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# Comparison of the direct antiglobulin test and the eluate technique for diagnosing haemolytic disease of the newborn

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ABSTRACT

*Objective:* The direct antiglobulin test (DAT) is an important tool for identification of haemolytic disease of the newborn (HDN) caused by erythrocyte immunization. Although this test has been used for decades, accurate insights into its diagnostic properties and optimal use in the diagnosis of HDN are limited. We aimed to gain more insight into the diagnostic properties of the DAT for HDN by comparing it with erythrocyte eluate screening.

*Design and methods:* DAT and erythrocyte eluate screening was performed in umbilical cord blood of neonates obtained from 317 consecutive deliveries. Clinical jaundice was scored 4–6 days after delivery for the determination of HDN.

*Results:* In 21 neonates a positive DAT and in 61 neonates a positive eluate screening was found, while only 4 cases of HDN were observed. For the overall population the positive predictive value (PPV) and specificity of the DAT for HDN were 10% and 93% respectively and in the population of neonates with abnormal post-partum jaundice population the PPV and specificity were both 100%. The DAT missed two cases of HDN. These missed cases were, however, positive in the erythrocyte eluate screening.

*Conclusion:* The detection of clinically irrelevant ABO immunization limits the specificity of the DAT and eluate for HDN in ABO-incompatible pregnancies. For optimal use, the DAT should be requested only in cases of jaundice and be interpreted in the context of ABO-incompatibility. Finally, a negative DAT does not rule out HDN. When clinical suspicion is high, an eluate should be added following a negative DAT.



## 1. Introduction

Classically, the direct antiglobulin test (DAT), or direct Coombs test, plays a central role in diagnosing haemolytic disease of the newborn (HDN) caused by foetal erythrocyte immunisation [1]. This condition is characterized by the presence of maternal blood group antibodies in the circulation of the foetus/neonate. These maternal antibodies are able to cross the placental blood barrier and are either directed against the A or B antigens of the regular ABO blood group, or antigens of one of the other blood group systems. HDN may lead to severe haemolysis, jaundice and kernicterus [1,2]. Adequate diagnostic

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tools are necessary to differentiate HDN by pregnancy immunisation from other erythrocyte-related causes of haemolysis and hepatic or infectious causes of jaundice. HDN by Rh D immunization is probably the best known variant due to its high incidence and severe clinical symptoms. Hence, several countries established programmes for the prevention and monitoring of Rh D immunization in pregnant Rh D negative women [1,2].

For diagnosis of HDN by pregnancy immunization, detection of neonatal erythrocytes sensitized with immunoglobins is a keystone diagnostic requirement. The DAT is a suitable test for this purpose. In the DAT assay, (neonatal) erythrocytes are incubated with polyclonal anti-human (IgG) antibodies. Erythrocytes sensitized with anti-erythrocyte immunoglobulin (IgG) agglutinate due to cross-linking of these erythrocytes. However the DAT does not determine the specificity of the bound antibody. For antibody identification, elution of erythrocyte bound antibody, followed by binding analysis of the antibody present in the eluate against test erythrocytes of well known erythrocyte blood group phenotype, is performed. Elution of the antibodies from sensitized erythrocytes can be performed using different approaches including ether or acid, or by freezing. Apart from identification of antibody specificity, elution techniques can also be used for screening of sensitized erythrocytes [3]. In general, elution techniques are more sensitive in detecting anti-erythrocyte antibodies compared to the DAT [4]. However, these techniques are often only available in specialized laboratories, require larger blood volumes and are laborious [3]. Especially for neonatal erythrocytes, a limited sensitivity of the DAT for anti-A (IgG) and anti-B (IgG) compared to other techniques has been reported [5].

In a recent case in our hospital, a negative DAT was found in a neonate with no other obvious cause of neonatal jaundice and a known neonatal/maternal blood group incompatibility (Apos/Oneg). Due to the high clinical suspicion of HDN by pregnancy immunization an eluate was prepared from neonatal erythrocytes and screened for the presence of anti-A and irregular erythrocyte blood group antibodies. This resulted in the identification of IgG anti-A antibodies. The discrepancy between the DAT and elution technique in this case triggered us to investigate the use of these techniques for the analysis of HDN. Despite the commonly accepted use of the DAT in the diagnosis of HDN, the sensitivity, specificity and predictive values of this test for HDN by immunization are poorly documented. In this study we investigated the predictive value of both the DAT and elution techniques in suspected and unsuspected HDN.

