HDFN, it is important to carry out a screening for red cell antibodies to all women within the first trimester of pregnancy, together with ABO/Rh typing [1,2]. When a positive antibody screen is obtained and a clinically significant red cell antibody is identified, antibody concentration should be evaluated and monitored every 4 weeks until 18 weeks gestation, thereafter every 2-4 weeks on the basis of clinical relevance [2]. Antibody titration is a semi-quantitative method, applied in transfusion medicine for the management and monitoring of HDFN risk. Titration test mainly consists in preparing twofold dilutions of the serum/plasma with selected red cells, to identify the antibody reactivity end point and to evaluate the relative amount of antibody present in the sample. Results are expressed as the reciprocal of the highest serum dilution that shows macroscopic agglutination. Besides, anti-D prophylaxis programmes, though hugely successful in reducing HDFN incidence, have yield the need to discriminate the passive or immune nature of detected anti-D [1]. Quantification of anti-D with continuous flow analysis (CFA) is being employed for the definition of anti-D nature [3], but an alternative method has been proposed with converting reaction grade on column agglutination techniques into a score value, namely Titre score (TS), that is expression of the avidity of the antibody [4].

In our laboratory, anti-D antibody titration is performed with the standard tube method [5] and fetal surveillance is activated for the critical titer value of 16 or if a twofold or greater increase in titer during pregnancy is detected. Tube testing is a manual method and therefore subject to numerous variables both in the execution phase and in results interpretation. Immunohematology laboratories are interested in implementing anti-D antibody titration test through the use of micro-columns in gel or with glass-beads on an automated instrument, in order to reduce results variability. Recent studies [6,7] have shown a reduced variance in performing antibody titrations by gel than tube, with titration results on average higher than in tube, by a factor of 1–2 titer dilutions or even greater, mainly with the use of double-dose reagent cells. On the other hand, consistent studies evaluating column titration on glass-beads and using single-dose reagent cells are to be set up.

The aim of our study was to compare results of anti-D titrations against R_0r cells between automated column agglutination technology on glass-beads and manual standard

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technique in tube. The reaction grades read by the system were reported and TS was also calculated, to evaluate its usefulness in determining whether anti-D was from immunoprophylaxis rather than from immune stimulation.

II. Patients and Methods

The population studied included 37 patients with anti-D from immunoprophylaxis (IP) and 30 patients with anti-D from active immunization (AI). Of each plasma sample, obtained by centrifugation from an EDTA sample, was prepared twofold dilutions series (in 1:1 to 1:1240) in physiological solution, for both the two different detection methods. For standard tube method in liquid phase (LP) [5], an amount equal to 60 µL of the 3% R_0r heterozygous cell from Panel 16 Immucor, panel A Ortho, or red blood cells resuspended in physiological solution was used. After incubation at 37° C for 60 minutes, 3 washes with physiological solution were performed and 100 µL of BIO-RAD anti-IgG serum were added. After performing an high-speed centrifugation for 15 seconds, test results were examined and macroscopically recorded. The titer was reported as the reciprocal of the highest dilution that produces 1+ macroscopic agglutination [5]. No potentiators as albumin, PEG or LISS and/or enzyme-treated red blood cells were used for test tube test. All tubes with negative result were tested by adding 1 drop of Coombs Control (IgG) coated red blood cells.

Column agglutination test on glass beads (CAT-gb) was carried out with Ortho Biovue® System IgG cassettes. Preparation of the dilutions, dispensing samples and reagent (40 µL of sample and 10 µL of cell 4 R_0r - Resolve Panel A, Ortho® at 3%), incubation at 37°C for 15 min, centrifugation and interpretation of results, were performed on the Ortho Vision platform.

For both methods, the observed strength of agglutination was assigned a number, and the sum of the score values of each positive reaction was calculated to evaluate the titre score (TS), another semiquantitative measurement of antibody reactivity [5]. To statistically determine the degree of agreement between FL and CAT methods, we constructed a Bland-Altman plot [8]. Furthermore, sensitivity and specificity of the CAT method were calculated in patients with immune antibodies to evaluate clinically significant threshold, given that this limit is fixed at 16 with standard tube technique.

III. Results

The results of anti-D titrations with standard tube method (LP) and CAT-gb method, showed a positive correlation on the general population, with r = 0.97. Anti-D titers were the same in 68.6% of samples (46/67), different for one dilution in 2.5% of samples (15/67), different for two dilutions in 9% of samples (6/67) while no difference was found for three or more dilutions.

