ANTI-D QUANTITATION BY CONTINUOUS FLOW ANALYSIS: COMPARATIVE STUDY OF TWO DIFFERENT METHODS ON TECHNICON/ALLIANCE INSTRUMENTS AND ON WHITE HORSE SCIENTIFIC SYSTEMS

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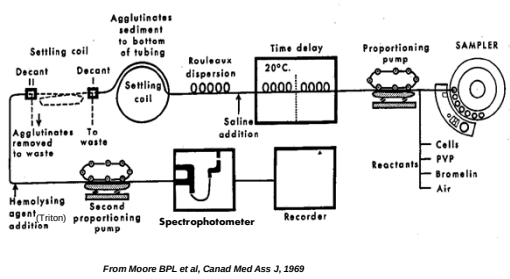
Background: **Anti-D quantitation by continuous flow analysis (CFA) on Autoanalyser is used for a long time in the the laboratory of the French National Center for Perinatal Hemobiology (CNHRP) for the management of allo-immunized pregnancies. Since several decades, this test has been performed with a method using polyvinylpyrrolidone (PVP) as "rouleaux" inducing agent on Technicon Autoanalysers and MS Alliance Evolution III systems.**
In 2014, the laboratory was equipped with Astoria Pacific systems (White Horse Scientific) to replace the Technicon Autoanalysers. A new method using methylcellulose (MC) instead of PVP was developed on these systems.
Aims: **The aim of this work was to compare the performance of the two methods (Alliance Evolution III (PVP) vs Astoria WHS (MC)).**

Methods: **Red blood cells of R^1R^2 phenotype were bromelinized prior to the test (« 2-stages »). In a continuous flow, "rouleaux" inducing agent was added to the cells. After incubation coils, dispersion of rouleaux was achieved by addition of isotonic saline in the circuit. Only immunological formed agglutinates remained. Agglutinated cells were then removed by decantation. Remaining red blood cells were lysed by Triton and supernatant content of hemoglobin was measured with a spectrophotometer at 550 nm. The International anti-D Standard (01/572) was used to calibrate a working secondary standard that allows the expression of the results in IU/ml.**

Alliance Evolution III flow diagram

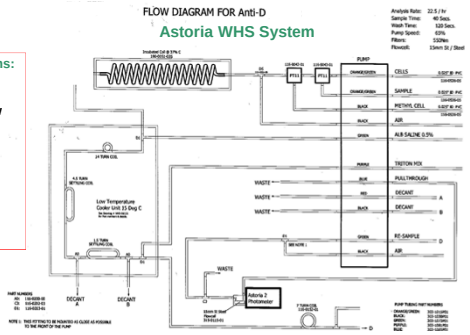


From Moore BPL et al, Canad Med Ass J, 1969

Differences between Alliance Evolution III and Astoria WHS Systems:

- **PVP vs MC as « rouleaux » inducing agent (= macromolecules inducing a high dielectric constant of the medium and thus a low zeta potential of the red blood cells)**
- **Incubation at 20+/- 3 vs 37 +/- 2° C**
- **Decantation at 20 +/- 3 vs 15 +/- 2° C**
- **Larger excess of antigen (higher dilution of sample): linear vs logarythmic curve *.**

FLOW DIAGRAM FOR Anti-D
Astoria WHS System



From White Horse Scientific

* Equation derived from the theoretical basis of anti-D continuous flow quantitation (Sipps Nisonoff Pressman (1958)):

$$\frac{1}{[Ab]_e([Ag]_e \cdot K')} + \frac{1}{[Ab]_t} = \frac{1}{[AgAb]}$$

where $[Ag]_e$ is the concentration of unbound antigen sites at equilibrium
$[Ab]_t$ is the total concentration of antibody sites
$[AgAb]$ is the concentration of bound sites
K' is the average binding constant
a is the heterogeneity index ($0 < a < 1$).

In a large antigen excess $[Ag]_e$ is high, then $[Ab]_t$ is nearly equal to [AgAb] (= measured value). If the concentration of antibody is increased, the zone of equivalence is reached and the straight line gives way to a logarythmic curve.

An international standard sample and a low and a high level of internal quality controls (IQC) were used to determine the intraassay and interassay imprecisions. Accuracy was determined based on z-score values obtained after retrospective dosage of external quality controls from the UK NHSBT AQQAS quality assurance scheme. Comparison of the results obtained from the same 75 clinical samples with both methods was carried out with Deming regression and measures of Bland-Altman bias.

