PO 11

Quantitation of anti-c by Continuous Flow AutoAnalyser and by Flow Cytometry: A Comparison.

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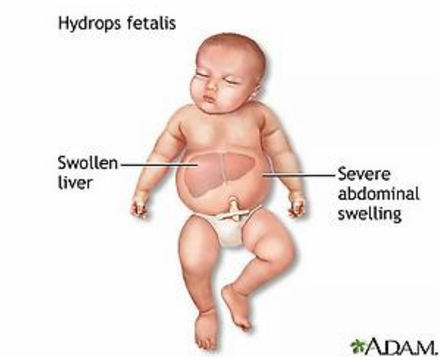
Introduction

Maternal anti-c allo-immunisation accounts for 10% of cases of *hydrops fetalis*, or haemolytic disease of the fetus and newborn (HDFN) in the UK. All expectant mothers are screened early in pregnancy for clinically significant antibodies.
Antibody levels in maternal blood must be accurately measured as these are used to decide which intervention, if any, is appropriate[1]. See table 1. Before this study, this was routinely evaluated out by IAT titre measurement. In the IBTS anti-D quantitation is carried out using the Continuous Flow AutoAnalyser (CFA). CFA quantitation is preferable to IAT titration, as it removes subjectivity, and is more reproducible and accurate. However, CFA is more labour intensive and requires a skilled operator. No recent studies have been published that correlate either IAT titres or CFA quantitation to the severity of anti-c mediated HDFN.
More recently the use of Flow Cytometry (FC) has been investigated as an alternative to CFA for anti-D quantitation [2]. FC has a higher throughput, better reproducibility and better reflects *in vivo* events[3] than CFA. Quantitation results are based solely on IgG antibody binding, which will give a better prediction of clinical outcome of HDFN.

The purpose of this study was to determine whether flow cytometry could present a more user friendly, accurate and reliable quantitation method, over CFA, for measuring anti-c levels.

Table 1: BCSH guidelines on referral to a Fetal Assesment Unit (FAU).

Anti-c Quantitation	Action to be taken
Less than 7.5 IU/ml	Continue to monitor
7.5 – 20 IU/ml	Risk of moderate HDFN, refer to specialist unit.
Over 20 IU/ml	Risk of severe HDFN, refer to specialist unit.



Methods

62 samples (from 28 patients) with anti-c at varying titre values, and six Antibody Quantitation Quality Assurance Scheme (AQQAS) samples were tested. Group O rr (dce/dce) red cells were used for both methods. Donor red cells were pooled to ensure consistent antigen density of the target red cells. Standard curves for each assay were obtained using the British Standard (BS) anti-c 84/628 (0.26 IU/ml) antibody. Bland-Altman statistical plot analysis was used to compare the two sets of data[4].

Anti-c Quantitation

Continuous Flow Autoanalyser (CFA)
The assay for quantitating anti-c by CFA is based on the method described by Marsh *et. al.* 1968[5]. Antibody samples form agglutinates with Orr red cells. After addition of methyl cellulose and incubation at 37˚ C, antibody-agglutinated cells fall out of suspension. The remaining red cells are lysed. Haemolysis is measured optically and is inversely proportional to the strength of the antibody. Antibody levels are determined by comparison to a standard curve.

Flow Cytometry (FC)
The assay for FC was adapted from that described by Austin *et. al.* in 1995[2]. Samples were analysed on a BD Biosciences FACSCanto II flow cytometer. FITC-labelled secondary anti-human IgG was used. Red cells were gated by using forward and side scatter parameters. FITC fluorescence was measured at 515-548nm. A total of 50,000 events were counted per sample. A standard curve was prepared at concentrations from 0.005 to 0.020 IU/ml BS anti-c. A linear correlation of ≥0.96 was required.

Results

The correlation between levels quantitated by CFA and FC was found to be 0.86 when considering all samples tested, see figure 2.

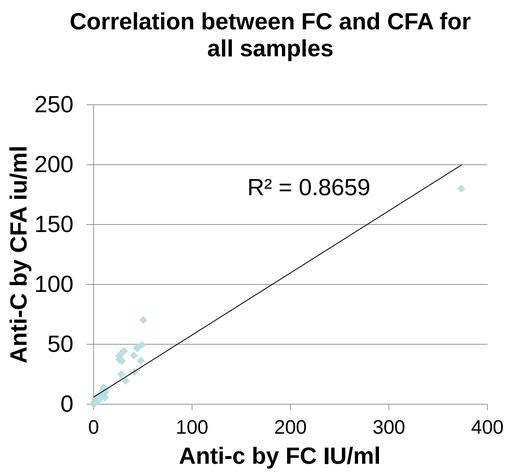


Figure 2: Correlation between CFA and FC quantitation levels for all patient samples tested.

The principal reason for measuring anti-c levels in maternal blood is to assess the risk of HDFN. Using the risk groups advised by the BCSH guidelines patients are divided into three categories which assess the need for referral to a Fetal Assesment Unit (FAU). In this study 55 of 62 (88%) agreement was observed between the mean values of the FC quantitation results and those produced by CFA in terms of identifying cases requiring referral to a FAU, see figure 3.

