Direct Antiglobulin Test in Predicting the Severity of Hyperbilirubinemia and Haemolytic Disease of the Newborn

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Abstract
This study investigated the efficacy of the Direct Antiglobulin Test (DAT) reaction grades and the severity of hyperbilirubinaemia, in order to assist with the prediction and treatment of HDN. However, a weak correlation was found in this study. DAT significance may be ambiguous and it is only when combined with clinical assessment may it aid in building a complete prognosis. This was supported from studies such as [1,2], who also believed that in isolation DAT significance is limited without other parameters such as, the Full Blood Count (FBC), Total Serum Bilirubin(TSB) , reticulocyte count and more importantly a clinical assessment.

A total of 287 neonates were identified and eligible for the study over a 3 year period, 126 were female of which 77.8% with a negative DAT, 15.9% weakly positive (1+), 4.8% with a 2+ reaction strength and 1.6% as 3+ reaction. In comparison there were 161 male neonates with similar results with a negative DAT at 70.2%, 1.9% had an inconclusive DAT and 13% had an 1+ DAT reaction. The male neonates were around 3 times more likely to have a 2+ DAT at 11.8% than the female neonates and almost twice more likely to have a 3+ DAT. Both genders had a p-value of 0.92. There was a low prevalence of DAT positive neonates. The frequency of negative DAT made up 73.5% of the study population with a skewness 1.879. In total there were 76 (26.4%) of which 14.3% had a weakly positive (1+) DAT.

The independent distribution of DAT positivity suggested non-parametric statistics application to the dataset. The DAT results were skewed (showing a disproportionate number of negative DAT results. However, this was also found in studies described by [3], who found only 37/303 neonates having a positive DAT.

The limitations to the study were the small number of patients with a positive DAT, only having 2 patients with a positive DAT >3. With it being an observational study, a correlation between the DAT positivity and hyperbilirubinaemia and phototherapy was sought, however this could not be used to determine causality within the cohort as there was so much variations between the subjects and a follow up with neonates was not justified post discharge.

Introduction
The Direct antiglobulin test (DAT) was first described in 1908 by an Italian Clinical Scientist called Carlo Moreschi [4], however became popularly known as the “Coombs test” in 1945 after it was described at Cambridge University by Robin Coombs, Arthur...
Mourant, and Rob Race [5]. Its clinical application is to detect the presence of immunoglobulins (IgG), complement or both, on the surface of erythrocytes in vivo. The DAT is valuable when diagnosing many immune conditions such as autoimmune haemolytic anaemia, drug induced haemolytic anaemia, haemolytic transfusion reactions and haemolytic disease of the newborn (HDN). [2,6] reported its use as having a limited sensitivity in predicting HDN but it is still frequently requested when HDN or jaundice is suspected. The neonatal period is defined as the first 28 days of life and for most newborns jaundice may not necessarily be an indication of an underlying disease and can be considered harmless. However, according to the NICE guidelines [7], jaundice can be one of the most common conditions also requiring medical attention, with around 80% preterm and 60% of term babies developing the condition in the first week of life. Initial assessment of prolonged neonatal jaundice is defined at Princess Alexandra Hospital as visible jaundice persisting beyond day 14 in term neonates and visible jaundice persisting beyond day 21 in preterm infants. The normal range for TSB is set between 0-21µmol/L. There are many causes for neonatal jaundice, such as a greater turnover of red blood cells as the haemoglobin levels drop significantly during the first 2-3 days of life, continuing for about 2 months, leading to hyperbilirubinaemia [8]. The newborn liver requires time to establish an arterial supply during the first week of life causing a reduction in the liver’s blood supply which contributes to reduced production of liver enzymes needed for the conversion of the lipid soluble bilirubin to a water-soluble version so that it can be excreted in bile and urine. This is performed by a process of glucuronidation by the enzymes Uridine 5’-diphospho-glucuronosyltransferase (UGT) which are underdeveloped in the neonate. HDN occurs when there is an feto-maternal erythrocyte incompatibility which leads to allo-immunisation causing the mother to develop antibodies to the foreign antigen derived from paternal antigens inherited by the neonate. The neonate erythrocytes sensitised by maternal IgG (subclasses 1 and 3) antibody are processed by their immature reticuloendothelial system which is incapable of conjugating enough of the bilirubin, a product of the destruction of the antibody coated red cells. The most immunogenic include antigens of the Rh and K systems. ABO HDN although rare is now becoming more prevalent than Rh HDN due to the introduction of the routine antenatal anti-D programme. This is reported largely limited to maternal blood group O having a newborn with blood group A or B as described by [9]. The occurrence of IgG anti-A or anti-B antibodies in type O mothers also explains why haemolysis caused by ABO incompatibility frequently occur during the first pregnancy without prior sensitization unlike an Rh incompatibility which occurs during subsequent pregnancies. [10] noted exclusively that breast-fed newborns also have a tendency for a type of jaundice due to the presence of beta glucuronidase in breast milk which increases the breakdown of conjugated bilirubin to unconjugated in their gut. Physiological jaundice can be considered normal due to the immaturity of the neonates’ liver, as mentioned above, where total bilirubin levels reach a peak by day 4 and reduces by day 14, it will not be seen on day 1 whereas pathological jaundice, can be seen on day 1, can persist beyond 14 days in a term neonate and 21 days in a premature neonate which will require further investigation [11]. The increased levels of unconjugated bilirubin can cause yellowing of the skin and sclera and when left untreated deposition of bilirubin in the brain can occur causing a condition known as kernicterus where permanent damage to the brain, hearing loss and cerebral palsy can occur [12]. Why are some newborns more susceptible to jaundice than others? Could this be attributed to postnatal age or gender where sex hormones may influence bilirubin conjugation [13] Or could it be due to co-morbidities of both the neonate and mother? An example of this was described by [14], they described a mother with Systemic Lupus Erythematosus (SLE) where transplacental autoantibodies caused the neonate to develop a self-limiting jaundice.

