Human platelet antigen (HPA) 1-5 and 15 typing in an Egyptian population using sequence specific primer PCR and TaqMan technology including studies of four discrepancies in the HPA 2 system.

Thesis (9th and 10th semester Medicine (stadium IV), University of Tromsø)

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Tromsø, August 28 2006

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1. Abstract

Genotyping of 196 DNA samples from a cohort of pregnant Egyptian women for HPA 1-5 and 15, using both sequence-specific primer (SSP)-polymerase chain reaction (PCR) and TaqMan technology was performed. When the results of the genotyping of the Egyptian cohort was compared with gene frequencies in a Caucasian population (1, 2), there was no difference in the frequencies of both a and b alleles in HPA 1-4 and 15, in HPA 5 a significant difference was found.

Four samples showed discrepancies in the HPA 2 system. In conventional PCR the samples were typed HPA 2bb, using TaqMan technology they were typed 2ab. The reason for this could be either a) a single nucleotide polymorphism (SNP) in the binding site for the primers used in SSP-PCR or the probes used in TaqMan or b) an error made during the performance of the PCR. The four samples in question were sequenced and this showed that they were all 2bb. The reason for the mistakes in TaqMan typing remains unknown.

2. Introduction

Human platelets are produced in the bone marrow by fragmentation of the cytoplasm of megakaryocytes. Platelets are discoid cells with a diameter of 1.5-3 μm. On the surface of platelets there are several glycoproteins (gp) including the receptors for platelet activating ligands. The gps are particularly important in the platelet reactions of adhesion and aggregation which are the initial events leading to platelet activation and plug formation during haemostasis (3).

Human platelet antigens (HPA) are a group of platelet specific antigens represented with two alleles (a and b). To date, 24 HPAs have been defined by immune sera of which 12 are grouped in 6 biallelic systems (HPA-1, -2, -3, -4, -5, and -15). The molecular basis of 22 of the 24 serologically defined antigens has been resolved. In all but 1 of the 22, the difference between self and non-self is defined by a single amino acid substitution owing to a single-nucleotide polymorphism (SNP) in the gene encoding the relevant membrane glycoprotein. (4, 5). Each allele can be analyzed using polymerase chain reaction (PCR) with either sequence specific primers (SSP-PCR) Skogen *et al* (6) or with TaqMan technology using allele specific probes (5).

HPAs are located on gps on the surface of the platelets (fig. 1-3). Following blood vessel injury, platelets adhere to the subendothelial connective tissue through von Willebrands factor (vWF) which binds to the gpIb/IX/V complex, where HPA 2 is also located. Under the influence of

shear stress platelets move along the surface of vessels until the gpIa/Iia (the site of HPA 5) engages collagen and halts translocation. Platelet activation is achieved by fibrinogen binding to the receptor on gpIIb/IIIa, where the majority of the HPAs described till now are located, inducing platelet aggregation (3).

In addition to gps, platelets also express blood group antigens A and B and human leukocyte antigen (HLA) class I, but not class II (3).
The main function of platelets is the formation of mechanical plugs during the normal haemostatic response to vascular injury, this is achieved through adhesion, secretion, aggregation and fusion and pro-coagulant activity (3).
Normally, there are between 150 and 450 x 10^9 platelets per liter of blood. If there are <100 x 10^9 platelets/L, there is an increased risk of bleeding, if there are less than 20 x 10^9 platelets /L, there is a risk for spontaneous bleeding (7).

About 1% of newborn have thrombocytopenia (8, 9). There are several causes for this, for example infection, prematurity, chromosome anomalities (e.g. Down's syndrome), syndromes (e.g. TAR-thrombocytopenia), maternal IgG autoantibodies or allantibodies transferred through placenta into the fetal circulation. Pregnancy, transfusion and transplantation may induce alloimmunization against HPAs (10).
Neonatal alloimmune thrombocytopenia (NAIT) is a result of an immunological process similar to the one that causes haemolytic disease of the newborn (HDN). The frequency of NAIT is 1:1000-2000 births, in comparison HDN has a frequency of 1:5000 births (personal communication, W Ouwehand, UK).
Fetal platelets have antigens on the surface of both paternal and maternal origin. If the paternal HPA phenotype differs from the maternal phenotype, the fetal platelets are expressing antigens that the mother may recognize as foreign if her immune system is exposed for the fetal antigen or the antigen expressed in another context (11). An example of this is vitronectin receptor (VNR). VNR on trophoblasts and gpIIa/IIIb on platelets share the same β integrin (β3). In the spiral arteries of a pregnant uterus, the smooth musculature and endothelium is replaced by invading trophoblasts from the developing fetus (placenta). VNR on these trophoblasts presenting the β3 allele of paternal origin can be phagocytosed by maternal antigen presenting cells (APCs) and be presented for maternal T-cells (12). Soluble antigens can be recognized by maternal B-cells. With T-cell help, the B-cell can differentiate into antibody producing plasmacells. Antibodies of IgG type can cross the placenta and bind to HPA on fetal platelets (12, 13). The platelets are then

removed from the circulation by the reticuloendothelial system (RES) in the spleen. If the bone marrow production of platelets cannot substitute the loss, the result is thrombocytopenia. Thrombocytopenia can lead to serious or even fatal bleeding in utero or after birth. The platelet count may continue to fall during the first 48 h after birth and the risk of intracranial bleeding is highest during this period (15).

If a woman develops anti-HPA 1a antibodies during her first pregnancy, the antibody titer will normally increase during the course of the pregnancy. If a woman, on the other hand, has developed a high level of anti-HPA 1a antibodies during a previous pregnancy, the antibody level will usually *fall* during the next pregnancy. This is a completely opposite reaction compared with the antibody response in Rhesus-immunization, where the antibody level will rise in subsequent pregnancies like in any other secondary immune response. The reason for this phenomenon is unknown, but may be related to an antigen specific suppression of the immune response. This suppression and tolerance of fetal antigens is a normal reaction in the fetal maternal interface. In women being immunized with fetal HPA 1a antigen the normal tolerance is broken.
In the subsequent pregnancy, the antibody level in the mother usually decreases. A possible explanation may be that anti-HPA 1a antibodies will neutralize HPA 1a positive trophoblasts replacing the smooth muscle and endothelium of the spiral arteries in the uterus, thereby preventing an antibody boost. In addition fetal HPA 1a positive platelets will absorb antibodies from the mother. As the fetus grows and the production of platelets increases, more and more maternal antibodies will be absorbed and the antibody titer will decrease (16). Several studies have reported a significant correlation between the maternal ant-HPA 1a antibody level and the severity of thrombocytopenia in the newborn (16, 17, 18).

In a Norwegian study including more than 100,000 pregnant women, the frequency of HPA 1bb was found to be 2.1%. The anti-HPA 1a frequency among these women was 10.6%. The total number of babies born with serious thrombocytopenia was 55, which of 2 babies suffered from intracranial haemorrhage (19). The chance of alloimmunization is strongly associated with maternal HLA class-II DRB3*0101 type (17).

Antenatal management of fetomaternal alloimmune thrombocytopenia focuses on preventing severe thrombocytopenia in the fetus. Available management options include administration of intravenous immunoglobulins and/or corticosteroids to the mother or intrauterine transfusion of antigen compatible platelets to the fetus. The last treatment option includes a 2% risk of fetal

death per procedure and is stopped in most centres (20). All options are costly and need to be assessed in terms of potential risk and benefit to both the mother and an individual fetus. However, there are insufficient data from randomised controlled trials to determine the optimal antenatal management of fetomaternal alloimmune thrombocytopenia. (21).

The mortality of NAIT is 10%. Intracranial hemorrhage (ICH) is the most devastating complication of NAIT, affecting approximately 20% of all proven cases, up to 50% of which occur antenatally (11). Currently, there is no screening program among pregnant women in Norway or any other country for detection of NAIT. Antenatal screening for the most common form of NAIT, due to anti HPA-1a is under consideration, but there is no established program at present (15). In a study done by Durand-Zaleski I *et al* (22), screening newborns for neonatal alloimmune thrombocytopenia showed to be more cost-effective than screening primiparous women. In a Norwegian study, a general screening program for all pregnant women has been found cost-effective (23).

