ORIGINAL ARTICLE

Comparison of tube and column agglutination technique for the quantification of blood group antibody anti-D by titration

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ABSTRACT

Objectives: Laboratory titration of blood group antibody anti-D is used to monitor immunized pregnancies. A titre of 32 is commonly used to trigger clinical procedures to investigate whether a fetus is suffering complications of haemolytic disease of the fetus and new-born. This so called "trigger titre" is based on historical tube methods. Column agglutination technology (CAT) has largely superseded tube for routine blood group investigations and is used by some laboratories for antibody titration. However, sensitivity differences exist between the two testing platforms and no trigger titre for clinical intervention has been established for column. Furthermore, titres suffer from a lack of reproducibility, produced by many factors including variation in equipment, consumables and techniques of different laboratory scientists. As a consequence, results of studies reporting sensitivity differences between tube and column titres vary widely. In this study, variations were minimised by having one scientist perform titration on the same samples at the same time, in tube and column. The work aims to add to the body of data available on sensitivity differences between CAT and tube for titration of anti-D, with a view to informing a trigger titre for CAT.

Methods: Twenty plasmapheresis donations containing anti-D were titrated under standardised conditions by one individual by indirect antiglobulin technique in tubes and columns. Reproducibility was assessed by titrating a reagent anti-D with each batch of plasmapheresis donations tested.

Results: Column titres of anti-D in plasmapheresis donations were on average 0.8 dilutions higher than tube, showing that column is more sensitive than tube. Reproducibility was high for both techniques, with titre varying ±1 dilution from the mode titre in both tube and column.

Conclusions: Consideration should be given to raising the trigger titre by one dilution when performed by CAT for titration of anti-D. However, it is key that laboratories communicate with clinicians to further evaluate whether the laboratory platform and the trigger titre are appropriate in their local setting.

Keywords: Anti-D, titration, column agglutination technology.

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INTRODUCTION

Haemolytic disease of the fetus and new-born (HDFN) is the destruction of red blood cells (RBC) of a fetus or neonate by antibodies produced by the mother (1). These antibodies are maternal immunoglobulin G (IgG) directed against fetal red cell antigens and include anti-D. It is important to monitor the quantity of anti-D in mothers throughout an immunized pregnancy, as this antibody has the capacity to cause severe clinical HDFN, and higher levels often correlate with greater disease severity (2,3).

There are different laboratory methods available to estimate the quantity of anti-D in a mother's plasma including titration, continuous-flow analysis and flow cytometry. All methods suffer from some degree of technical variation leading to a lack of reproducibility both within and between laboratories (4-6), but titration remains the most used method worldwide.

Titration is performed by preparing a series of dilutions of mother's plasma, with dilutions tested against RBC which express the target antigen, in a haemagglutination technique capable of detecting IgG. The end point is the highest dilution (representing the lowest concentration of antibody) which can agglutinate RBC expressing the antigen. A titre of 32 is commonly regarded as the "trigger titre"; the level at or above which clinical investigations should be performed to ascertain the health of the fetus (7,8). The traditional method for titration is the manual tube technique. Dilutions of patient plasma are incubated in tubes with a suspension of antigen positive RBC, then the RBC are washed to remove unbound immunoglobulins naturally present in the patient plasma. Anti-human globulin (AHG) is added to allow agglutination of IgG-coated RBC. The tube is briefly centrifuged and examined and graded for haemagglutination.

In contrast, column agglutination technology (CAT) is performed in a microcolumn filled with either dextran-acrylamide gel or glass bead matrix (9,10). AHG is already in the column and no washing is required because the unbound immunoglobulins remain in the supernatant and do not contact the AHG. In a positive reaction, red cells will agglutinate and are trapped in the matrix during centrifugation, whilst non-agglutinated cells form a pellet of free cells at the bottom of the column.

Lack of reproducibility is an accuracy limiting factor in manual titration methods (5) and can be largely attributed to technique variability between personnel, with the practice of doubling dilutions magnifying any inaccuracy in the volume delivered across the titre series (1). CAT titres show improved reproducibility compared to tube (5) and CAT is easily automated, which further improves reproducibility (11,12) and removes the subjectivity associated with reading tubes.