METHOD	Sample	N	Mean (IU/ml)	S.D (IU/ml)	CV Intraassay (%)
Astoria WHS (MC)	International stantard (batch 2015)	26	6	0,4	**6,1**
Alliance Evolution III (PVP)	International stantard (batch 2011)	20	6,6	0,5	**7,2**

METHOD	Sample	N	Mean (IU/ml)	S.D. (IU/ml)	CV Interassay (%)
Astoria WHS (MC)	IQC 1	32	5,3	0,9	**17,3**
	IQC 2	29	0,12	0,02	**15,2**
Alliance Evolution III (PVP)	IQC 1	112	5,2	0,9	**16,8**
	IQC 2	95	0,14	0,02	**18,3**

Fig-1 Intraassay and interassay imprecision of the 2 methods

S.D = standard deviation
CV = Coefficient of vairation

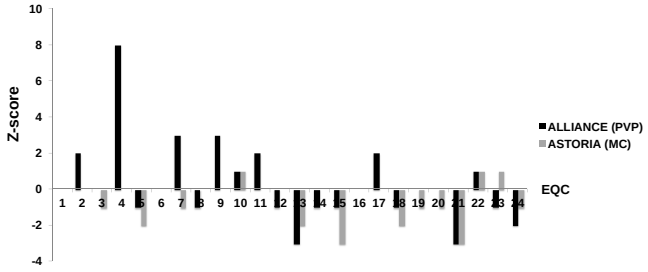
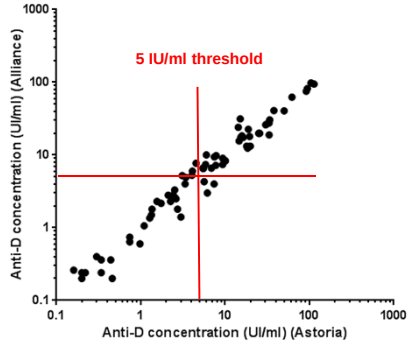


Fig-2 Accuracy: z-score values obtained with each method after retrospective dosage of 24 frozen external quality control (EQC).

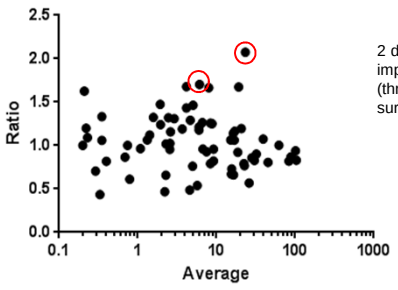
Means of the z-score per sample (absolute number):
Astoria WHS (MC) : 1.0 / Alliance Evolution III (PVP) : 1.4

Results: **For MC and PVP methods, the intraassay imprecision was determined on the international standard level and shows comparable means (6 versus 6.6 IU/ml) and coefficients of variation (CV) (6 versus 7 %). The interassay imprecision was calculated on the low and high level of IQC and shows comparable means (0,12 and 5.3 versus 0,14 and 5.2 IU/ml) and CV (15 and 17 versus 18 and 17 %). The accuracy was determined on 24 frozen AQQAS samples and the means of the calculated z-score were respectively 1 and 1.4.**



For 5 samples:
anti-D concentrations were > 5 IU/ml (threshold for ultrasonographical surveillance) in one method and not in the other. No clinical impact : none of the corresponding fetus develop fetal anemia

Fig-3 Deming regression between the 2 methods
N = 72 (3 values >> 200 IU/ml excluded to avoid bias). Equation: Y = 0,87X +0,86.
Correlation r = 0,984 p<0,0001



2 diverging points with no clinical impact (values > 5 IU/ml (threshold for ultrasonographical surveillance))

Fig-4 Bland-Altman ratio between the 2 methods
N= 75 Ratio = 1,05. 95% limits of agreement : 0,42 to 1,68

The Deming regression equation obtained on 72 clinical samples was Y=0.87X+0,86 (r= 0.984). The mean bland-Altman bias was 1.05.

Summary / Conclusions: **Both methods show comparable results in terms of performance criteria and a good correlation coefficient. The threshold concentration of 5 IU of anti-D /mL used to determine when clinicians should monitor the pregnancy by ultrasonography because of a risk of fetal anemia remains the same for both methods. The high scattering of Bland Altman ratio is probably due to technical differences ("rouleaux" inducing agent used, auto-analyzer specificities) and to the fact that methylcellulose method works in a larger excess of antigen with linear hemagglutination curves instead of logarithmic ones. Thus, the biological follow-up of each anti-D allo-immunized pregnancy has always to be done with the same method.**