SSP-PCR

Sequence specific primer polymerase chain reaction (SSP-PCR) can amplify specific sequences or add sequences as primers to cloned DNA. There are three steps in PCR (illustrated in figure 4):
Step one: Denaturation. The DNA fragment to be amplified is mixed with a large excess of sequence specific primer in addition to a very heat-stable DNA polymerase (Taq polymerase) and a supply of all four nucleotides. The mixture is heated to about 98 °C, at this temperature the DNA fragment dissociates into single strands.
Step two: Annealing of primers. The mixture is cooled down to about 60 °C and the single DNA strands reassociate into double strands. Since there is an excess of primer, each strand of DNA base-pairs with a complimentary primer flanking the region to be amplified, leaving the rest of the fragment single-stranded.
Step three: Primer extension. Using the primer, the Taq polymerase copies the rest of the fragment as if it were replicating DNA. When it is done, the primer has been lengthened into a complimentary copy of the entire single-stranded fragment. Because both strands of the fragment are replicated, there are now two copies of the original fragment.
Steps1-3 are repeated, each time doubling the amount of DNA copies. After 20 cycles, a single fragment produces more than one million (2^{20}) copies (24).

TaqMan technology

The TaqMan technology detects the specific PCR products as it is amplified during PCR by using fluorenscent probes. The process is illustrated in figure 5.

The oligo-nucleotide probe is labeled with two different fluorescent dyes, the 5' terminus reporter dye and the 3' terminus quenching dye. When the probe is intact, energy is transferred from the short-wavelength reporter fluorophore to the long-wavelength Quencher fluorophore on the other end, so that the reporter signal is quenched. The close proximity of the dye and quencher results in negligible fluorescence when the probe is intact.

During the extension phase of PCR, the probe is cleaved by 5' nuclease activity of Taq DNA polymerase.

The cleavage of the probe removes the probe so that it not inhibits the PCR, and separates the reporter dye from the quencher dye, and the reporter dye signal emission increase is measured. The amount of fluorescence emitted is proportional to the amount of DNA fragment that has been synthesized and can be used to calculate the initial copy number of target DNA present in the reaction.

3. Aim

As part of a screening project in the Department of Immunology and Transfusion Medicine, UNN/University of Tromsoe, platelet antigen genotyping was performed using SSP-PCR and detection by gel electrophoresis. The same samples were also genotyped using TaqMan technology. Out of the 196 samples being studied, we found four discprepancies between the genotyping using SSP PCR and TaqMan technology. The aim of the study was to find the explanation for these discrepancies and to compare the gene frequency of the different alleles to gene frequencies in other populations studied.

4. Material and methods

4.i Patient samples

The Department of Immunology and Transfusion Medicine, UiTø, received 196 DNA samples from a random cohort of pregnant Egyptian women in June 2004. The patient samples were from Shabrawishy Hospital in Cairo, Egypt.

4.ii SSP-PCR

By using SSP-PCR, genotyping of platelet antigens HPA 1-4 and 15 was performed (6).
In brief, PCR amplifications were conducted in 96-well PCR plates containing double-distilled autoclaved water and a mastermix with SSP (for primer sequences see table 1). Both aa-, bb- and ab-controls were used when genotyping HPA 1-3 and 15, but only aa- and ab-controls when genotyping HPA 4. PCR conditions were as follows:

*__HPA 1, 2 and 4__: pre-PCR 94 °C for 5 min, then 30 cycles of 94 °C for 20 sec, 65 °C for 1 min and 72 °C for 1 min. Post-PCR 10 min at 72 °C, 4 °C for ∞.

*__HPA 15__: pre-PCR 94 °C for 5 min, then 30 cycles of 94 °C for 20 sec, 61 °C for 1 min and 72 °C for 1 min. Post-PCR 10 min at 72 °C, 4 °C for ∞.

*__HPA 3 (and 5)__: pre-PCR 94 °C for 5 min. Then 5 cycles of 94 °C for 20 sec, 70 °C for 1 min and 72 °C for 1 min followed by 21 cycles of 94 °C for 20 sec, 65 °C for 1 min and 72 °C for 1 min. After that 5 cycles of 94 °C for 20 sec, 50 °C for 1 min and 72 °C for 1 min. Post-PCR 10 min at 72 °C and 4 °C for ∞.

PCR products were detected by gel electrophoresis. To make the agarose gel, a 1.6% solution with agarose in TAE-buffer (Tris Acetate EDTA buffer) was made. One drop of ethidium bromide per 50 ml of agarose was added. The agarose gel had 4 rows of 26 wells each. To detect PCR products, the electrophoresis ran on 140 V for 35 minutes. The gels were then transilluminated by ultraviolet light and photographed. They were then analyzed by comparing the bands of the control samples with the bands of the patient samples.

4.iii TaqMan technology

The TaqMan procedure used in this study use two allele-specific probes labelled with reporter dyes VIC (allele 1) and FAM (allele 2), respectively, and contain a nonfluorescent quencher. In addition, the probes also are fitted with a minor groove binder to keep the probe length to a minimum and maximize the effect of the one-base mismatch (25).

The same DNA samples which were received from Cairo in June 2004 and genotyped using SSP-PCR, were genotyped again using TaqMan technology. (I performed this in Willem Ouwehand's laboratory in Cambridge, UK). First, the concentration of DNA in each sample was measured using NanoDrop (NanoDrop-1000 Spectrophotometer, NanoDrop Technologies). The samples were thereafter diluted with water to a standard concentration of DNA (5 ng/ul). PCR

amplifications were conducted in 96-well PCR plates. 5 ul DNA were added per well, columns 11 and 12 were left empty for no template controls. The master mix was made containing 773 ul universal PCR master mix, 14 ul forward primer (100 nM), 14 ul reverse primer (100 nM), 21 ul FAM probe (15 pM), 21 ul VIC probe (15 pM) and 187 ul water per 96-well plate. 10 ul master mix was added per well, including at least 4 no template wells (for primer and probe sequences see tables 2 and 3). The plate was then sealed with optical film and put on the PCR block. PCR conditions varied according to the probe: pre-PCR 50 °C for 2 minutes, 95 °C for 10 minutes, then 40 cycles of 95 °C for 15 seconds and 56 °C (HPA 1, 3, 5 and 15)/64 °C (HPA 2) for 1 minute. Hold at 4 °C for ∞. The results were analyzed using TaqMan technology and SDS 2.2.1 software.

4.iv DNA sequencing

First, the known DNA sequence for glycoprotein (gp) IIa, exon 3, which contains the SSPs and probes used for the HPA 2 genotyping, was found using NCBI nucleotide-nucleotide BLAST search (26). In addition, the single nucleotide polymorphisms (SNPs) already described were also found. The information was plotted into BioEdit Sequence Alignment Editor. New forward and reverse primers, which cover the area on gpIIa exon 3, where a possible SNP might be, were ordered (http://www.medprobe.com) (Table 4).

Using the new sequence specific primers, PCR was performed using the following protocol: 5 ul PCR buffer 10x, 5 ul forward primer (2 uM), 5 ul reverse primer (2 uM), 1 ul dNTP (10 mM) and 0,5 ul HotStarTaq (5 U/ul). Aa-, bb-, ab- and negative controls were used. PCR conditions were as follows: pre-PCR 95 °C for 15 seconds, then 40 cycles of 94 °C for 30 seconds, 56 °C for 40 seconds, 72 °C for 40 seconds. Post-PCR 72 °C for 10 minutes. Qualitative analysis was done by gel electrophoresis on a 1.6% agarose gel, which ran for 30 minutes (140 V). The gel was transilluminated by ultraviolet light and then photographed.

Comparing the bands to a 100 base pair ladder showed that the PCR products were about 600 base pairs long, which was the correct length. The bands were cut out from the agarose gel and purification was done using GFX PCR purification kit. The end products were then sequenced using an ABI 3130 X1 Genetic Analyzer (Applied Biosystems).

4.v Control genotyping

Control genotyping was performed using TaqMan technology, this time the genotyping was done in the laboratory at the Dep. of Immunology and Transfusion Medicine in Tromsoe adapted after

the method used in Cambridge. PCR was done using Abi Prism 7900HT Fast Real-Time PCR System (Applied Biosystems) and analyzed using SDS 2.2.2 software.

