# Haemolytic disease of the newborn

# INTRODUCTION

## Outline of the Project

The selected gene is KEL gene, which plays a role in Haemolytic Disease of the Newborn (HDN). The specific exon that I will focus on is exon 6, which is known as K1. This usually affects neonates during pregnancy or from transfusions given to the mother, hence causing the disease HDN. I will amplify this gene via PCR and commenting on its effectiveness in identifying polymorphisms. In addition, I will also be researching latest techniques used in identifying KEL1 gene in foetuses.

## What is HDN?

HDN was once a major cause of fetal loss and death among neonates. HDN was first discovered in 1609 by a French midwife who delivered twins, one baby was swollen died just after birth. The other baby developed jaundice, which lead to death several days later. Many cases continued to develop for the following 300 years, leading to many deaths in newborns (Dean, 2005).

The principal cause was not identified until the 1950s, which was known to be the newborn’s red blood cells (RBCs) being attacked by antibodies from the mother. This occurs during the mother’s pregnancy, while the baby is still in the womb (Dean, 2005). This is due to the transfer of antibodies IgG1 and IgG3 sub-types across the placental barrier from the mother to the fetus. These antibodies then cause destruction of fetal RBCs or erythroid progenitors, which may continue after birth (Finning et al, 2007).

Then in the 1960s, trails were being conducted in the United States (U. S) and the United Kingdom (U. K) to develop therapeutic strategies. They tested

the use of antibodies as a therapy, which could remove antibodies that cause HDN from the mother's circulation. The trail proved to prevent HDN from occurring by using these therapeutic antibodies during the pregnancy. By the 1970s, routine antenatal care was given to all mothers who were found to be at risk of HDN and were given the preventative treatment. This has lead to a huge decrease in the prevalence of HDN, particularly death (Dean, 2005).

## How is HDN caused and different causes of HDN

In pregnancy, it is normal for the mother's antibodies to be transported across the placenta and enter the fetal circulation. This occurs because when the baby is born they only have a primitive immune system, therefore, the presence of maternal antibodies ensure they survive while their immune system is developing. However, the disadvantage of this protection is that by targeting fetal RBCs, maternal antibodies can also cause HDN (Dean, 2005).

A prevalent cause of HDN is incompatibility of the Rh blood group between the mother and fetus. Haemolytic disease is mostly triggered by the D antigen, however, other antigens such as c, C, E and e, can also cause problems (Dean, 2005).

The pregnancies that are at risk of HDN are an Rh D-negative mother who is carrying an Rh D-positive child, in which the child inherited the D antigen from the father. This causes the mother to produce an immune response to the fetal D antigen by forming IgG antibodies against it (anti-D) (Dean, 2005).

Incompatibility of the ABO blood group can also cause HDN. It occurs when a mother with blood type O becomes pregnant with a fetus that has a different blood type, such as type A, B or AB. The mother’s serum contains naturally occurring anti-A and anti-B of the IgG class and can hence cross the placenta and haemolyse fetal RBCs. However, this is usually less severe than Rh incompatibility (Dean, 2005).

Other causes of HDN include antibodies against antigens of the Kell blood group (e. g. anti-K and anti-k), Kidd blood group (e. g. anti-JKa and anti-Jkb), Duffy blood group (e. g. anti-Fya), MNS and s blood group antibodies. However, these are less common compared to the others (Dean, 2005).

## The Kell blood group

The Kell blood group system is complex and has many antigens which are highly immunogenic. These antigens (particularly KEL1) are second to RhD as a cause of HDN at triggering an immune reaction (Li et al, 2008). As mentioned previously, antibodies that target Kell antigens can cause HDN, as well as, transfusion reactions. Even though, HDN due to Kell immunization is rare, cases cause severe fetal anaemia as maternal anti-Kell target fetal RBC precursors, suppressing the fetal production of RBCs (Dean, 2005).

The Kell blood group system was discovered in 1946 and was named after Mrs. Kelleher, a patient whose anti-Kell antibodies lead to HDN in her newborn. Since this finding, 25 Kell antigens have been established, which are expressed in different frequencies in diverse populations (as seen in Table 1). However, the original K antigen is still important in transfusion

medicine and HDN (Dean, 2005). Furthermore, Kell is considered to be one of the major human RBC group (Wagner et al, 2004).