Although surveys show some labs now use CAT in preference to tube for antibody titration (7), CAT has not been universally adopted due to sensitivity differences between tube and CAT. CAT is more sensitive than tube for detection of some antibodies, notably Rh antibodies (13-17). Due to this sensitivity difference, and a lack of large prospective studies comparing CAT titre results with clinical outcomes, no universally accepted trigger titre has been established for CAT.

This study was an opportunity to compare 20 plasmas in real time using standardised reagents in techniques performed by one individual, thus providing optimum conditions for reproducibility. Reproducibility was assessed by titrating a reagent anti-D with each batch of testing. The aim was to assess if titres were consistently higher, lower or the same when conducted in tube and gel-based CAT, with a view to adding to the data to establish a CAT trigger titre for anti-D levels in pregnant women.

MATERIALS AND METHODS

The cohort consisted of 20 plasmapheresis donations from New Zealand blood donors, supplied by New Zealand Blood Service (NZBS) with permission (NZBS 2020/04). Donations were all from Rh(D) negative individuals with anti-D. The mechanism for immunisation in these donors is unknown. Plasmapheresis bags (average volume 565mL) were received frozen and stored at minus 20 degrees Celsius for up to two years. Bags were

thawed at 4°C. Once thawed, the bags were emptied into a glass beaker, mixed and clotted with 5mL of thrombin (Siemens). After addition of the thrombin, the bags were mixed by inversion and left at 4°C overnight. After mixing again, sieving through muslin was performed to remove clotted material. The filtered serum was then aliquoted into 20mL vials with one vial used immediately for this study. These clotted plasmapheresis donations containing anti-D were tested in batches of two, together with a reagent antiserum (Epiclone IgG + IgM anti-D) as a control to assess reproducibility between batches.

All testing was performed by one operator, using the same electronic pipette (Rainin E250) and the same reagents by the same method (18). The same set of master dilutions was used to titrate both tube and CAT methods. Master serial two-fold dilutions were prepared for each sample using standard protocols in total volumes of 250µL, using phosphate buffered saline (PBS) as diluent. For each plasmapheresis donation, a dilution series was prepared up to a dilution factor of 2048.

For the tube method, 100µL of each master dilution was mixed with 50µL of 3% red cells (R_2R_2 DiaCell II cells (Biorad) and incubated for 1 hour at 37°C. Cells were washed four times with PBS before two drops of poly-specific antihuman globulin (AHG) reagent (Epiclone Anti-IgG-C3d polyspecific green) were added to the dry red cell button. Tubes were centrifuged and examined for agglutination from highest to lowest dilution and graded using the standard 0-4 grading scale (18). Washing adequacy was checked with Coombs Control cells (Biorad, Coombs-Control 4%).

For the CAT method, a 0.8% solution of cells was prepared by adding 2.67mL of the 3% DiaCell II R_2R_2 (BioRad) to 7.33mL Cellstab (BioRad). Fifty microlitres of 0.8% red cells and 25µL of each master dilution were added to columns of Biorad Liss/Coombs gel cards. Cards were incubated for 15 minutes at 37°C and then centrifuged before reading for agglutination and graded using 0-4 grading scale (18). For both tube and CAT methods, the titre was determined as the reciprocal of the highest dilution at which grade 1 agglutination was observed (18).

The number of additional dilutions for CAT compared to tube was calculated for each plasmapheresis donation. For example, where a titre was 8 by tube, and 16 by CAT, this was calculated as plus 1. Where a titre was 8 by tube and 4 by CAT, this was calculated as minus 1. Where the titre was the same by tube and CAT, the calculated additional dilutions equalled zero. This data was used to calculate the average difference in dilutions between CAT and tube.

In addition, a paired t-test was calculated to assess if the titre pairs were significantly different by either method, with raw titre results converted to base 2 logarithm to reduce the large numerical difference between one raw titre value and the next (19).