4.vi Statistical analysis

The gene frequencies are distributed according to Hardy Weinberg equilibrium: $a^2+2ab+b^2=1$.
The statistical analysis was done using 2-proportion large sample z-test.

One can use a large sample z-test if:
n1 * p1 (1 - p1) > 5 and n2 * p2 (1 - p2) > 5

n1 = the number of samples in population 1 p1 = the gene frequency in population 1
n2 = the number of samples in population 2 p2 = the gene frequency in population 2

H_0: there is no difference between the two populations.
H_1: there is a difference between the two populations.

$$\text{Mean } p = \frac{(p1*n1) + (p2*n2)}{n1 + n2}$$

$$\text{Test}_{obs}: z = \frac{(p_1 - p_2)}{\sqrt{(p(1-p))} * \sqrt{(1/n_1 + 1/n_2)}}$$

According to the standard normal distribution: if $|z| > 1.96$ then $p < 0.05$.
If $p < 0.05$, the H_0 is rejected and the conclusion is that there is a significant difference between the two populations.

5. Results

The results of the genotyping of 196 DNA samples from a cohort of Egyptian pregnant women using SSP-PCR and detection by gel electrophoresis are shown in table 6. The results of genotyping of the same samples using TaqMan technology are shown in table 7. Figures 6-9 shows the results of the SSP-PCR of the four DNA samples which were typed HPA 2bb using SSP-PCR and HPA 2ab using TaqMan technology. The result of the TaqMan analysis of the

same DNA samples are showed in diagram 1, the diagram also shows the result of two samples which are genotyped to HPA 2aa using both SSP-PCR and TaqMan technology and two no template controls for comparison. No samples in the HPA 2 system were genotyped HPA 2bb using TaqMan technology. The gene sequences of the samples which concludes they are all HPA 2bb are showed in figures 10-17.

The gene frequency of HPA 1a and HPA 1b was found to be 0.792 and 0.208, respectively, using both SSP-PCR and TaqMan techonology. The gene frequency of HPA 2a and 2b was 0.865 and 0.135, respectively. In the HPA 3 system, the frequency of the a and b allele was 0.655 and 0.345, respectively. There were no b alleles in the HPA 4 system, the frequency of HPA 4a was 1.0. The HPA 5 and 15 systems were only typed using TaqMan technology, the gene frequencies in the HPA 5 system showed a frequency of 0.843 for the a allele and 0.157 for the b allele. For the HPA 15 system the gene frequency of the a allele was 0.530 and for the b allele 0.470.

The results of the sequencing of the four DNA samples, shows that they are all HPA 2bb. The second genotyping of the samples by TaqMan technology also shows that all the samples are HPA 2bb.

Statistical analysis of the gene frequencies found in Egypt compared to Caucasian gene frequency (1, 2), shows that there is a significant difference in the HPA 5 system ($0{,}005 > p > 0{,}002$), there is a lower frequency of the HPA 5a allele, and thus a higher frequency of the HPA 5b allele, in Egyptian than Caucasian populations. No significant difference in gene frequency was found in the HPA 1, 2, 3, 4, 15 systems (table 8).

6. Discussion

Out of the 196 DNA samples genotyped in the HPA 1, 2 and 3 systems, all results were consistent in both SSP-PCR and TaqMan technology except for four samples. These were typed HPA 2bb using SSP-PCR and HPA 2ab using TaqMan technology.

There are several possible explanations for the discrepancies found. The initial hypothesis was that there was a mistake in SSP-PCR and that the correct genotype was HPA 2ab. This is because studies have showed that TaqMan technology is faster, more reliable and reproducible, compared to the standard SSP-PCR (27). The DNA sequencing showed, however, that all four samples in

question were HPA 2bb, which means that the results of the SSP-PCR showed the correct genotypes.

A study done by Kjaer KM *et al* (28) showed similar results in the HPA 1 system. They found that upon repeated testing, two samples consistently came out as HPA 1bb in SSP-PCR and HPA 1ab in 5' NA (nuclease assay). DNA sequencing revealed a polymorphism located in an intron that corresponds to the consensus primer used for the SSP-PCR HPA 1a typing. A similar defect in the area for the HPA 2a probe used in the TaqMan typing could cause the incorrect results. This could be the reason that the HPA 2a probe bound to the DNA fragment even though there was no a allele. Again, the DNA sequencing showed that there was no SNP, the sequence for all four samples was HPA 2bb, no SNPs were found.

Other studies have concluded with complete concordance of results for all samples tested by SSP-PCR and 5' NA and that genotyping of HPA 1, 2, 3, 5 and 15 by 5' NA ensures superior detection of all alleles (29).

The erroneous results using TaqMan technology could also be because of contamination during the genotyping. There is little chance of this as the samples in question were typed on different PCR plates on different days. The no template controls on all PCR plates were negative. This also excludes the possibility that the HPA 2a probe was non-functional, thereby automatically transmitting an HPA 2a signal.

There is so far no explanation as to why the TaqMan results were incorrect and further studies is needed to be done in order to find the exact reason for this.

In Caucasian populations HPA 1a is the most frequently implicated antigen in NAIT (30). Anti-HPA-5b accounts for only 4.3 percent of all NAIT cases. NAIT due to anti-HPA-5b is thought to be milder and have fewer complications than NAIT caused by anti-HPA-1a because of the lower number of HPA-5b antigenic sites per platelet (31). Little is known about the frequencies of the different HPAs and their role in NAIT in Egypt. According to this study, there is a significant difference in the frequency of HPA 5 in Caucasian and Egyptian populations. To this date, however, there has been no investigation as to which antibody is most often implicated in NAIT in Egypt.

7. Conclusion

Genotyping of HPA 1, 2 and 3 using SSP-PCR and TaqMan technology respectively, showed a high consistency in results. Out of 196 DNA samples studied, only four samples showed different results in the HPA 2 system. After DNA sequencing of these samples, the conclusion

was that the genotyping done by SSP-PCR was correct, all four samples were HPA 2bb, not HPA 2ab as showed by TaqMan technology. The reason for the erroneous results remains unknown. When comparing the gene frequencies found in the Egyptian cohort with gene frequencies in Caucasian population (1, 2), there was a significant higher frequency of the HPA 5b allele and a significantly lower frequency of the HPA 5a allele ($0{,}005 > p > 0{,}002$) in the Egyptian population than in the Caucasian population. For HPA 1, 2, 3, 4 and 15, there was no significant difference in gene frequency.

8.

HPA 1a	**5'**-CTT-ACA-GGC-CCT-GCC-TCT-**3'**
HPA 1b	**5'**-CTT-ACA-GGC-CCT-GCC-TCC-**3'**
HPA 1c	**5'**-CCT-GCT-TCA-GGT-CTC-TCC-**3'**
HPA 2a	**5'**-CCC-CCA-GGG-CTC-CTG-AC-**3'**
HPA 2b	**5'**-CCC-CCA-GGG-CTC-CTG-AT-**5'**
HPA 2c	**5'**-GCA-GCC-AGC-GAC-GAA-AAT-A-**3'**
HPA 3a	**5'**-GAC-TG7-GGG-CTG-CCC-AT-**3'**
HPA 3b	**5'**-GAC-TG7-GGG-CTG-CCC-AG-**3'**
HPA 3c	**5'**-GTG-CTC-CCA-GGG-ACC-AAG-**3'**
HPA 4a	**5'**-CTG-GCC-ACC-CAG-ATG-CG-**3'**
HPA 4b	**5'**-CTG-GCC-ACC-CAG-ATG-CA-**3'**
HPA 4c	**5'**-GGT-AGA-AAG-GAG-CTA-TAG-TTT-GGC-**3'**

Table 1: SSP-PCR primer sequences (the 7 in the sequences of HPA 3a and b primers stands for the inert base inosine. Inosine makes the association between the primer and the DNA sequence to be copied weaker, but the specification is better).

9.