**Table 1: The Kell antigens and the distribution among the population (Redman et al, 1999).**

| Low incidence | High incidence |
|---|---|
| KEL1 (K) | |
| KEL2 (k) | |
| KEL3 (Kpa) | |
| KEL4 (Kpb) | |
| KEL17 (Wka) | |
| KEL11 (K11) | |
| KEL23 (K24) | |
| KEL14 (K14) | |
| – | |
| KEL5 (Ku) | |
| KEL10 (Ula) | |
| – | |
| – | |

KEL12 (K12)

–

KEL13 (K13)

–

KEL16 (K16)

–

KEL18 (K18)

–

KEL19 (K19)

–

KEL20 (Km)

–

KEL22 (K22)

KEL23

–

KEL25 (VLAN)

–

–

–

KEL26 (TOU)

–

Kx

Distribution of low incidence antigens

KEL1

9% in Caucasians, 2% in Blacks, 12% in Iranian Jews

KEL3

2% in Caucasians

KEL6

0. 01% in Caucasians, 20% in Blacks

KEL10

Less than 0. 01% in most populations, 2. 6% in Finns, 0. 46% in Japanese

KEL17

About 0. 3% in most populations

KEL21

Less than 0. 01% in most populations, 0. 32% in Japanese

KEL23

Less than 0. 01% in most populations

KEL24

Less than 0. 01% in most populations

KEL25

Only found in 1 family

The antigens classified as high incidence occur in over 90% of the population

## The KEL gene

KEL is inherited in an autosomal codominant manner and differs in different populations (Lee, 1997). KEL is located on chromosome 7q33 and consists of 19 exons in the range of 63 and 288bp and spanning approximately 21. 5Kb. The size of the introns range from 93bp to approximately 6kb. Exon 1 has an untranslated region consisting of GATA-1 and Spl binding sites, with a single codon for a potential initiation methionine. The upstream region (ÿ176) contains a CACCC box and two other presumed GATA-1-binding sites. The nucleotide region, ÿ176 to ÿ1, placed in front of a reporter gene, shows promoter activity, however complete analysis has not been conducted. The KEL coding region, except exon 1 that initiates methionine, resides in exon 2 to exon 19. Exon 2 is also likely to initiate methionine at nucleotide 178, amino acid residue 20. Neither two presumed translation initiation in KEL are classic Kozak sequences. The single membrane spanning region of Kell

protein is encoded in exon 3 and the zinc-binding catalytic site is in exon 16. The base sequences in exons 16 and 19 are the most homologous with the other members of the M13, Neprilysin and zinc endopeptidases family (Redman et al, 1999).

The Kell/XK complex, which shows amino acid substitution in different Kell phenotypes. The transmembrane domains of Kell and XK subunits are shown as cylinders. Cysteine residues are shown as (C), but some cysteines in XK are not shown. The disulphide linkage between Kell Cys72 and XK Cys347 is illustrated. The amino acid changes in Kell protein, resulting from point mutations in KEL of different phenotypes are shown. The C-terminal domain of Kell, which has the highest homology with other M13 zinc endopeptidases, is shaded, and the zinc binding, enzymatic active site (HELLH) is marked. N-glycosylation sites in Kell are designated “ Y.” The hollow Y corresponds to the N-linked sugar that is not present in KEL1 protein (Lee et al, 2000).

## KEL1

KEL1 (K) is the strongest immunogenic Kell antigen and is the common cause of antibody production in mismatched blood transfusions, and maternal alloimmunization, which causes severe anaemia in neonates (Lee et al, 2000). The KEL1/KEL2 (K/k) DNA polymorphism is a single base substitution (C to T) in exon 6 of the KEL gene, causing exchange of threonine to methionine at residue 193 (Li et al, 2008). This amino acid substitution voids a compromised N-glycosylation site and the Kell protein in red cells of KEL: 1,-2 phenotype has 4, rather than 5, N-linked oligosaccharides (Lee, 1997).

The KEL1 gene is inherited in an autosomal codominant approach. If the pregnant woman is KEL1-negative and has a partner that is KEL1/KEL2 heterozygous, then the fetus has a 50% chance of being KEL1-positive, placing it at risk (Lee, 1997).

## Anti-Kell and HDN

Anti-Kell is an important cause of HDN. It seems to occur in mothers who have had several blood transfusions in the past, but also arises in mothers who have been sensitized to the Kell antigen in previous pregnancies (Dean, 2005).

Anti-K is prevalent in approximately 1 in 1000 pregnant women, and about 40% of K+ babies of women with anti-K are affected with severe anaemia. K has a frequency of about 9% in Caucasian but is much less in Africans (1. 5%) and rare in Eastern Asian and in Native Americans. The k/K polymorphism results from a 698C> T single-nucleotide polymorphism (SNP) in KEL encoding a T193M substitution in the Kell glycoprotein. The pathogenesis of HDN caused by anti-K differs from anti-D. This is because it is much harder to predict, as there is little association between anti-K titre and severity of the disease and anti-K HDN is linked with lower concentrations of amniotic fluid bilirubin than in anti-D HDN of corresponding severity and postnatal hyperbilirubinaemia is not present in babies with anaemia caused by anti-K. Also, there is decreased reticulocytosis and erythroblastosis in the anti-K disease. Fetal anaemia in anti-K HDN is due to suppression of erythropoiesis. The Kell glycoprotein emerges on erythroid progenitors, by macrophages in the fetal liver, before they develop into haemoglobinized erythroblasts (Finning et al, 2007). As RBC precursors

