Automated antibody identification and titration tests using solid phase red cell adherence allows to determine the prophylactic or immune nature of circulating anti-D (RH1) in pregnancy

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Background: **Since 1970, generalization of antenatal immunoprophylaxis by anti-D immunoglobulins (RhIg) injections made the interpretation of anti-red blood cell antibodies screening during pregnancy more complex. In 1999, the laboratory of the CNRHP set up the anti-D microtitration test that allows to quantitate low levels of anti-D (between 0.008 and 0.12 IU/ml). It consists of a gel titration test, using a 0.03 IU/ml anti-D standard and papain-treated D positive cells. Comparison of found anti-D concentrations with concentrations expected according to the dose and date of RhIg injection based on pharmacokinetic curves, allows us to conclude on the prophylactic and/or immune nature of the circulating anti-D.**

Anti-D microtitration test used in the CNRHP laboratory

Brossard Y et al, Feuillets de Biologie 2002 (French)

Indirect antiglobulin test , gel method, saline medium, using RH:1,-2,-3,4,5 (D+C-E-c+e+) papain-treated RBC and a polyclonal anti-D standard correlated to the WHO international anti-D standard

Anti-D concentration = Inverse of the last positive dilution of the sample X Concentration of the standard with a similar reaction intensity

Here : 8 x 0,008 = **0.06 IU/ml**

Theoretical anti-D concentration in the plasma after RhIg administration

Time after injection	Concentration after 1000 IU (200µg) anti-D administration (IV route)	Concentration after 1500 IU (300µg) anti-D administration (IV route)
48h	30 ng/ml (0,15 IU/ml)	45 ng/ml (0,23 IU/ml)
1 week	24 ng/ml (0,12 IU/ml)	36 ng/ml (0,18 IU/ml)
3 weeks	15 ng/ml (0,08 IU/ml)	22,5 ng/ml (0,11 IU/ml)
6 weeks	7,6 ng/ml (0,04 IU/ml)	11,4 ng/ml (0,06 IU/ml)
9 weeks	3,8 ng/ml (0,02 IU/ml)	5,7 ng/ml (0,03 IU/ml)
12 weeks	1,8 ng/ml (0,01 IU/ml)	2,7 ng/ml (0,015 IU/ml)
15 weeks	0,9 ng/ml (0,005 IU/ml)	1,35 ng/ml (0,007 IU/ml)

Every year, in the French Hemovigilance System, as well as in the UK SHOT report, there are several cases of unexpected severe hemolytic disease of the fetus and newborn (HDFN) reported, due to immune anti-D that were erroneously assumed to be prophylactic

In the example 0.06 IU/ml → corresponds to a 1500 IU RhIg injection about 6 weeks earlier

Measurement uncertainty : 0,5 dilution

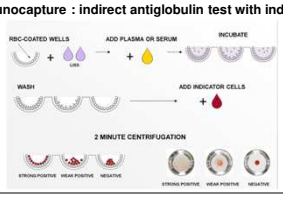
So if RhIg injection was done more than 9 weeks before: suspicion of weak anti-D allo-immunization despite prophylaxis → anti-D quantification every 2 weeks to detect an increase in concentration and to anticipate clinical manifestations of HDFN

Aims: **We performed a study on Neo Iris (Werfen (Immucor)) to determine if a quantitative approach based on agglutination values calculated by the analyzer in a solid phase red cell adherence identification test could help to determine the nature of anti-D. We also wanted to evaluate the potential of the automated anti-D titration test as a substitution method for the microtitration test.**

Solid phase red cell adherence automated method Identification test

Neo IRIS (Werfen Immucor)

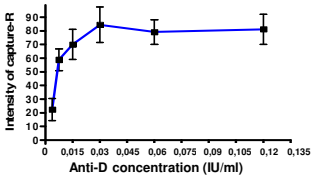
Immunocapture : indirect antiglobulin test with indicator cells (Capture ID test)



For the anti-RBC identification test, the automaton automatically calculates a /99 value for each well depending on the agglutination strenght (threshold of positivity = 20).

A

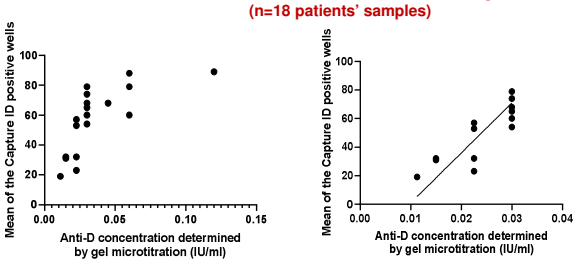
Mean agglutination values (/99) in the D positive wells of the Identification test (Capture ID) for standards ranging from 0.008 t0 0.12 IU/ml



At delivery, about 14 weeks after the 1500 IU RhIg injection at 28 GW, the patient's expected anti-D concentration is less than 0.01 IU/ml: the intensity of the expected /99 mean value is below 70

B

Correlation between the /99 mean values of the D positive wells in the Capture ID identification test and the anti-D concentration found in the gel microtitration test (n=18 patients' samples)



*** Spearman correlation : mean of the /99 values of the D positive wells vs anti-D concentration determined by gel microtitration, n= 18, **p = 0,0014, r=0.69** [0,34-0.88]

Linearity between 0,008 and 0,03 IU/ml: Deming **p = 0,0003** **Y = 3505X-34 (IU/ml) or Y = 17,5X-34 (ng/ml)**

A good correlation was found between the mean of the /99 agglutination intensity values in the D positive wells of the identification test and the gel microtitration values. Linearity was observed between 0.008 and 0.03 UI/ml.