	Primer sequences
HPA 1	**Forward:** **5'** – CTG ATT GCT GGA CTT CTC TTT GG – **3'**
	Reverse: **5'** – AGC AGA TTC TCC TTC AGG TCA CA – **3'**
HPA 2	**Forward:** **5'** – CTG AAA GGC AAT GAG CTG AAG AC – **3'**
	Reverse: **5'** – CCA GAC TGA GCT TCT CCA GCT T – **3'**
HPA 3	**Forward:** **5'** – TGG GCC TGA CCA CTC CTT T – **3'**
	Reverse: **5'** – TGA TGG GCC GGG TGA A – **3'**
HPA 5	**Forward:** **5'** – GAC CTA AAG AAA GAG GAA GGA AGA GTC T – **3'**
	Reverse: **5'** – ATG CAA GTT AAA TTA CCA GTA CTA AAG CAA – **3'**
HPA 15	**Forward:** **5'** – TGT ATC AGT TCT TGG TTT TGT GAT GTT – **3'**
	Reverse: **5'** – CCA AGA AGT GAT AGA ATC AGG TAC AGT TAC – **3'**

Table 2: TaqMan primers

10.

	MGB (minor groove binding) probe sequences (3' NFQ (non fluorescent quencher))
HPA 1	**HPA 1a probe (5' FAM labelled):** **5'** – CTG CCT C**T**G GGC TC – **3'**
	HPA 1b probe (5' VIC labelled): **5'** – CTG CCT C**C**G GGC TC – **3'**
HPA 2	**HPA 2a probe (5' FAM labelled):** **5'** – CTC CTG A**C**G CCC ACA – **3'**
	HPA 2b probe (5' VIC labelled): **5'** – CTC CTG A**T**G CCC ACA – **3'**
HPA 3	**HPA 3a probe (5' FAM labelled):** **5'** – TGC CCA **T**CC CCA GCC – **3'**
	HPA 3b probe (5' VIC labelled): **5'** – CTG CCC A**G**C CCC AG – **3'**
HPA 5	**HPA 5a probe (5' FAM labelled):** **5'** – TTA CTA TCA AA**G** AGG TAA AAA – **3'**
	HPA 5b probe (5' VIC labelled): **5'** – TGT TTA CTA TCA AA**A** AGG TAA A – **3'**
HPA 15	**HPA 15a probe (5' FAM labelled):** **5'** – CTT CAG TT**C** CAG GAT T – **3'**
	HPA 15b probe (5' VIC labelled): **5'** – CTT CAG TT**A** CAG GAT TT – **3'**

Table 3: TaqMan probes

11.

	Primer sequences
HPA 2	**Forward: 5'**-TAG-GGC-AGA-CAC-TGC-CTG-C-**3'**
	Reverse: 5'-GGA-ACT-TGA-CCA-CAG-TCC-TTG-TG-**3'**

Table 4: Sequences of the SSP-PCR primers used for sequencing of the four samples showing discrepancy between the genotyping using SSP-PCR and TaqMan techonolgy.

12.

System	Antigen	Glycoprotein	CD
HPA 1	HPA 1a HPA 1b	GPIIIa	CD61
HPA 2	HPA 2a HPA 2b	GPIbα	CD42b
HPA 3	HPA 3a HPA 3b	GPIIb	CD41
HPA 4	HPA 4a HPA 4b	GPIIIa	CD61
HPA 5	HPA 5a HPA 5b	GPIa	CD49b
	HPA 6bw	GPIIIa	CD61
	HPA 7bw	GPIIIa	CD61
	HPA 8bw	GPIIIa	CD61
	HPA 9bw	GPIIb	CD41
	HPA 10bw	GPIIIa	CD61
	HPA 11bw	GPIIIa	CD61
	HPA 12bw	GPIbβ	CD42c
	HPA 13bw	GPIa	CD49b
	HPA 14bw	GPIIIa	CD61
HPA 15	HPA 15a HPA 15b	CD109	CD109
	HPA 16bw	GPIIIa	CD61

Table 5: Human platelet antigens (HPAs) (Metcalfe P, Watkins NA, Ouwehand WH, Kaplan C, Newman P, Kekomaki R *et al.* Nomenclature of human platelet antigens. Vox Sang. 2003 Oct;85(3):240-5.)

13.

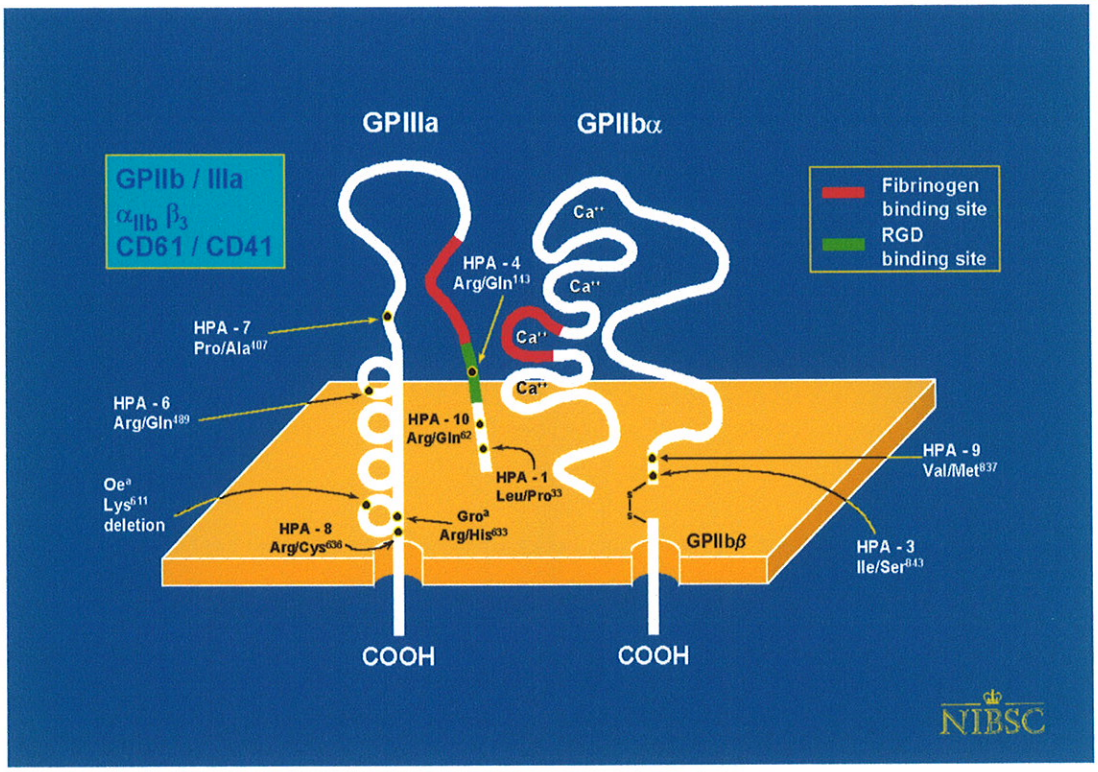

Figure 1: GPIIb/IIIa (http://www.nibsc.ac.uk/aboutus/platelets.asp?id=31)