## 2. Patients and methods

### 2.1. Patients and study set-up

Neonates from 317 consecutive deliveries in our hospital were included in this study. Informed consent was obtained from each participant.

ABOD blood type of the mother was determined previously for the Dutch prophylactic anti-D programme. In this programme, all Rh D negative pregnant women received anti-Rh D treatment with polyclonal anti-Rh D (IgG, CDL Behring) in week 30 of pregnancy.

Post-partum, 7 mL of venous lithium–heparin anticoagulated blood from the mother and 7 mL lithium–heparin anticoagulated umbilical cord blood were collected. Umbilical cord blood was analysed for ABOD and DAT by column (Low Ionic Strength Solution (LISS), Bio-Rad Laboratories, Hercules, CA, USA), and Polyethylene-glycol (PEG) methods. Erythrocyte eluates were prepared by freeze and ether elution methods. Eluates were screened for irregular blood group antibodies using a 3 cell panel and, in cases of maternal/neonatal ABO-incompatibility, also for the presence of IgG anti-A or anti-B antibodies. In the event of positive screening of the eluate, venous blood of the mother was used for irregular antibody identification. In addition, titres of anti-blood group antibodies were determined in umbilical cord and maternal plasma.

In order to avoid invasive blood collection for measurement of bilirubin, a non-invasive jaundice score was used. The jaundice was scored 4–6 days postpartum, using the following self-defined criteria: jaundice of the face, chest, sclera and mouth/tongue was scored as absent (0 points), little (1 point), clearly present (2 points). The minimal jaundice score was 0 and the maximal score was 8. A jaundice score of $\geq 4$ was considered as abnormal post partum jaundice. HDN by foetal erythrocyte immunization was defined as: a jaundice score of $\geq 4$ as well as serological evidence for erythrocyte immunization by DAT or elution techniques, in the absence of other causes of abnormal post partum jaundice.

### 2.2. ABOD blood group determination

The ABO-Rh D blood groups of the neonate and the mother were determined by a direct antiglobulin technique (salt phase-enhanced) using agglutination of monoclonal antibodies against A1, B, D and enhanced D for forward ABO grouping and A1 and B test erythrocytes for reverse ABO grouping. Antibodies and test erythrocytes were purchased from Sanquin (Amsterdam, The Netherlands).

### 2.3. DAT

The DAT was performed manually in a manual agglutination mode and in the LISS/column mode. For the manual agglutination mode, erythrocytes were washed 3 times with cold 0.9% NaCl. A 3% erythrocyte solution was obtained by adding 33 drops (1.2 mL) of 0.9% NaCl to 1 drop of washed erythrocytes. Next, 2 drops of polyspecific anti-human IgG (Sanquin,

Amsterdam, The Netherlands) was mixed with 1 drop of 3% erythrocyte solution and centrifuged (120*g* for 20 s). Next, red cells were gently resuspended and agglutination was scored macroscopically and categorized as negative (−), weak (+/−) or positive (1+, 2+, 3+, 4+).

The LISS/column mode was performed with LISS/Coombs cards (Bio-Rad Laboratories, Hercules, CA, USA) containing both anti-IgG and anti-C3d antibodies, according to the manufacturer's instructions. Agglutination was scored on a similar positivity scale to the manual agglutination method.

### 2.4. Antibody elution methods

Antibody elution from umbilical cord erythrocytes was performed by both freeze and ether elution methods. Erythrocytes were washed 6 times with cold 0.9% NaCl. The supernatant of the last washing was used as negative control.

For the freeze elution method, 5 drops of packed washed erythrocytes were mixed with 1 drop of 22% bovine serum albumin (BSA) (Sanquin, Amsterdam, The Netherlands) and frozen for at least 10 min at −40 °C. After thawing at 37 °C and centrifugation (800*g*, 10 min) the supernatant was collected and analyzed for antibodies.