To assess reproducibility of titres of the control Epiclone anti-D antisera, the mode titre was established, and the number of dilutions falling either side of the mode was counted.

RESULTS

The end point titres of the plasmapheresis donations performed by tube and CAT agglutination are presented in Table 1 and Figure 1. Titres ranged from 1 to 512 by tube, and 2 to 1024 by CAT. Of the 20 samples tested, five (25%) had the same titre by CAT and tube. Eleven (55%) of the samples showed a one dilution higher result and three (15%) showed a two dilution higher result in CAT compared to tube. One sample (5%) showed a one dilution higher result in tube. On average, CAT was 0.8 dilutions higher than tube. When comparing differences between individual pairs of titre results on the same sample the difference is statistically significant ($p = 0.0002$). The results of the control anti-D used to assess batch to batch reproducibility are shown in Table 2. Titres for both tube and CAT varied by a maximum of ±1 dilution of the mode, which was 512 for both tube and CAT.

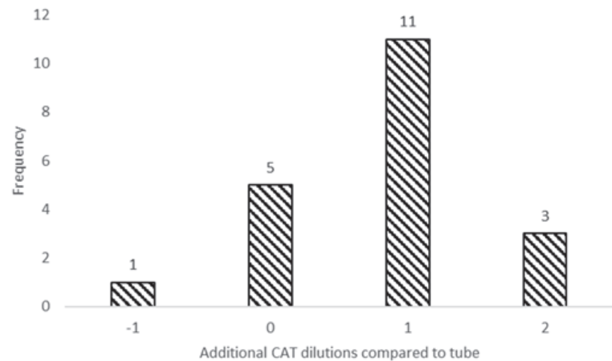

Figure 1. Additional CAT dilutions compared to tube dilutions. The number of additional dilutions for CAT compared to tube was calculated for each plasmapheresis donation. For example, where a titre was 8 by tube, and 16 by CAT, this was calculated as plus 1. Where a titre was 8 by tube and 4 by CAT, this was calculated as minus 1. Where the titre was the same by tube and CAT, the calculated additional dilutions equalled zero.

Table 1. Comparison of titres of anti-D in 20 plasmapheresis donations by manual methods of tube and CAT. Numbers represent the number of samples per titre.

		CAT titre											
Tube titre		1*	2	4	8	16	32	64	128	256	512	1024	2048
	Log base 2	0	1	2	3	4	5	6	7	8	9	10	11
1*	0	-	-	1	-	-	-	-	-	-	-	-	-
2	1	-	1	2	-	-	-	-	-	-	-	-	-
4	2	-	-	1	-	-	-	-	-	-	-	-	-
8	3	-	-	1	-	1	-	-	-	-	-	-	-
16	4	-	-	-	-	-	-	-	-	-	-	-	-
32	5	-	-	-	-	-	-	-	-	-	-	-	-
64	6	-	-	-	-	-	-	-	4	1	-	-	-
128	7	-	-	-	-	-	-	-	-	1	-	-	-
256	8	-	-	-	-	-	-	-	-	2	1	1	-
512	9	-	-	-	-	-	-	-	-	-	1	2	-
1024	10	-	-	-	-	-	-	-	-	-	-	-	-
2048	11	-	-	-	-	-	-	-	-	-	-	-	-

1 = undiluted sample; highlighted cells show when tube and CAT titres are identical*

Table 2. Results of titres of control (Epiclone anti-D) tested on 10 occasions by tube and CAT.

Titre	Tube	CAT
256 (log 8)	4	3
512 (log 9)	5	6
1024 (log 10)	1	1

DISCUSSION

This study confirms that in most cases CAT yields higher titres of anti-D than tube does. This finding emphasizes the need for communication between clinicians and laboratory scientists, to discuss and further investigate the impact of this finding on clinical outcomes for pregnancies in mothers immunized with anti-D.

Column has largely overtaken tube as the platform of choice for methods using haemagglutination as the end point for standard blood grouping methods. Compared to tube, column technology has several advantages; stable and reproducible reactions, ease of automation, and no requirement for washing of cells in the antiglobulin technique. However, many laboratories have not adopted column for titration methods of antibody quantification, due to a lack of internationally established trigger titre for column.