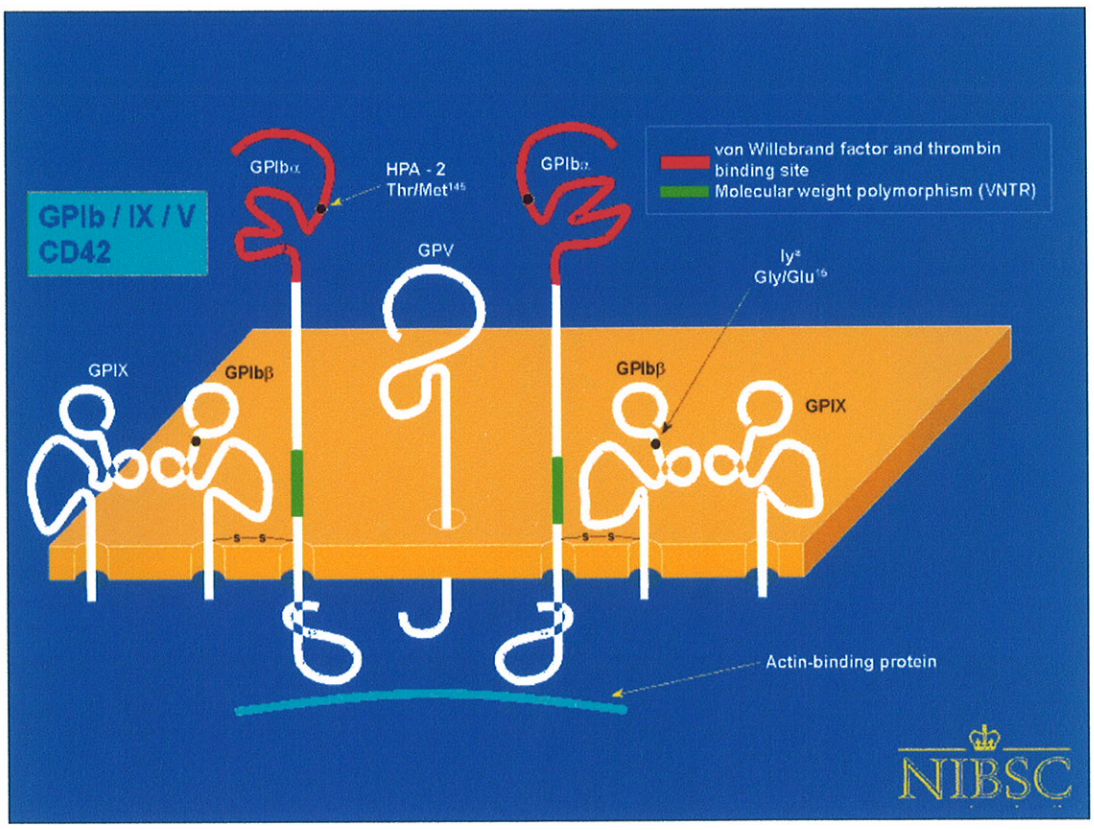

Figure 2: GPIb (http://www.nibsc.ac.uk/aboutus/platelets.asp?id=31)

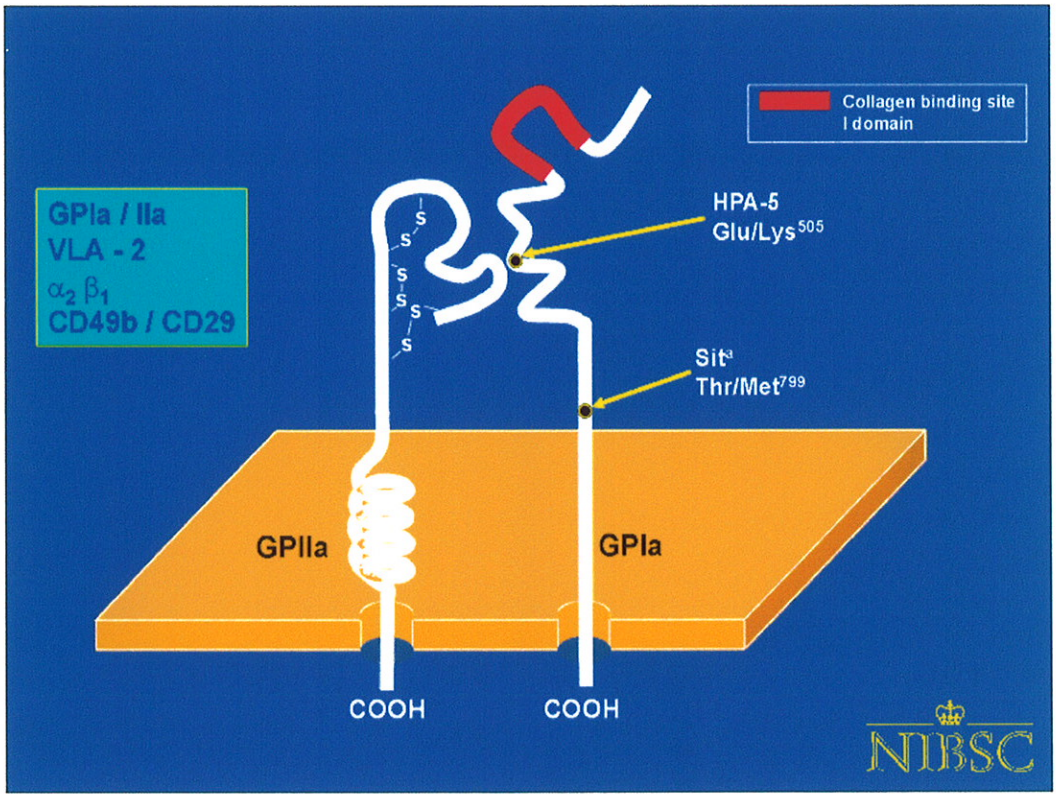

Figure 3: GP Ia/IIa (http://www.nibsc.ac.uk/aboutus/platelets.asp?id=31)

14.

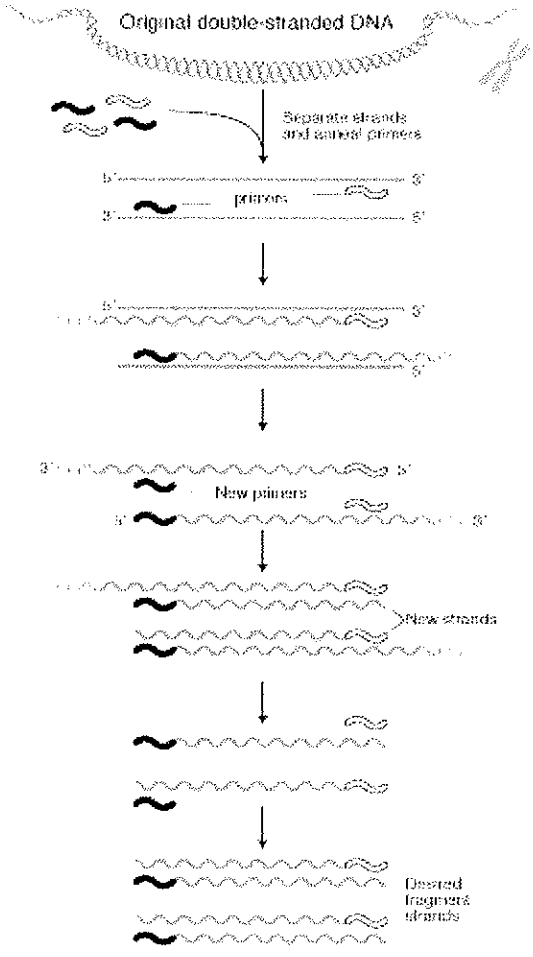

Figure 4: illustration of the polymerase chain reaction

(http://www.genome.gov, National Human Genome Research Institute, Division of Intramural research)

15.

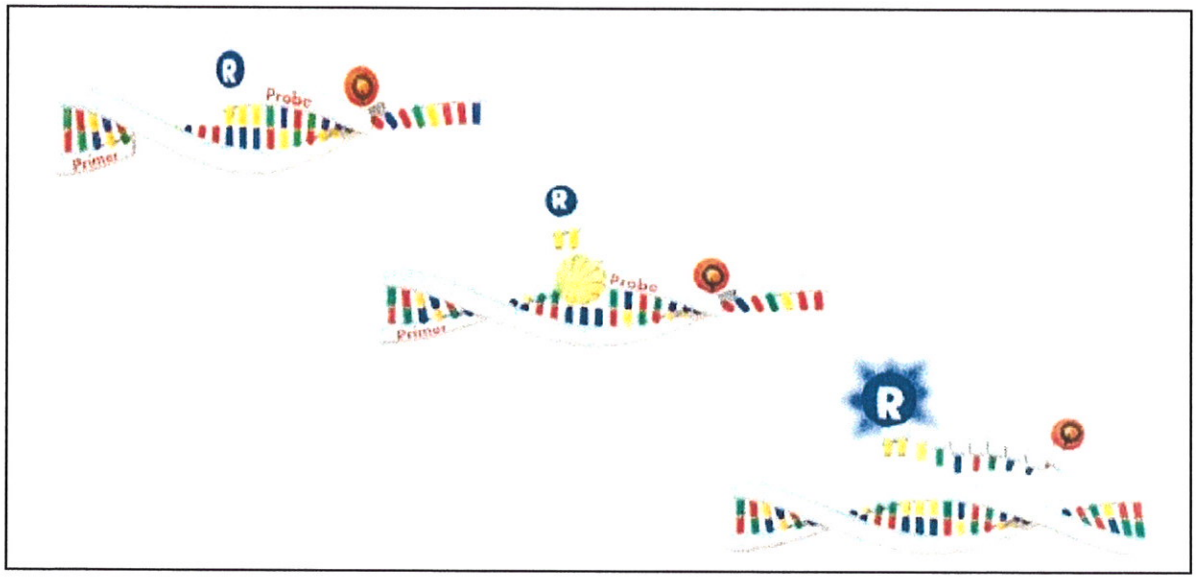

Figure 5: The TaqMan Technology. A dual-dyed probe is cleaved by the Taq DNA-polymerase. The probe is cleaved and when the quencher is not in close proximity of the reporter, the emission from the reporter can be measured. (http://www.appliedbiosystems.com/)

16.