contain no haemoglobin, the release of bilirubin is reduced during haemolysis, therefore jaundice is not as common, but severe anaemia may occur (Dean, 2005). This is supported by in vitro studies which have shown that antibodies to Kell inhibit growth of KEL1 progenitor cells. The mechanism by which antibodies to Kell suppress erythropoiesis is unknown. However, studies of progenitor cells have also demonstrated that Kell surface antigens are expressed ahead of Rh, Landsteiner± Weiner, glycophorin A, band 3, Lutheran and Duffy. This suggests that antibodies to Kell may react at an early stage of erythropoiesis where the progenitor cells are first committed to erythroid differentiation, suppressing further growth. As the endothelins are mitogenic and may have an effect on maturation of erythroid precursors, other possibilities are that the endothelins-3-converting enzyme activity of Kell may be involved in the process and antibodies to Kell may change its capacity to process endothelins (Redman et al, 1999).

## Management of HDN in women with KEL1 fetus

In order to prevent HDN, it is necessary for the detection of the fetal KEL1 gene (Li et al, 2008. In pregnant women with anti-KEL1 and who have a partner with KEL: 1, 2, prediction of KEL1 phenotype from tests on fetal DNA allows obstetricians to adjust management of the pregnancy based on whether the fetus is at risk of developing HDN. This DNA can be acquired from amniotic fluid, chorionic villi, or maternal plasma, which requires the analysis of polymerisation chain reaction (PCR) products with the restriction enzyme Bsm1, PCR including an allele specific primer, or allelic discrimination by Taqman technology (Poole et al, 2006).

Three methods have mainly been used over the past decade for prediction of KEL1 phenotype or KEL1/2 genotype from genomic DNA.

1. PCR with a KEL1 allele-specific reverse primer followed by agarose gel electrophoresis (Poole et al, 2006). This is performed using an antisense K1-specific allele-specific PCR primer specific for exon 6 of the KEL gene and a sense primer specific for exon 5. An internal control primer is also included.

The PCR is performed with a final volume of 50µl containing the following: 25ng each of Kell sense and anti-sense ASP-primers, 1ng each of Rh exon 5 internal control primers, 10mM tris-Cl pH 8. 3, 500mM KCl, 3. 0mM MgCl2, 1. 25mM each dNTP and 1. 25U Taq DNA polymerase. Then the following cycles conditions are required: 5 min at 94°C followed by 30 cycles at 94°C/1 min, 70°C/1 min and 72°C 1/min 30s, then lastly 72°C extension step for 5 min (Avent & Martin, 1996).

2. Direct sequencing of the region of KEL exon 6 including the KEL1/2 polymorphism. A 384-bp product in amplified by PCR with the following primers: KellseqFor, GCTTCCTAGAGGAATCCAAG, and KellseqRev, TATCACACAGGTGTCCTCTTCC. The annealing temperature was 70°C for 1 cycle and then reduced by 2°C for each of 8 cycles, followed by 35 cycles at 52°C. After electrophoresis of the amplified product in a 1. 5% agarose gel, the band is cut out of the gel and the DNA removed. Sequencing reactions are conducted with a ready reaction kit and KellseqFor or KellseqRev primers for forward and reverse reactions, respectively and an automated sequencer is used to carry out the sequencing (Poole et al, 2006).

3. Allelic discrimination by Taqman PCR. This involves PCR amplification of the region of the KEL gene including the KEL1/2 polymorphism and the amplification of two Taqman fluorescent probes, each carrying different reporter dyes and each specific foe either the KEL1 or the KEL2 allele. The sequences of the primers are the following: KELL_ADF, GGAGGCTGGCGCATCTC; and KELL_ADR, GAGAGGCAGGATGAGGTCCA. The KEL1 specific and KEL2 specific probes, which integrate the reporter dyes FAM and VIC, respectively, are TAACCGAATGCTGAGACTTCTGATGAGTCAG and TAACCGAACGCTGAGACTTCTGATGAGCAG. Amplification happens in a 96-well plate. Incubation at 50°C for 2 minutes and then 95°C for 10 minutes is followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fluorescence is then read by a sequence detector. Relative endpoint fluorescence from the two reporter dyes us calculated and scored as homozygous for KEL1 or KEL2 or heterozygous for both (Poole et al, 2006).

PCR techniques for determining blood groups using fetal DNA isolated from maternal plasma have allowed the application of non-invasive methods in a simple and reliable method (AraÚjo et al, 2005). Furthermore, this is the method I will be using in order to amplify K gene and identify any polymorphisms, therefore, this will determine how effective the technique really is.

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