Performance of an automated antibody titration method using solid phase red cell adherence.

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In France, the Immunohematology decree of May 15, 2018 paved the way for the use of other antibody titration techniques than the reference tube technique. The tube method is largely manual and is known to show great inter-laboratory results variability depending on multiple parameters. The new developed titration techniques could be automated, and better technical performance are expected. We wanted to assess the performance of solid phase red cell adherence plate titration method on the Neo Iris automated system (Werfen, Immucor) and to compare it with the performance established for the reference tube method, used in our lab since decades. Another objective of the study was to try to determine plate titer thresholds for the main antibody specificities responsible for hemolytic disease of the fetus and newborn to trigger fetal monitoring by ultrasounds and measurements of the peak systolic velocity in the middle cerebral artery.

Solid phase red cell adherence automated titration method (immunocapture)

Automated dilutions of patients sera (from, ½ to 1/256) in Low ionic strenght solution (LISS).
Distribution of the dilutions in the microplate whose wells are coated with RBC membranes (see below for the phenotypes)
Incubation. Wash.
Addition of anti-IgG revealing RBC (Capture R cells) Centrifugation.

Reading of the titer by the automaton camera

Red blood cells (RBS) phenotypes
For anti-D titration : D+ C+ E- c- e+ (Panoscreen I)
For anti-c titration : D- C+ E- c+ e+ (Pansocreen Extend I)
For anti-E titration : D- C- E+ c+ e+ (Oanoscreen Extend II)
For anti-K titration : K+ k+ (Panoscreen Extend III)

Duration of the test for 1 plate : 87 min.
Minimal sample : 500 µl

Neo IRIS (Werfen Immucor)

Report of results:

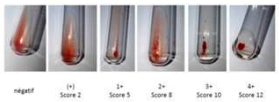
Sample ID	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	Titer	Pattern	Flags
2443541941900139507									Positive	44	
2453561925000139613									Negative	-	
18104445	0.00	0.00	0.00	0.00	4.10	0.00	0.00	0.00	<2 or negative	———	
18104497	0.00	0.00	0.00	1.80	5.20	0.70	0.00	0.00	<2 or negative	———	
1804029-2	99.0	99.0	86.8	60.5	0.00	0.80	0.00	0.00	16	4432—	
17101090	0.00	0.00	0.00	0.10	0.00	4.30	0.00	0.00	<2 or negative	———	
18102558	1.80	0.00	0.00	1.20	0.00	0.00	0.00	0.00	<2 or negative	———	
18102467	98.7	99.0	99.0	99.0	99.0	99.0	95.5	73.1	>=256	44444443	
18103730	0.00	0.00	0.00	0.00	2.30	4.50	0.00	0.00	<2 or negative	———	
17102805	23.2	13.7	0.00	0.00	0.00	1.70	0.00	0.00	2	1———	
19102051	99.0	99.0	99.0	90.2	77.0	13.6	0.00	0.00	32	4443—	
18102756	28.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2	1———	
18102327	11.7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	<2 or negative	———	

For each well:

- Automatic calculation of a /99 value depending on the agglutination strenght
Positive threshold = 20 → automatic calculation of the titer (inverse of the last positive dilution)
- Automatic calculation of « Pattern » values = intensity of the hemagglutination reaction reported on a /4 scale (as for the tube or gel method). Possible calculation of the Marsh score (see tube method)

Tube titration method used in the CNRHP laboratory

Automated dilution of fresh samples (starting dilution :100 µl of samples + 100 µl NaCl 0,9%) (Tecan Freedom Clinical Base)
Reagent = fresh red blood cells (RBC) (< 15 days). Phenotypes: RH:1,2,3,4,5 (D+C+E+c+e+) for anti-D, anti-c and anti-E titrations / K+k+ for anti-K titration
Red blood cell concentration used :4% in 0,9% NaCl
Incubation of 50µl of red blood cells + 100 µl of diluted samples at 37° C +/- 2 ° C for 60 +/- 15 min
Washes: 3 times
Testing phase: Antiglobulin (Diagast AGH Maestria IgG) diluted to 1/3, added at room temperature (22 +/- 3° C)
Centrifugation and read:
Manual reading End point : (+), macroscopic:



Calculation of the Marsh scores :
4+ = 12
3+ = 10
2+ = 8
1+ = 5
(+) = 2

Results:

An home-made anti-D internal quality control (IQC) prepared and calibrated using the international anti-D Standard (16/332) was used to determine the intra-assay and inter-assay imprecisions, regarding the score and the titer results.