HPA genotype	n	Genotype frequency	HPA allele	Gene frequency
HPA 1a/1a	190	0,632	HPA 1a	0,792
HPA 1a/1b		0,319	HPA 1b	0,208
HPA 1b/1b		0,0486		
HPA 2a/2a	191	0,751	HPA 2a	0,865
HPA 2a/2b		0,228	HPA 2b	0,135
HPA 2a/2b		0,0207		
HPA 3a/3a	187	0,443	HPA 3a	0,655
HPA 3a/3b		0,422	HPA 3b	0,345
HPA 3b/3b		0,134		
HPA 4a/4a	188	1,00	HPA 4a	1,00
HPA 4a/4b		0,00	HPA 4b	0,00
HPA 4b/4b		0,00		

Table 6: Gene and genotype frequencies using SSP-PCR

HPA genotype	n	Genotype frequency	HPA allele	Gene frequency
HPA 1a/1a	190	0,637	HPA 1a	0,795
HPA 1a/1b		0,316	HPA 1b	0,205
HPA 1b/1b		0,0474		
HPA 2a/2a	191	0,738	HPA 2a	0,869
HPA 2a/2b		0,262	HPA 2b	0,131
HPA 2a/2b		0,00		
HPA 3a/3a	187	0,444	HPA 3a	0,658
HPA 3a/3b		0,428	HPA 3b	0,342
HPA 3b/3b		0,128		
HPA 5a/5a	188	0,707	HPA 5a	0,843
HPA 5a/5b		0,271	HPA 5b	0,157
HPA 5b/5b		0,0213		
HPA 15a/15a	186	0,285	HPA 15a	0,530
HPA 15a/15b		0,489	HPA 15b	0,470
HPA 15b/15b		0,226		

Table 7: Gene and genotype frequencies using TaqMan technology

17.

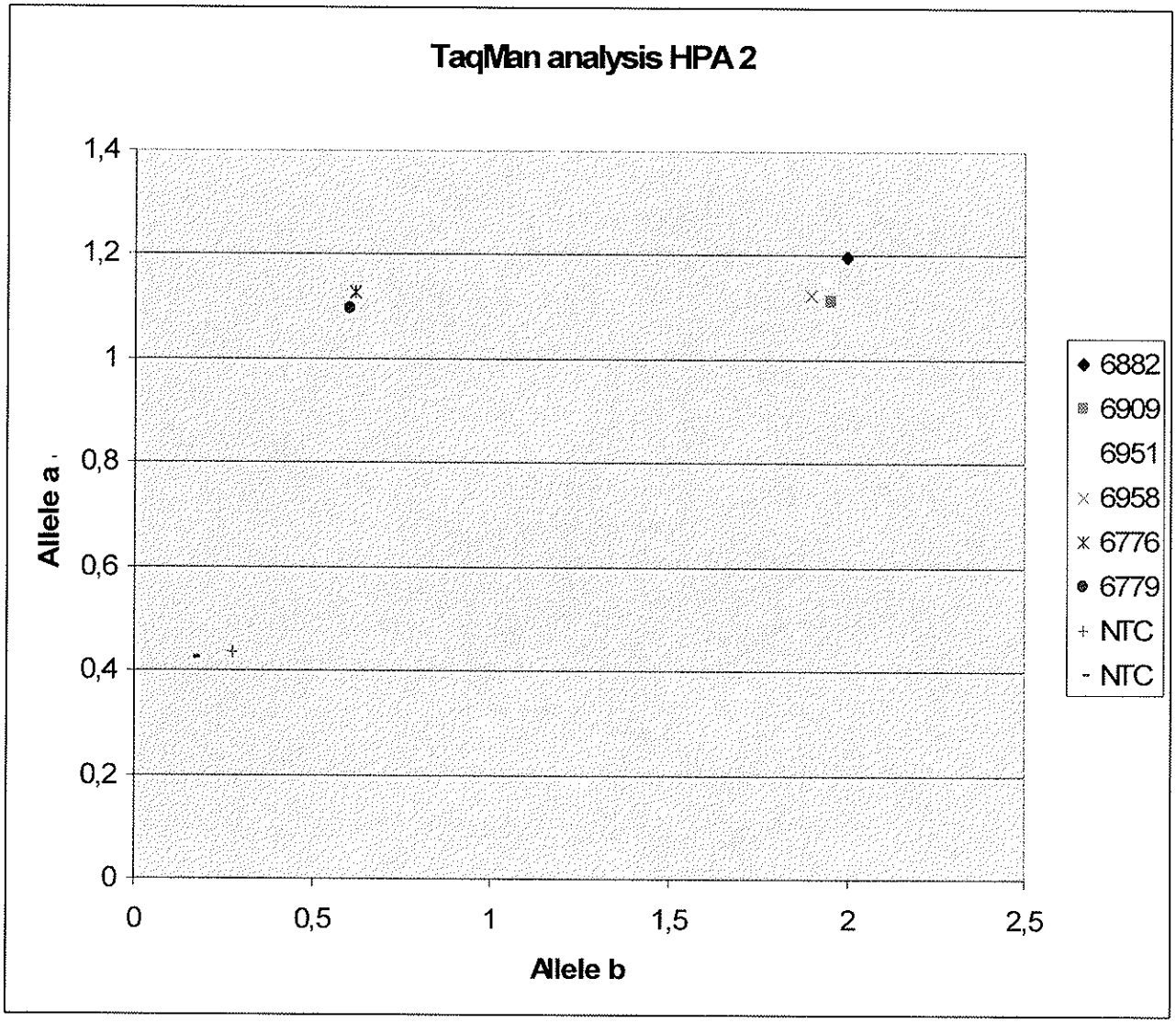

Diagram 1: Results from TaqMan analysis of the four DNA samples which showed discrepancies in the HPA 2 system (6882, 6909, 6951 and 6958), two DNA samples which were typed HPA 2aa using both SSP-PCR and TaqMan technology (6776 and 6779) and two no template controls (NTC).

18.

D 1	5	5	0	5	5	a	4	4	6882	2	5	15	−
2	5	5	0	5	5	b	4	4	6882	2	5	15	+
3	5	5	0	5	5	a	4	4	6883	2	5	15	+
4	5	5	0	5	5	b	4	4	6883	2	5	15	−

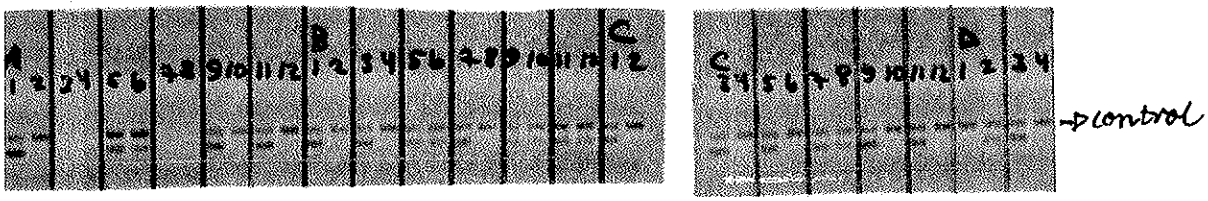

Figure 6: Result of electrophoresis of SSP-PCR of sample 6882 in the HPA 2 system showed in wells D1-2 (HPA 2bb).