**Methodology**

**Determining Sample Size**

Sample size was initially determined over a period 12 months 2022-2023, which was 75 samples, this was small to provide any statistical power therefore was increased to 36 months. The population size of Princess Alexandra Hospital (PAH), Harlow for 2023 approximated to 92,149 according to http://population.city/united-kingdom/harlow/ so the sample size was calculated based on this figure using www.calculator.net at a 95% confidence level, a 5% margin of error and a population proportion of 50% giving a sample size of 383. This means 383 or more measurements/surveys are needed to have a confidence level of 95% that the real value is within ±5% of the measured/surveyed value. This study was approved by the Patient Safety and Quality Team at Princess Alexandra NHS Hospital (approval number: 4076). To protect the anonymity of the subjects in this study clinical data obtained for this study were numerically coded for anonymity.

**Inclusion and Exclusion Criteria**

The inclusion criteria for this study were neonates diagnosed with jaundice with a total serum bilirubin level (TSB) and DAT requested over a 3-year period.
Neonates known to have a haemoglobinopathy and other hereditary conditions such as G6PD, Gilbert’s syndrome and Criggler-Najjar syndrome [15], which increase bilirubin levels due to an impairment in bilirubin conjugation and plays a role in pre hepatic jaundice will be ineligible and excluded from this study.

Confounding Factors
This was not a controlled study; the resulting sample size was quite small compared to other studies. Both of these limitations may produce a greater level of sampling error but can be outweighed by systematic error. To ensure confirmability, which explains the sampling error but can be outweighed by systematic error, the researcher’s attempt to check study findings this was supported by a second member of staff checking to provide data confirmation of accuracy which could enhance the aptness of the study.

Method of Testing
The inclusion criteria for this study were neonates diagnosed with jaundice with a total serum bilirubin level (TSB) and DAT requested over a 3-year period. The inclusion criteria for this study were neonates known to have a haemoglobinopathy and other hereditary conditions such as G6PD, Gilbert’s syndrome and Criggler-Najjar syndrome [15], which increase bilirubin levels due to an impairment in bilirubin conjugation and plays a role in pre hepatic jaundice will be ineligible and excluded from this study.

Number of neonates had an inconclusive DAT and 13% had a 1+ DAT. Both genders had a p-value of 0.92. There was a low prevalence of DAT positive neonates in this study. The degree of negative DAT made up 73.5% of the study population with a skewness 1.879. In total there were 76 (26.4%) of which 14.3% had a weakly positive (1+) DAT. There were 8 cases where an alloantibodies was implicated were anti-Cα and anti-M needing double phototherapy, anti-M and anti-Fy(a) requiring single phototherapy, anti-c where sunlight and regular feed was sufficient, anti Le(a) resolved, anti-D with triple phototherapy and anti-D where folic acid was given. 7 out of 8 were male and 4 out of 8 had a maternal incompatibility. 4 out of 8 had a positive DAT all with a 2+ reaction.