Figure 1: Intra- and interassay imprecisions determined on IQC for different titration methods

CV% for titers have been calculated using the dilution rank with (i.e titer of 2 = dilution rank 1, titer of 4 = dilution rank 2, titer of 8 = dilution rank 3, titer of 16 = dilution rank 4, titer of 32 = dilution rank 5, titer of 64 = dilution rank 6, titer of 128 = dilution rank 7, titer of 256 = dilution rank 8, titer of 512 = dilution rank 9, titer of 1024 = dilution rank 10, titer of 2048 = dilution rak 11

** Calculation made depending on the dilution ranks*
*** Non applicable. Due to a maximal value of 256 in this method, the CV value of the titer could not be accurately calculated*

Titration method	Intra-assay imprecision				Inter-assay imprecision			
	N=	Mean of the titer	Titer* (CV %)	Score* (CV %)	N=	Titer* (CV %)	Score* (CV %)	
Tube (manual dilutions)	19	32	9,2	11,5	29	10,3	20,9	
Tube (automated dilutions)	26	32	0	6,1	30	3,7	6,4	
Gel (automated dilutions)	20	512	0	1,25	9	0	1,9	
Solid phase red cell adherence (automated dilutions)	7	> 256	N/A**	4	5	N/A**	5	

For intra- and inter-assay imprecision, better results were obtained with the automated solid phase red cell adherence plate titration method compared to the tube method with manual dilutions. Imprecisions were similar to those of the tube method with automated dilutions. Studies on more samples are required to confirm these results.

Patients samples for testing were chosen during the 12-months assay period, regarding the specificity of the antibodies and the tube titer in order to cover a wide range of situations. Comparison of the results obtained from the same clinical samples with both methods was carried out.
Titers obtained on tube and plate methods were compared for 131 samples from pregnant women: 37 anti-D, 41 anti-c, 31 anti-E, 22 anti-K.

Antibody specificity	n (total)	n with a lower titer value with the plate method	n with an equal titer value with the plate method	n with a higher titer value with the plate method	Mean of the titer value differences (plate - tube) (in dilutions number))	Standard deviations of the titer value differences (plate - tube) (in dilutions number))
anti-D	37	0	4	33	**2,3**	1,27
anti-c	41	4	12	25	**1,5**	1,61
anti-E	31	2	8	21	**2**	1,55
anti-K	22	3	7	12	**0,6**	1,05

Figure 2: Comparison of the titer results obtained with the semi-automated tube method used at the CNRHP and the solid phase red cell adherence automated method (131 samples)

For anti-D (RH1), anti-E (RH3) and anti-c (RH4), titers were on average 2.3, 2 and 1.5 times higher respectively in the plate technique than in the tube technique.

For anti-K (KEL1), differences in titers were less marked, averaging less than 1 dilution (0.6).

A Anti-D n=37

Tube titer	Solid phase red cell adherence titer									
	<2	2	4	8	16	32	64	128	256	> 256
<2	1		3	3		1				
2					2		1			
4			1			3				
8						1	3			
16							1			
32							3	1		2
64										5
128										2
256										2
> 256										2

B Anti-K n=22

Tube titer	Solid phase red cell adherence titer									
	<2	2	4	8	16	32	64	128	256	> 256
<2	1		1							
2	1		1							
4		2	1							
8				1	1					
16							2			
32							3			
64							1	1		1
128								1		
256										2
> 256										2

Figure 3: Comparison of the titer results obtained with the semi-automated tube method used at the CNRHP and the solid phase red cell adherence automated method for each anti-D [A] and anti-K [B] samples.

For anti-D samples, the titer threshold of 16 in the tube method used in our lab to trigger fetal ultrasound surveillance to detect fetal anemia, seems to correspond to a titer threshold of around 64 in the solid phase red cell adherence automated method.

For anti-K samples, the titer threshold of 16 in the tube method seems to be also applicable in the solid phase red cell adherence automated method.

Conclusion :

Automated anti-red blood cell antibodies titration by solid phase red cell adherence plate method on the Neo Iris shows similar inter-assay imprecision CVs compared to the tube method. But better imprecision CVs could probably be obtained using only fresh titration and revealing cells. Less inter-laboratory variability could be expected with this method due to automation.
Comparison of plate and tube titers show significant differences, with disparities depending on samples and antibody specificities. For anti-RH antibodies, titer results with the automated plate method are often higher than those with the tube method. For anti-K antibodies, results are more similar. In order to be able to establish right threshold titers using the plate technique, linked to a risk of severe fetal or neonatal hemolytic disease, further studies on a larger number of samples need to be envisaged and results require to be validated with clinical data from pregnancy outcomes.