H1	5	5	0	5	5	a	4	4	6906	2	5	15	+
2	5	5	0	5	5	b	4	4	6906	2	5	15	−
3	5	5	0	5	5	a	4	4	6907	2	5	15	+
4	5	5	0	5	5	b	4	4	6907	2	5	15	−
5	5	5	0	5	5	a	4	4	6908	2	5	15	+
6	5	5	0	5	5	b	4	4	6908	2	5	15	−
7	5	5	0	5	5	a	4	4	6909	2	5	15	−
8	5	5	0	5	5	b	4	4	6909	2	5	15	+

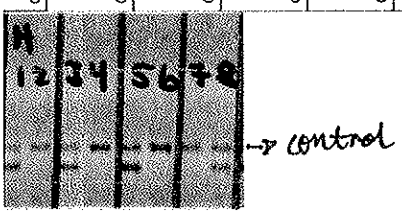

Figure 7: Result of electrophoresis of SSP-PCR of sample 6909 in the HPA 2 system showed in wells H7-8 (HPA 2bb).

H1	1	5	5	0	5	5	a	4	4	6950	2	5	15	+
	2	5	5	0	5	5	b	4	4	6950	2	5	15	−
	3	5	5	0	5	5	a	4	4	6951	2	5	15	−
	4	5	5	0	5	5	b	4	4	6951	2	5	15	+

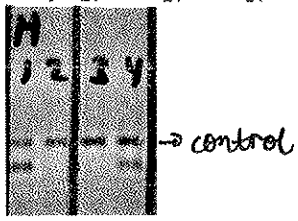

Figure 8: Result of electrophoresis of SSP-PCR of sample 6951 in the HPA 2 system showed in wells H3-4 (HPA 2bb).

B 1	5	5	0	5	5	a	4	4	6958	2	5	15	-	bb
2	5	5	0	5	5	b	4	4	6958	2	5	15	+	

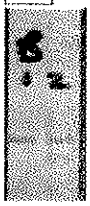

Figure 9: Result of electrophoresis of SSP-PCR of sample 6958 in the HPA 2 system showed in wells B1-2 (HPA 2bb).

19.

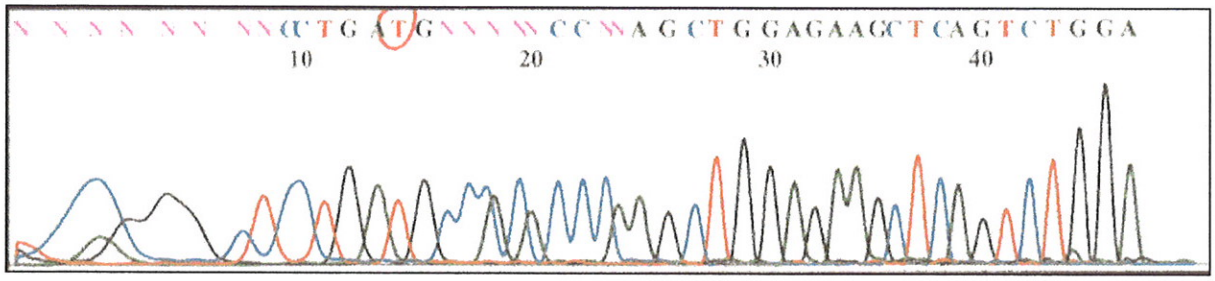

Figure 10: Result of sequencing of sample 6882 (forward primer).

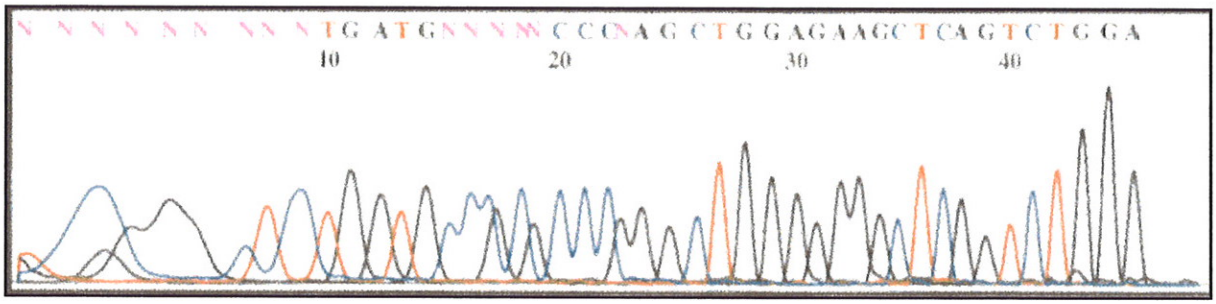

Figure 11: Result of sequencing of sample 6909 (forward primer).

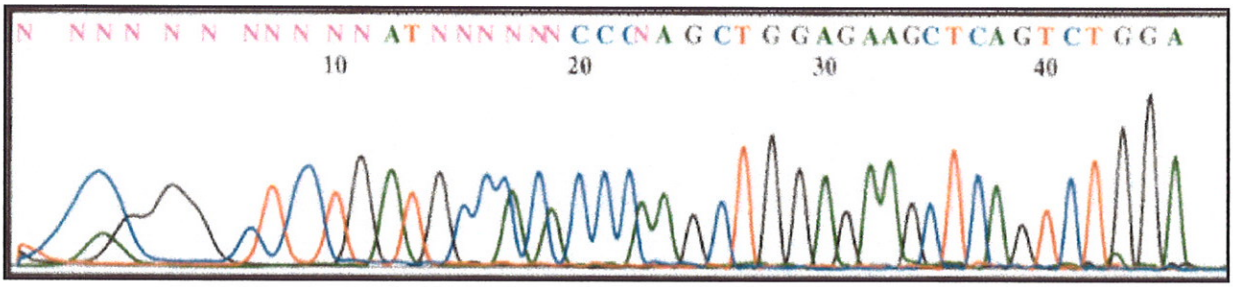

Figure 12: Result of sequencing of sample 6951 (forward primer).

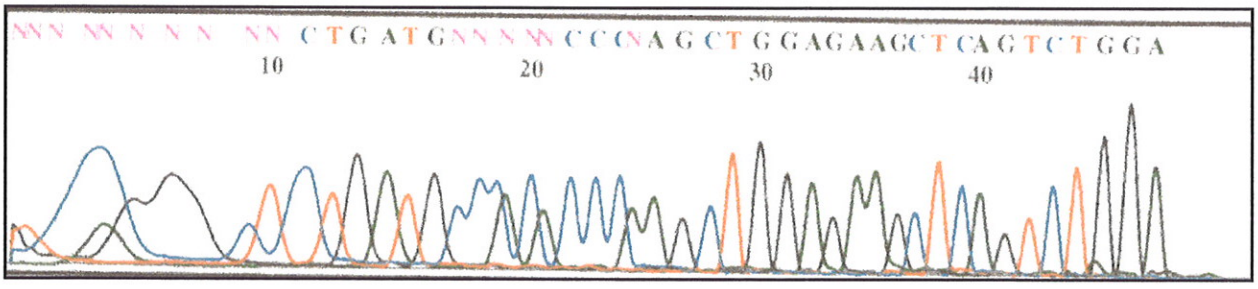

Figure 13: Result of sequencing of sample 6958 (forward primer).

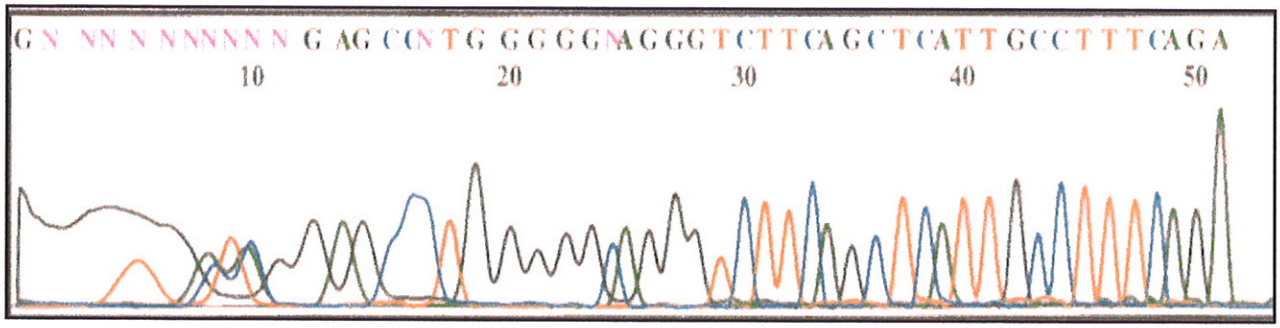

Figure 14: Result of sequencing of sample 6882 (reverse primer).