Mother/infant ABO blood group incompatibility was recorded in 150/287 (52%) of the study population. This included, in order of the most common ABO incompatibility O/A in 65(43.3%), A/O in 29(19.3%), O/B in 21(14%), B/O in 19(12.7%), AB/A in 5(3.3%), A/AB in 4(2.7%), B/AB in 4 (2.7%), and AB/B in 3(2%). The range for gestational age in weeks were 25 to 41 with a mean age of 36.90 SD ± 3,129 chi-square p-value .124 and rs=.119:p=.044. The birth weight was measured in grams, there were 2 missing data points with a minimum weight 1208gms and a maximum 4998gms. The mean weight was 3094.75 and SD ± 801.61 Chi-square p-value 0.63 and rs=0.167:p=0.005. The majority of neonates were breast fed for term and plus term infants however if medical interventions were needed and pre term neonates an artificial feed was used p-value .83 rs =0.079: p = 0.183. In all cases the variables had a weak positive association with the DAT with a low Chi-square probability that this was of significance. The range of TSB results were min. 8 and max. 595 with a mean of 248.17 and an SD ±106.59. A DAT was performed predominantly when the TSB was 201-300 µmol/L. The DAT was requested rarely when the TSB was between 0-20 µmol/L and when the TSB was greater than 300 µmol/L. Chi square p=0.01 showed that there was a high probability of a meaningful difference however the Spearman rho rs=0.024:p=0.682 indicated a weak positive relationship that did not have a statistically significant association between the two variables. 51.9% of neonates showed an ABO incompatibility with their mother. The results showed 95(63.8%) had a negative DAT. Among this group, the DAT was found to be positive 36.3% of the time. The most common was the weakly positive (1+) at 28(18.8%), Chi-square p value= 0.001 indicating a high probability of a meaningful difference. There was a weak negative strength of association r_s= -0.238: p=0.001, as shown using Spearman correlation. Conversely, Rh...
incompatibility was found in 57 (19.9%) of the study population. 39 (68.4%) had a negative DAT. Three neonates had an inconclusive result, there was a difference of 1 between a DAT 1+ (14%) and 2+(15.8%). Chi-square p-value 0.17, rs = -0.059; p = 0.322 showing there was a low probability of a meaningful difference and there was no association between them. The Kruskal-Wallis test was used to determine whether the distribution of the DAT was the same across the different categories of therapy used at the PAH (n=287) rs=0.519, p=0.607, this indicated an insignificant difference. Single phototherapy was the therapy of choice irrespective of the DAT strength and for a negative or weakly positive DAT the full spectrum of therapy was used. Table 1 rs=0.089; p=0.135 indicating a very weak positive correlation that was not statistically significant. The Pearson Chi-square p=0.122 showed there was a low probability of a meaningful difference between the DAT strengths in relation to the therapy used.