In patients with IP anti-D, antibody titration values were lower, as expected, with a maximum titration value of 4 in LP and 8 in CAT-gb. Titers were the same in 75.7% of the samples (28/37), different for a dilution in 21.6% of the samples (8/37) and only 1/37 sample showed two dilutions of difference. In patients with AI, anti-D antibody concentrations were found to be more variously distributed, with a median of 8 in LP and 12 in CAT-gb and a range from 1 to a maximum titration value of 512 in both LP and CAT-gb. Titers were the same in 60% of samples (18/30), different for one dilution in 23.3% of samples (7/30) and for two dilutions in 16.6% of samples (5/30).

The grade of agreement between the two methods was assessed and the bias in the mean differences was computed. In the IP antibody group (n=37) anti-D column titration results were 0.27 additional dilutions greater than in LP (95% CI = -0.73 to 1.27) (Figure 1). In AI group patients (n=30), column titration results were 0.50 additional dilutions greater than in LP (95% CI = -1.11 to 2.11) (Figure 2).

In patients with IP anti-D, TS showed a median of 3 in LP with a range between 3 and 23 and a median of 6 in CAT-gb with a range between 3 and 33. In patients with anti-D from AI, TS showed median and ranges higher both in LP and CAT: in LP median was 29.5 with ranges between 3 and 90, while in CAT-gb median was 40 with ranges between 3 and 103 (Figure 3). Only in two cases TS in CAT-gb was lower than TS in LP.

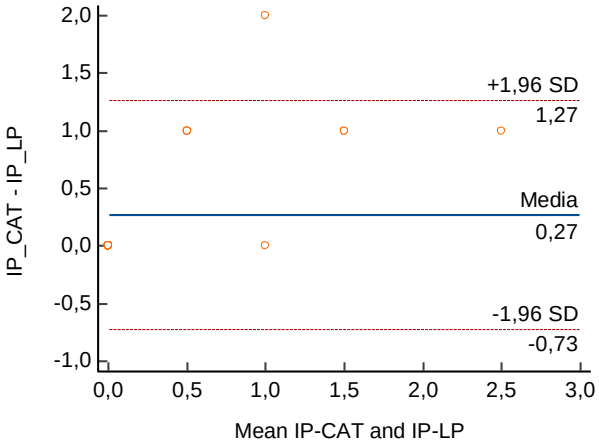

Fig. 1. Bland-Altman plot of anti-D titration in IP anti-D patients with LP *vs* CAT-gb methods.

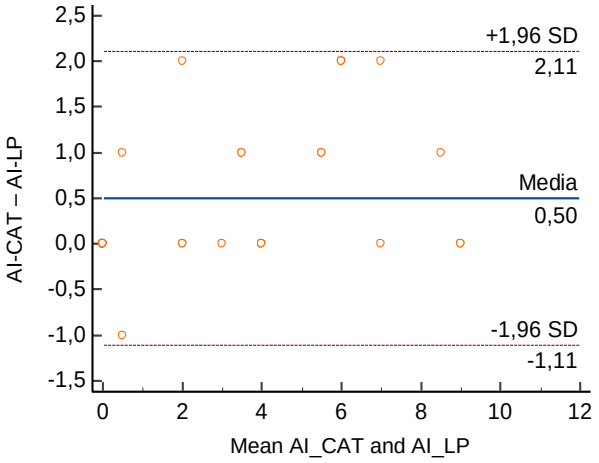

Fig. 2. Bland-Altman plot of anti-D titration in AI anti-D patients with LP *vs* CAT-gb methods.

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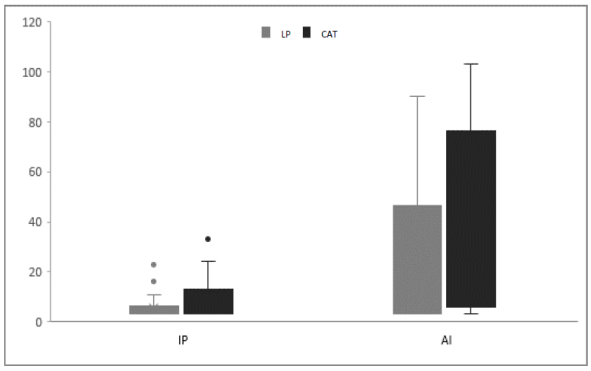

Fig. 3. Titre Score median and ranges in IP and AI anti-D patients with LP and CAT-gb methods.