Ether eluates were prepared by addition of 1 part 0.9% NaCl and 3 parts diethyl ether to 2 parts of packed and washed erythrocytes. This was mixed for 1 min and incubated for 30 min at 37 °C. After centrifugation (800*g*, 10 min) the lower layer was collected. The last step was repeated and the remaining fraction (eluate) was analyzed for antibodies.

### 2.5. Identification of anti-ABO (IgG) and irregular blood group antibodies

In cases of ABO-incompatible pregnancies, eluates were analysed for IgG anti-A and anti-B antibodies by use of A1 and B cells (Sanquin) respectively. When positive, 1:1 serial dilutions of neonatal plasma were obtained for IgG anti-A and/or anti-B titre determination. In addition, maternal plasma was analysed for relevant IgG anti-ABO antibodies and antibody titres. Maternal plasma was diluted 1:1 with 0.01 M dithiothreitol (DTT) in 0.9% NaCl and incubated for 15 min at 37 °C. Monoclonal Rh D IgM antibodies treated with and without 0.01 M DTT tested on Rh D positive erythrocytes were used as controls. Antibody titres from maternal blood were determined by testing different dilutions (1:2–1:15,000) in a salt-phase indirect antiglobulin technique using agglutination of A1 or B positive erythrocytes as detection technique. In this technique antibodies against A1 or B were agglutinated by using polyspecific anti-human globulin directed against IgG and C3d according to the manufacturer's recommendations (Sanquin; Amsterdam, The Netherlands).

For detection of irregular antibodies, eluates were screened using a panel of 3 erythrocyte screening cells (Sanquin) in a PEG-phase indirect antiglobulin test. When positive, antibody identification was performed in maternal heparin plasma using an 11-cell antibody identification panel (Sanquin).

## 3. Results

### 3.1. Clinical findings

Jaundice was scored in 282 of 317 neonates. In 33 cases the jaundice-score was $\geq 4$. In 9 of these, the abnormal postpartum jaundice score coincided with a positive identification of sensitized neonatal erythrocytes. In 4 of these only anti-D was found on neonatal erythrocytes which could be ascribed to prophylactic administered anti-Rh D during pregnancy. For this reason these were not classified as HDN by pregnancy immunisation. In 1 of the 9 suspected cases the jaundice was most likely caused by infection, therefore this neonate was also excluded. All together, based on our criteria, 4 cases of HDN were found in this cohort (Table 1).

### 3.2. Serological findings

In the group of ABO compatible pregnancies (238), one case of positive DAT and positive eluate was found. The antibody specificity was anti-E and this neonate had a jaundice score of 0. An overview of the results found in the ABOD-incompatible pregnancies is presented in Table 2.

**Table 1**
Characteristics of four neonates diagnosed with HDN.

| | Delivery | Jaundice score | DAT | Eluate | Ab | PT | Bilirubin (μmol/L) | Ab titre mother | Ab titre neonate |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Vacuum extraction | 7 | – | 1+ | anti-A | – | 159 | 1:128 | – |
| 2 | Forceps extraction | 5 | 1+ | 2+ | anti-A | – | 164 | 1:4000 | 1:2 |
| 3 | Spontaneous partus | 4 | – | 1+ | anti-A | – | ND | 1:128 | 1:1 |
| 4 | Spontaneous partus | 8 | 2+ | 2+ | anti-A | + | 297 | 1:1024 | 1:32 |

Ab: antibody specificity; PT: phototherapy; ND: not determined.

**Table 2**
Serological findings for ABOD incompatible pregnancies.

| | ABO-incompatibility | | | | | | ABO-compatible | Rhesus-D |
|---|---|---|---|---|---|---|---|---|
| **Mother** | **0** | **0** | **A** | **A** | **B** | **B** | **(other than anti-D)** | **–** |
| **Neonate** | **A** | **B** | **B** | **AB** | **A** | **AB** | | + |
| **Total number** | 46 | 14 | 5 | 6 | 3 | 5 | 238 | 31 |
| **Pos DAT** | 18 | 1 | 0 | 0 | 0 | 0 | 1[a] | 2[b] |
| **Pos eluate** | 37 | 4 | 0 | 0 | 1 | 1 | 1[a] | 18[b] |
| **Neg DAT** | 28 | 13 | 5 | 6 | 3 | 5 | 237 | 29 |
| **Neg eluate** | 9 | 10 | 5 | 6 | 2 | 4 | 237 | 13 |
| **jaundice score ≥ 4** | 4 | 2 | 2 | 2 | 0 | 1 | 22 | 5 |
| **HDN** | 3 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |

For each type of ABO-incompatible and Rhesus D incompatible pregnancy, the number of positive DAT, eluate, jaundice score and HDN are presented.
[a] Caused by anti-E.
[b] 1 × Anti-A antibodies present in DAT and 2 × in eluate. For all negative eluates, DAT was also negative.

In 19 (24%) of the 79 ABO-incompatible pregnancies a positive DAT was found and in 43 (54%) of these pregnancies a positive eluate was observed. Especially in the group of blood group 0 mothers with a neonate blood group A (AO-antagonism), a high incidence of positive DAT and eluate was found; 39% (18/46) and 80% (37/46) respectively.

All observed anti-Rh D antibodies could be ascribed to prophylactic anti-Rh D. These antibodies were either not demonstrable or demonstrable in low titres in maternal serum.

### 3.3. Predictive values for HDN

In order to investigate the diagnostic properties of the DAT and eluate, the predictive values, sensitivity and specificity were calculated for the total population, for neonates with a jaundice score of ≥4 and for ABO-incompatible pregnancies (Table 3). All but two positive DAT were caused by anti-A IgG or anti-B IgG antibodies. The majority of the positive eluate findings were caused by anti-A IgG or anti-B IgG antibodies and prophylactically administered anti-D. As a consequence, the predictive values and sensitivity/specificity presented, especially for the DAT are almost exclusively related to ABO-blood group incompatibility.

The positive predictive value (PPV) calculated from the total population was ≤10% for DAT and eluate. In addition, a limited specificity of 93% and 80% for DAT and eluate respectively was found due to the frequent presence of anti-A (and

**Table 3**
Diagnostic accuracy of DAT and eluate.

| | Total population | Jaundice score ≥4 | ABO incompatible |
|---|---|---|---|
| **HDN− and DAT− (*n*)** | 259 | 29 | 53 |
| **HDN+ and DAT+ (*n*)** | 2 | 2 | 2 |
| **HDN− and DAT+ (*n*)** | 19 | 0 | 17 |
| **HDN+ and DAT− (*n*)** | 2 | 2 | 2 |
| **Sensitivity DAT (%)** | 50 | 50 | 50 |
| **Specificity DAT (%)** | 93 | 100 | 76 |
| **PPV DAT (%)** | 10 | 100 | 11 |
| **NPV DAT (%)** | 99 | 94 | 96 |
| **HDN− and Eluate− (*n*)** | 222 | 28 | 31 |
| **HDN+ and Eluate+ (*n*)** | 4 | 4 | 4 |
| **HDN− and Eluate+ (*n*)** | 56 | 1 | 39 |
| **HDN+ and Eluate− (*n*)** | 0 | 0 | 0 |
| **Sensitivity eluate (%)** | 100 | 100 | 100 |
| **Specificity eluate (%)** | 80 | 97 | 44 |
| **PPV eluate (%)** | 7 | 80 | 9 |
| **NPV eluate (%)** | 100 | 100 | 100 |
| **Missing values[a]** | 35 | NA | 5 |
| **Total (*n*)** | 282 | 33 | 74 |

Table includes numbers (*n*) of true and false positive and negative findings, sensitivity, specificity and predictive values for HDN. Parameters were calculated for the total population, neonates with a jaundice score of ≥4 and ABO-incompatible pregnancies.
NA: not applicable.
[a] Missing values are caused by the absence of a jaundice score.

anti-B) antibodies in ABO-incompatible pregnancies. These values were calculated when both tests were used in a screening set-up for HDN in the overall population. To illustrate the relevance of ABO-incompatibility, the predictive values and sensitivity and specificity are also calculated for this population. In general clinical practice a DAT is most often performed in cases of a clinical suspicion of HDN. Therefore, the diagnostic properties of the DAT and eluate, were also calculated for the population with a jaundice score of $\geq 4$. These results are all presented in Table 3.