Discussions

This study was conducted with a view to re-design and implement a new a practice in accordance with the BSH guidelines of reporting the DAT reaction strength to aid in predicting the degree of hyperbilirubinaemia and HDN at the Blood Transfusion laboratory, PAH. The first step was to identify neonates where a total serum bilirubin level and DAT request, we aimed for a sample size of 383 neonates. However, the sample size was not met at 287 neonates, the more data collected, the more abridged the data became leading to a less reliable estimate and a decrease in statistical confidence. The independent distribution of DAT positivity suggested non parametric statistics application to the dataset. The DAT results were skewed showing a disproportionate number of negative DAT results versus the hyperbilirubinaemia, however this was also found in studies described by [3], who found only 37/303 neonates having a positive DAT. Further differentiation of the reaction strengths additionally reduced the test population. The variation between the TSB concentrations and the positivity of the DAT supported the concept of its use as an indicator of immune mediated haemolysis only when haemolysis is robust or at least sufficient to produce an imbalance between bilirubin production and bilirubin clearance promoting hyperbilirubinaemia development [16]. This study showed the DAT identified 26.5% of neonates with TSB needing further investigations. The DAT had no discriminatory capacity to distinguish between positive class and negative class indicating that the test provided little value when predicting hyperbilirubinaemia 201-300µmol/L. The decision to compare the DAT with datapoints of TSB 201-300µmol/L was due to there being more positive DAT results at that level, this could have been further explored and possible provide more incite with a precision recall curve as described by [17]. It is best used when the dataset is highly skewed with more negative DATs than positive with a greater degree of imbalance therefore using positive and negative predictive values. This study was from a single Trust and a bigger sample size and the collaboration with other Trusts would give this study general credence and power. The homogenous of the study, sample size and the distribution of the results will require further exploration. Timing of the TSB taken could have impacted DAT positivity, except it was discovered TSB >400µmol/L rarely required a DAT as immediate treatment was required to prevent kernicterus, as this was a retrospective study, this data was missed which could have provided some useful insight. Additionally a significant observation from many publications such as [18], highlighted the importance of the timing of testing as they found readmission to be between two and five days which coincided with ABO HDN and this was generally assumed when hyperbilirubinaemia or neonatal anaemia was discovered [19]. The DAT was rarely requested when the TSB was 0-20µmol/L or when the TSB was greater than 300µmol/L with single phototherapy being the most utilised therapy, there was never a need for an exchange transfusion at PAH trust. [18] mentioned a variation of TSB amongst neonates to be based on skin tone and body region which raised the importance of the strength of multiple investigations as one test alone cannot define causality or the gravity of the condition. ABO antibodies found in the plasma of blood group O individuals include anti-A, anti-B and anti-A,B these antibodies are predominantly IgM and weaker IgG, whereas the anti A,B is primarily IgG therefore neonates of group O mothers are at a higher risk for HDN as the larger IgM pentamer cannot cross the placenta. DAT positivity due to ABO incompatibility was found mainly in O/A setting with a DAT 1+ (weakly positive), similarly to most other studies. Low expression of A and B antigens over neonatal red blood cells may be the reason for the low DAT positivity. Moreover ABO antigens are expressed widely over tissues such as endothelial and epithelial cells which could result in a decrease in binding of these antibodies to RBCs also a naturally lower expression of A and B antigens on the neonatal red cells therefore leading to a less severe haemolysis[20]. This study also indicated that there was a significant stronger association with the DAT predicting ABO HDN than Rh HDN with a p value of .01, this could have been due to the impact of the routine antenatal anti-D prophylaxis and simply there is more of a variation between .This was also substantiated by the Mann Whitney U test where there was a moderate size difference between the ABO compatible and incompatible in relation to the DAT whereas the Mann-Whitney U test showed a
smaller size difference between the Rh incompatible and compatible samples showing a statistically very weak negative correlation. This could ratify the need for development of a preventative model for an ABO prophylaxis, as mentioned by [21]. However, it’s need may be inconsequential due to ABO HDN being mild in nature. The difference between the ABO and Rh HDN significance was unprecedented, as an assumption was made that the mechanism of DAT positivity would be the same for both variables although it is possible that there could have been an underlying factor being the source of variability, which could also be due to the use of anti-D prophylaxis. [22], compared the DATs ability to detect haemolysis and jaundice with the end tidal carbon monoxide concentration and showed it to be non-invasive and more sensitive than the DAT with a high positive predictive value for significant jaundice, however the cause of this test not being readily available at PAH was not established.

The DAT was the same across all categories of therapy based on the Kruskal-Wallis test. There was no correlation between the therapy and the strength of the DAT as first proposed. Conversely the findings of [2] demonstrated a positive correlation between the DAT positivity of cord blood and phototherapy, which could support future work in comparing the DAT of the cord and venous sample as if they were comparable it could prevent the need for neonatal blood sample to prevent early discharge and allow early prevention. [23,24] research substantiated the findings of this study that there were no relationship between the DAT and therapy. [23] described the primary outcome was to determine the association of the positive DAT with a p=0.271 and did not govern a higher TSB level. The risk associated with kernicterus is due to the levels of unconjugated bilirubin, the assessment of the total serum bilirubin did not show the conjugated/unconjugated ratio (unconjugated normal range is 3-13 µmol/L). The unconjugated bilirubin level can be broken down by phototherapy but incapable of the breakdown of conjugated bilirubin. When the conjugated bilirubin is more than 20% of the TSB this is suggestive of a liver disease [25]. This was deemed relevant, due to the opinion that some neonates having unnecessary treatments based on cases that would spontaneously resolve without intervention [26], as the results from the study showed 50/287 (17%) resolution. There was a greater degree of variance in treatment with a negative DAT than the treatment option became less varied as the DAT reaction strength increased. Incidentally the distribution of bilirubin levels was assessed to determine whether the levels were the same across the categories of therapy also using the Kruskal-Wallis test and was found to not be the same p= 0.025, showing it to be more clinically significant. The presumption being if the results showed a weak relationship between the DAT and TSB that it would also have a weak relationship with therapy. The impact of breastfeeding is the likelihood of dehydration in the neonate leading to excessive weight loss associated with hyperbilirubinaemia especially within the first 72 hours post partum, which was not considered within the study. The dehydration inhibits the excretion of bilirubin in urine by decreasing gut motility [27]. Similarly to [27], it was difficult to establish a distinction between the methods of feeding as 26% of mothers adopted mixed feeding causing ambiguity within the feeding technique group. Having a stricter cut-off could not have been clearly defined as the decision to breast feed is not only a scientific reasoning but personal too. The data including gestation and feeding technique although significant, showed more relevance when determining the significance for the clinical aspect of managing jaundice of the newborn as described in the NICE guidelines and was shown to have a weak association based on the Spearman’s rho. Prematurity was not highlighted in this study as the Trust’s policy would be to transfer patients to a specialised centre. Additionally pre-term Infants would generally have a further underlying condition and have had interventions prior to delivery such as intrauterine transfusion and therefore there could be a dilution of the alloimmunisation and an increase in donor blood.