The TS scores in IP patients, obtained with both methods, were always lower than 35. In AI patients we also evaluated the sensitivity and specificity of the CAT-gb to try to define a critical range. Given that this limit is fixed at >= 16 with LP, if the range is kept at 16 also for the CAT-gb, the sensitivity was 100% and the specificity 88.2%. If the CAT-gb range was raised to 32, the sensitivity decreased to 76.9%, while the specificity remained at 100%.

IV. Discussion

Identification of antibody specificity and accurate titration are essential for HDFN management, in case of positive indirect antiglobulin test. Antibody titration can be performed with different methods, but it's characterized by an intrinsic variability [9] and lack of standardization [10]; alternative novel techniques can be used to mitigate discrepancies noted with current methods.

A recent review about anti-D titration [7] evaluated the relationship between results obtained in test tubes and in gel. In the majority of the reviewed studies, titrations performed in gel were twofold higher than those observed in tube, and in the most cases double dose test cells were used (R1R1, R2R2, R1R2).

One of the methods developed recently for titration is column agglutination test on glass-beads (BioVue®). This method was compared to conventional tube agglutination test to evaluate the antibody concentration on anti-D immunoglobulin preparations in quality control laboratories: results showed that the BioVue column agglutination test is comparable to conventional tube agglutination, with titers on the BioVue® column consistently twofold higher than those of the conventional tube test, with similar sensitivity and specificity [11]. The study concluded that the BioVue Column Agglutination Technology test method can replace tube agglutination method for quality control purposes.

Column agglutination technology on glass beads is an attractive alternative to traditional tube method for antibody titration on patient samples, because of the advantages of small sample size, greater uniformity between repeat tests, and decreased operator dependence, but we could not find consistent studies on its routine application in immunohematology laboratory.

In our work titration results with automatized BioVue Column Agglutination Technology were compared with those obtained by parallel serial dilution with the tube technique against the same single-dose phenotype 3% red cells and Antihuman IgG, following AABB standard method. On the Ortho Vision Analyzer platform, the test with microcolumn glass-beads was set according to a method comparable to AABB standards: same zygosity of the test red blood cells, use of anti-IgG serum, absence of enhancing means, such as albumin, low-ionic strength saline (LISS) or enzyme treated RBCs. R_0r heterozygous test cells were chosen to mimic fetal condition in a RhD negative pregnant mother, as suggested by Italian guidelines [2].

Our data show a good correlation in anti-D titration results against single-dose reagent cells between automated column agglutination technology on glass-beads and standard technique in tube

in patients with passive or immune anti-D. In our comparative study, titration levels gave identical results in 68.6% of the total test population, and different for at most two dilutions in the remaining percentage of cases, with a tendency for higher results with anti-D titration in CAT-gb than with standard method, as expected due to the higher sensitivity of the method and as generally reported in the literature for column methods.

The grade of agreement between the two methods was particularly good in patients with low titer anti-D with supposed passive nature.

In patients with AI, our preliminary data on anti-D titration suggested not to switch from a 16-32 titre to determine the activation of fetal surveillance and to maintain 100% sensitivity with automated column agglutination technology on glass-beads.

In our comparative work, we also evaluated the difference between Titre score, obtained with both methods: TS represents additional information relating to the strength of agglutination and the avidity of the antibody, useful for understanding whether we are in presence of a dangerous titer, for discriminating between passive or immune antibodies and for follow-up in pregnancy.

In a multicenter comparative study [4] an assessment of the significance of the automated TS on Biovue® microcolumn was conducted as an alternative method to CFA, that is the UK standard for discrimining passive or immune anti-D in pregnancy. In this comparative study, samples tested with CFA were also tested with an automatic anti-D test on Ortho Vision and then converted to TS. Automated serial dilutions were performed on the Ortho Vision platform and heterozygous cells (R1r 0.8%) and BioVue Anti-IgG Ortho Clinical Diagnostics cassettes were used. The results of this study demonstrated that an automatic TS can reliably predict the nature of anti-D detected in pregnancy as passive when using a TS of 35.

Similarly, in our study, which differed in type and concentration of the heterozygous cell (R_0r 3%), TS scores in patients with passive antibodies, obtained with both methods compared were always lower than 35.

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Further studies are needed to assess the critical threshold and to evaluate the suitability of CAT-gb for titration of antibodies different from anti-D.

Conflict of interest

There are no conflict of interest

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