## 4. Discussion

In this study, anti-erythrocyte antibodies detected by eluate and/or DAT were mainly observed in ABO-incompatible pregnancies. All observed anti-D antibodies could be explained by prophylactic administered anti-Rh D. Since only one other antibody (anti-E) was detected in our population, ABO-incompatible pregnancy was by far the major cause of positive DAT and eluate screening. Despite the fact that this observation is specific for this population, due to the Dutch programme for prevention of anti-D immunisation and the cEK compatible transfusion policy in our hospital for women under 45 years, it does illustrate the relevance of ABO-incompatible pregnancies for finding a positive DAT and/or eluate screening.

In the group of A/O-incompatible pregnancies 37 (80%)occurrences of an anti-A antibody were found on the neonatal erythrocyte using eluate screening and 18 occurrences (39%) using DAT, while only 4 cases (9%) of this group presented with abnormal post partum jaundice. This discrepancy illustrates the high sensitivity of both techniques for detecting neonatal erythrocytes sensitized with anti-A and anti-B. Especially for A/O-incompatible pregnancies it appears that some degree of sensitization of neonatal erythrocytes with maternal IgG anti-A occurs regularly. The question arises as to the clinical relevance of this phenomenon. Lower haematocrit values have been reported for the group of ABO-incompatible as well as DAT-positive neonates [6,7]. This suggests that, despite the lack of obvious clinical jaundice, some degree of haemolysis could very well be present. Unfortunately, we were unable to investigate the relevance of this observation in our study, since no material was available for biochemical analysis of haemolysis and haemoglobin. The observed phenomenon of "subclinical" erythrocyte sensitization results in a low positive predictive value and specificity for both DAT as well as eluate screening [8]. Screening for HDN by DAT results in many false positive results. One should be aware that in cases of ABO-incompatible pregnancies large percentages of positive neonatal DAT results are observed in the absence of clinical jaundice. The use of DAT for screening purposes therefore remains controversial and will result in a large amount of false positive results [8–11]. For diagnosing HDN in ABO-incompatible pregnancies, additional information such as neonatal haemolysis and high titres of antibody present in the mother could be helpful to improve the diagnostic accuracy.

The calculated sensitivity of DAT and eluate screening for HDN in this population lacks accuracy due to the limited statistical power caused by the low incidence of HDN [12,13]. In 317 consecutive pregnancies only 4 (1.3%) cases of HDN were observed according to our criteria. However, this study gives accurate insights in the specificity of the DAT and eluate screening for HDN. The requirement of serological evidence of erythrocyte sensitization for determination of HDN by pregnancy immunisation together with the low incidence in this group (approximately 10%) is responsible for the high NPV of the DAT and a NPV for eluate screening of 100%. The latter is caused by the higher analytical sensitivity of eluate screening compared to the DAT for anti-A and anti-B. For detection of other blood group antagonism caused by irregular blood group systems, the DAT probably has a better analytical sensitivity which is more comparable to the analytical sensitivity of eluate screening. This can be explained by the low neonatal erythrocyte expression of the A- and B-antigens [15].

The PPV for HDN in the jaundice group is much higher compared to the overall population due to the increased incidence, but is still imprecise due to the small number of positive HDN cases.

Other groups have reported a higher sensitivity in a comparable population (86%) and postulate that in cases of DAT negative HDN, other causes of hyperbilirubinemia should be sought [8,14]. In our study the DAT missed 2 cases of HDN. This might also be explained by the higher analytical sensitivity of the eluate technique for detecting erythrocyte-bound antibodies together with coincidence of other non-specified causes of jaundice [3,5]. The more sensitive eluate screening technique was not used in these previous studies.

## 5. Conclusion

The DAT has limited sensitivity for HDN and is therefore an invaluable screening tool for HDN. In addition, a positive DAT should be interpreted in the context of ABO-incompatibility because of its limited specificity in these pregnancies. In cases of a strong clinical suspicion for HDN together with a negative DAT, a second, more sensitive test such as eluate screening should be performed in order to rule out or confirm the diagnosis.

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## Conflict of interest

None for all authors.

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