CFA [anti-c] (IU/ml)

FC [anti-c] (IU/ml)	< 7.5	7.5 - 20.0	>20
< 7.5	39	2	0
7.5 - 20.0	1	4	1
> 20.0	0	0	15

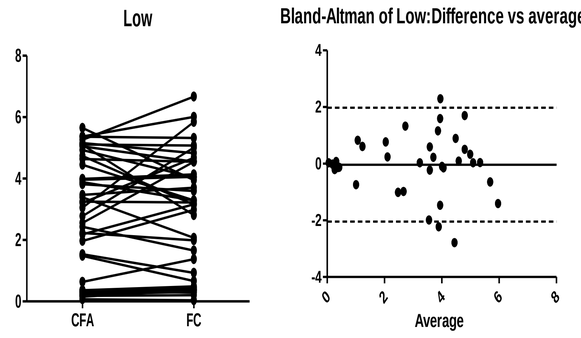
FAU referral status agreement
FAU referral status agreement but severity prediction incorrect
FAU Disagreement in referral status

Figure 3: Comparison of the agreement between CFA and FC in terms of FAU referral.

Bland-Altman Analysis

Bland-Altman plots are used to measure the performance of a new technique in comparison to an established standard. For the purposes of this analysis, the CFA quantitation result was used to categorise the samples, and FC results were compared to these. Results for 61 out of 62 samples tested are shown. Quantitation values are separated into three risk categories, as per BCSH guidelines, for FAU referral.

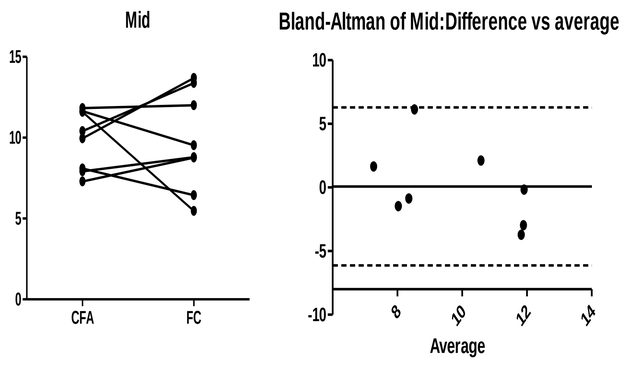
Low quantitation value, less than 7.5 IU/ml – Continue to monitor pregnancy:
Samples in the lower range show good correlation with no overlap of samples from one category to another.



Bias & Agreement

Bias	-0.033500
SD of bias	1.02519
95% Limits of Agreement	
From	-2.04288
To	1.97588

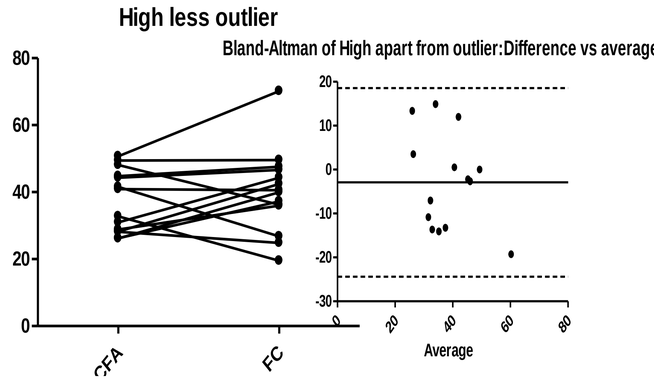
Mid range quantitation value 7.5 to 20 IU/ml – Risk of moderate HDFN, refer to FAU.
Samples form this category shows overlap between middle and lower categories. All samples are within the 95% confidence interval but the limits of agreement are higher than for the lower category and the sample size is smaller.



Bias and Agreement

Bias	0.0812501
SD of bias	3.16799
95% Limits of Agreement	
From	-6.12802
To	6.29052

High range quantitation value over 20 IU/ml – Risk of severe HDFN, refer to FAU.
Good correlation is seen for most samples in this category. All samples lie within the 95% confidence interval, but this category has the highest limits of agreement. As the quantitation levels rise, the differences between results obtained by CFA and FC increase, as can be seen from the SD of bias. The bias for this category is also the largest, showing the most variance of these results. For statistical reasons, one sample was omitted as an outlier.



Bias and Agreement

Bias	10.2040
SD of bias	51.8882
95% Limits of Agreement	
From	-91.4969
To	111.905

Discussion & Conclusion

Statistical analysis of the results of this work show good overall correlation between quantitation values obtained from CFA and FC analysis. This shows that FC offers a potentially viable alternative to CFA for determining maternal anti-c levels. The results obtained during the course of this study showed reliable repeatability and the inclusion of AQQAS samples showed that the results obtained were accurate. Similar patterns of correlation have been observed by other groups working with anti-D[6].
The IBTS introduced anti-c quantitation by CFA as part of routine testing in February 2011. Flow cytometry has been shown to offer a feasible alternative to CFA, but more work needs to be done before this can be considered as a replacement. Ideally, this study should be expanded to include more samples. Information on the clinical outcome of patients would lend better predictability of HDFN severity from quantitation values. Ethical and logistical constraints make this task difficult as it requires collaboration from a wide range of disciplines from laboratory staff to medical, nursing and administrative staff. Ethical considerations must also be taken into account as patient consent may be necessary.
The future of fetal care may move towards molecular techniques such as MCA Doppler Ultrasonography or fetal genotyping. However, there will always be an important role for anti-c quantitation to complement hospital based diagnoses and to allow physicians predict HDFN severity more accurately.

References

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