For the clinical application of antenatal monitoring, maternal Rh antibody titres which reach and exceed 32, and titres which increase throughout the pregnancy are used as triggers to inform clinicians that the baby may be at risk of HDFN (7,8). The trigger value of 32 is historical, based on correlations established in manual tube technology, and has not been reviewed or updated to reflect the use of manual or automated CAT. It is of concern that despite this lack of review some laboratories do use CAT as their routine titration platform (7, 17, 20, 21).

In cases where a titre triggers clinical investigation, fetal middle cerebral artery peak systolic velocity measurements (MCA PSV) are performed and depending on the result of this non-invasive screening test, more invasive measures may be needed to ascertain the presence and degree of fetal anaemia (1). MCA PSV can yield false positive results (22), leading to invasive measures which are not without risk to the fetus (1). It is therefore important that the trigger titre used for referral for clinical investigations correlates well with clinical outcome.

It is well established that CAT is more sensitive than tube for detection of Rh antibodies (13-17). Several studies have found CAT titres of anti-D to be higher than tube, with some between one and two dilutions higher on average across a study cohort (13,14,20,23), and in some cases between three and five dilutions higher by CAT (11,21). One study found a 0.5 average dilution higher in CAT than tube (24). Inter-laboratory study variables in these studies included Rh(D) phenotype, cell diluents, incubation times, cell suspension strengths and number of tube washes.

This work had access to twenty plasmapheresis donations which could be tested in one study in a standardised environment, and aimed to assess if titres of red cell antibody anti-D were consistently higher, lower or the same when tested by manual techniques in tube and CAT haemagglutination. Results were highly reproducible (±1 dilution of the mode value). For the 20 plasmapheresis donations, CAT titres were on average 0.8 dilutions higher than tube.

The results of this study showed a lower difference in additional dilutions in CAT compared to tube (average of 0.8 dilutions higher in CAT than tube) than other studies (11,13,14,20,21,23), where the mean across studies was 2.7 dilutions higher in CAT than tube (17). One reason for this could be that in this study all agglutination reactions were read by a second experienced scientist immediately after being read by the person conducting the tests. Sometimes, weak reactions in tube can be misinterpreted as negative (25), particularly if the scientist is not reading tube reactions very often, or lacks experience with tube reading (26). In this case tube titres can be reported falsely low.

Based on correlation with clinical HDFN in 17 cases of anti-D immunized pregnancies and laboratory results, Steiner et al suggested that a titre of 64 could be used as the trigger for gel-based CAT (13). This was supported by work of Thakur et al (14). This study further supports that 64 could be used as the gel CAT trigger, as CAT titres were on average 0.8 dilutions higher than tube. The figure of 0.8 is close to 1, and it is impossible to raise a titre trigger by 0.8, therefore it seems reasonable to round this to the nearest whole number of 1. However, it should be noted that one sample had a one dilution higher result in tube rather than CAT, and further work to correlate CAT and tube titres with clinical outcome is essential (11,14,17,27). A lack of communication between clinical and laboratory teams has hampered progress in this arena, and efforts must be made in further studies to overcome this communication barrier, to ensure involvement from both scientists and obstetric clinicians.

In routine practice, titres are considered acceptably reproducible if results of the same sample are within one (±1) dilution (27), as was the case in this study. This study demonstrated high (but not absolute) reproducibility because one scientist did all the manual pipetting. In clinical practice it is not practical for one person to do all the work across the laboratory, and therefore it is strongly recommended that if CAT titres become the norm, that they be performed on an automated platform, where an accurately calibrated robot performs the pipetting steps. One reason for lack of absolute reproducibility in this study could be that the indicator cells, whilst always of the same Rh type (R_2R_2), were not from the same individual, and individuals of the same Rh type can have differing numbers of RhD antigens (28).

CONCLUSION

The trigger titre for monitoring a fetus at risk of HDFN due to anti-D should be reviewed in laboratories using CAT as their routine method.

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