The limitations to the study were the small number of patients with a positive DAT only having 2 patients with a positive DAT >3. With it being an observational study, a correlation between the DAT positivity and hyperbilirubinaemia and phototherapy was sought, however this could not be used to determine causality within the cohort as there was so much variations between the subjects and a follow up with patients was not justified post discharge. Stratifications were undertaken by subgrouping based on the similarities within the data variables, however due to the small sample size this reduced the data sample size further and as such I believe this caused a loss in statistical power.

**Conclusion**

A DAT demonstrates the presence of IgG antibody bound to the surface of the newborns red cells a precondition for immune mediated haemolysis, without the presence of this it would be difficult to say whether this was an immune dependent process therefore useful in determining whether the cause of haemolysis is immune or non-immune. It has been reported that IgG subclasses 1 and 3 are the predominant immunoglobulins detected in HDFN. HDFN was more severe when both subclasses were present than when only IgG1 as proposed in studies such as [28,29]. Contrary to this, [30] found IgG1 and IgG3 of anti-D had no predictive value by themselves.
and therefore could not predict HDFN but concluded the subclasses should be further explored for evaluating the HDFN severity. A literature review found no such studies in the United Kingdom, however it is evident this data is relevant, as this showed consistency with the composition of the Rhophylac anti-D immunoglobulin IgG1=84.1%, IgG2=7.6%, IgG3=8.1% and IgG4=1.0%, with clear evidence of its effectiveness in routine antenatal anti-D prophylaxis programme in providing protection in RhD negative women. Anti-D prophylaxis can result in a positive DAT in the neonate who may not necessarily be at risk of haemolysis. This has further been minimised by the introduction of the cfDNA RhD screening described in the BSH guidelines (White et al., 2016), adopted by PAH. By determining the fetal RhD status at 11+2 weeks prophylactic anti-D would not need to be administered if the fetus was predicted as being RhD negative, limiting the exposure to a blood derived product and testing postnatally. The NHSGGC guidelines [31] direct reporting weakly positive DAT as positive to be redundant, unless requested by the Clinician due to there being significant jaundice. The risk of morbidities could be avoided with robust antenatal management and quantitation or titration monitoring of maternal antibodies that can lead to HDFN and base this on the premise that if the maternal antibodies have not reached a moderate to high risk to the fetus up to 28 weeks gestation there will be no increased risk postnatally for anaemia. For this to be discussed as a quality improvement for the PAH trust further study and risk assessments would need to be performed however this could aid in earlier hospital discharge and unwarranted testing and interventions, providing a cost saving. The severity of HDN varies depending on the properties of the maternal antibody, the level of antibody in maternal blood and duration of exposure of the foetus to the antibody NICE (2010). Consequently, where there is evidence of haemolysis or hyperbilirubinaemia the aetiology could be due to an antibody to a low frequency antigen coating the neonate’s red cells not detected during maternal antibody screening, such as anti-Wr(a) which can cause severe HDFN. The utilisation of such tests as the end tidal carbon monoxide concentrations a topical non-invasive screening tool of haemolysis reported as having a positive correlation with higher positive predicted values for hyperbilirubinaemia [32,33], this data has been consistent for 20 years but still not readily available at Princess Alexandra Hospital NHS Trust. The study design was to determine the efficacy of the DAT reaction grade in determining the severity of hyperbilirubinaemia, whether it could aid in the prediction of HDN and to determine if this would have an impact on therapy selection, however only a weak correlation was found. DAT significance may be ambiguous and it is only when combined with clinical assessment may it aid in building a complete prognosis, thus whether we report the reaction strength to the clinicians or not, there would be no diagnostic, cost implications nor impact on treatment. This was supported from studies such [1,2], who also believed in isolation its significance is limited without other parameters such as, the FBC, TSB, reticulocyte count and more importantly a clinical assessment.

Declaration of Competing Interest

The co-authors do not have any conflict of interest to declare.

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