C

Strategy used in the CNRHP laboratory on D negative patients samples received at delivery (for Rh disease prevention tests)

D positive wells = wells 1,2,3,4 and 14 of the Capture ID identification test

ID Patient	Position	Resultat	Puit 1	Puit 2	Puit 3	Puit 4	Puit 5	Puit 6	Puit 7	Puit 8	Puit 9	Puit 10	Puit 11	Puit 12	Puit 13	Puit 14	Ctrl Pos	Ctrl Neg
0,03IU/ml standard	1	Positive	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-
			43.9	72.7	39.5	56.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	65.3	91.7	0.00
240600445303	3	Negative	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
			9.40	16.2	17.1	13.6	0.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.90	99.9	0.00
240600236903	5	Negative	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
			1.60	11.0	8.00	5.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.70	99.9	0.00
240600250003	7	Negative	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	99.9	0.00
240600205903	9	Negative	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
			0.00	4.70	5.90	5.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	99.9	0.00
240600259903	11	No_Int	-	?	-	-	-	-	-	-	-	-	-	-	-	-	+	-
			13.3	21.0	17.7	19.9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	15.2	99.9	0.00

Patients

Use of an 0,03 IU/ml standard: Mean of the /99 values of the D positive wells for the standard = 56

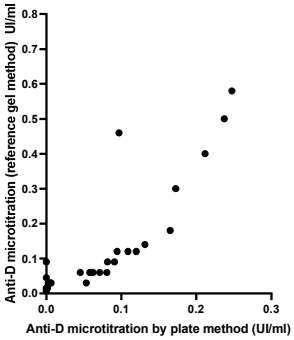
For patient N° 240600259903 : Mean of the /99 values of the D positive wells = 17 < mean of the 0,03 IU/ml standard

The patient has an anti-D concentration < 0,03 IU/ml at delivery. She received 1500 IU/ml of RhIg at 28GW and delivered at 40GW. The found concentration corresponds to the expected concentration (see table above).
→ We can confirm that the prophylactic nature of the anti-D.

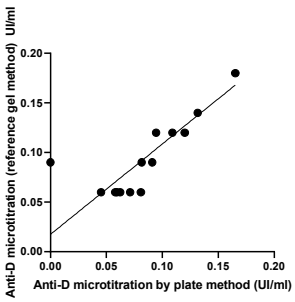
Solid phase red cell adherence automated method Anti-D titration test

Automated dilutions of the 0,12 IU/ml anti-D standard and 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 D+ C+ E- c- e+ (Panoscreen V) RBC membranes. Incubation.
Wash.
Addition of anti-IgG revealing RBC (Capture R cells) Centrifugation.
Reading of the hemagglutination reaction by the automaton camera

Using a 0.12 UI/ml anti-D standard, 2 quality controls and 33 patient's samples with anti-D concentrations ranging from 0.011 to 0.58 UI/ml were analyzed in the titration test. Scores (/99) were calculated adding /99 values for each dilution wells showing agglutination. Samples /99 scores were reported to the 12 IU/ml standard /99 score and anti-D concentration could be calculated for each sample and compared to those found by the gel microtitration test



*** Spearman correlation : anti-D microtitration by gel reference method vs anti-D microtitration by plate method, n= 33, **p < 0,0001, r=0.91** [0,82-0.96]



Linearity between 0,008 and 0,03 IU/ml: Deming **p = 0,0008** **Y = 0.9114X+0,018**

Adaptation of the titration test to anti-D microtitration

Sample ID:	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	Titer	/99 score
24423724470113 1584	-	-	-	-	-	-	-	-	Positive	
24523624310059 1089	-	-	-	-	-	-	-	-	Negative	
732312035036	4.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	<2 or negative	
732312034993	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	<2 or negative	
231203469801	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	<2 or negative	
231203460101	99.0	89.5	2.10	0.00	0.00	0.00	0.00	0.00	4	
231203435801	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	<2 or negative	
231203397001	11.2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	<2 or negative	
732312035215	86.3	77.2	2.50	0.00	0.00	0.00	0.00	0.00	4	
QSUCHP (0,0fUl/mL)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	<2 or negative	
3UCHP (0,06UI/ml)	80.8	66.9	0.00	0.00	0.00	0.00	0.00	0.00	4	
6UCHP (0,12 UI/ml)	99.0	99.0	49.6	0.00	0.00	0.00	0.00	0.00	8	
TEMTTIT	99.0	99.0	99.0	99.0	99.0	99.0	92.0	16.2	128	

Patient 86,3+77,2+2,5 = 166
Control 1 0
Control 2 80,8+66,9 = 147,7
Standard 99+99+49,6 = 247,6

Anti-D concentration = /99 score of the sample (or control) / 99 score of the 0,12 IU/ml standard X 0,12

Control 1 < detection limite
Control 2 = (147,7/247,6) X 0,12 = **0.07 IU/ml**
Patient = (166/247,6) x 0,12 = **0,08 IU/ml**

Anti-D titration test using /99 scores and a 0.12 IU/ml standard to report values gave concentrations that were well correlated to the reference gel microtitration test concentrations. Values were comparable and linearity was obtained between 0.06 and 0.18 IU/ml.

Conclusion :

Comparison of the sample's agglutination values (/99) in the positive wells of the solid phase red cell adherence identification test with the mean values of the 0.03 IU/ml anti-D standard allows to have a quantitative approach that could be useful at delivery, as anti-D concentrations 9 to 13 weeks after routine antenatal prophylaxis are expected to be within the linearity range.

In other cases, when targeted RhIg prophylaxis has been done a few weeks before sampling, an anti-D titration test could be performed with calculation of the /99 scores and report to the 0.12 UI/ml anti-D standard /99 score. It allows to calculate accurate anti-D concentrations and ensure they are within the expected range based on RhIg dose and injection date.

If the sample's found values are too high, anti-D immunization despite prophylaxis should be considered.