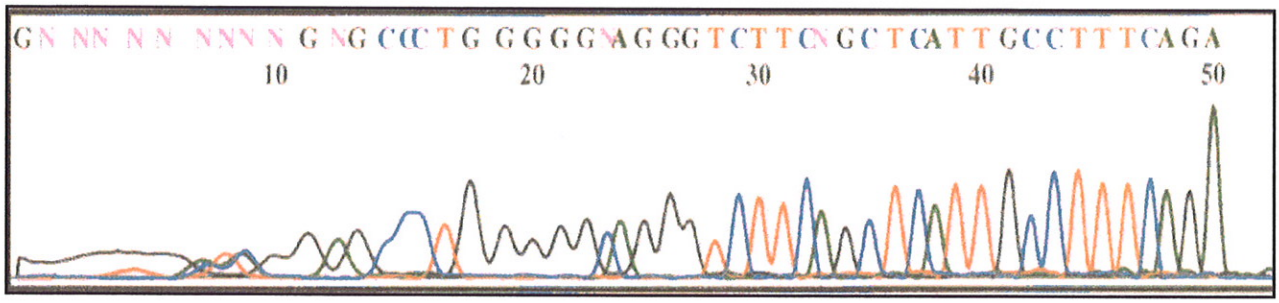

Figure 15: Result of sequencing of sample 6909 (reverse primer).

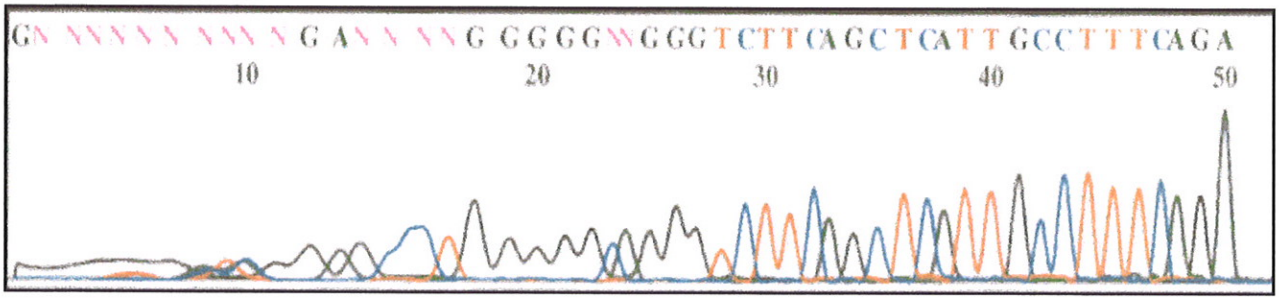

Figure 16: Result of sequencing of sample 6951 (reverse primer).

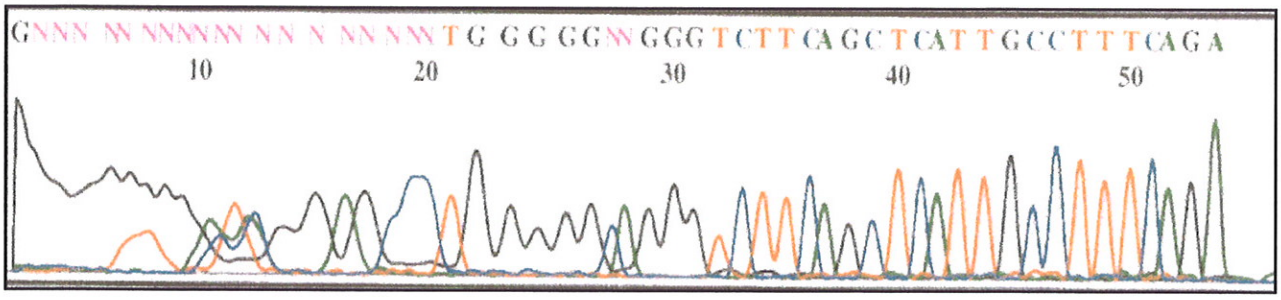

Figure 17: Result of sequencing of sample 6958 (reverse primer).

18.

		Egypt	Caucasian	p-value
HPA 1	*a*	0,792	0,83	0,5 > p > 0,20
	b	0,208	0,17	
HPA 2	*a*	0,865	0,92	0,20 > p > 0,10
	b	0,135	0,08	
HPA 3	*a*	0,655	0,63	0,5 > p > 0,20
	b	0,345	0,37	
HPA 5	*a*	0,843	0,92	0,005 > p > 0,002
	b	0,157	0,08	
HPA 15	*a*	0,530	0,50	p > 0,5
	b	0,470	0,50	

Table 8: Statistical analysis of HPA gene frequencies in an Egyptian and Caucasian population (30, 31)

19. References

(1) Steffensen R, Kaczan E, Varming K, Jersild C. Frequency of platelet-specific alloantigens in a Danish population. Tissue Antigens. 1996 Aug;48(2):93-6.

(2) European bioinformatics Institute: http://www.ebi.ac.uk/ipd/hpa/freqs_1.html

(3) Hoffbrand AV, Pettit JE, Moss PAH. Essential Haematology, Fourth edition, 2001, Blackwell Science Ltd, 236-237.

(4) Metcalfe P, Cavanagh G, Hurd C, Ouwehand WH. HPA Genotyping by PCR-SSP: Report of 4 Exercises. *Vox Sanguinis 1999;77:40-43.*

(5) Bugert P, McBride S, Smith G, Dugrillon A, Klüter H, Ouwehand WH, Metcalfe P. Microarray-based genotyping for blood groups: comparison of gene array and 5'-nuclease assay techniques with human platelet antigen as a model. Transfusion. 2005 May;45(5):654-9.

(6) Skogen B, Bellissimo DB, Hessner MJ, Santoso S, Aster RH, Newman PJ, McFarland JG. Rapid determination of platelet alloantigen genotypes by polymerase chain reaction using allele-specific primers. Transfusion. 1994;34:955-960.

(7) Norsk elektronisk legemiddelhåndbok, http://www.legehandboka.no

(8) Dreyfus M, Kaplan C, Verdy E, Schlegel N, Durand-Zaleski I, Tchernia G. Frequency of immune thrombocytopenia in newborns: a prospective study. Immune Thrombocytopenia Working Group. Blood. 1997 Jun 15;89(12):4402-6.

(9) Kaplan C. Immune thrombocytopenia in the fetus and the newborn: diagnosis and therapy, Transfus Clin Biol. 2001 Jun;8(3):311-4.

(10) http://www.haem.cam.ac.uk/transmed/pages/ouwehand.html#pi

(11) Johnson JA, Ryan G, al-Musa A, Farkas S, Blanchette VS. Prenatal diagnosis and management of neonatal alloimmune thrombocytopenia. Semin Perinatol. 1997 Feb;21(1):45-52.

(12) Zhou Y, Fisher SJ, Janatpour M, Genbacey O, Dejana E, Wheelock M *et* al. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest* 1997; **99:** 2139–51.

(13) Malek A., Sager R, Lang AB, Schneider H. Protein transport across the in vitro perfused human placenta, Am J Reprod Immunol. 1997 Oct;38(4):263-71.

(14) Palfi M., Selbing A.. Placental transport of maternal immunoglobin G, Am J Reprod Immunol. 1998 Jan;39(1):24-6.

(15) Ahya R, Turner ML, Urbaniak SJ; SNAIT Study Team. Fetomaternal alloimmune thrombocytopenia. Transfus Apher Sci. 2001 Oct;25(2):139-45.

(16) Bertrand G, Martageix C, Jallu V, Vitry F, Kaplan C. Predictive value of sequential maternal anti-HPA-1a antibody concentrations for the severity of fetal alloimmune thrombocytopenia. J Thromb Haemost. 2006 Mar;4(3):628-37.
(17) Williamson LM, Hackett G, Rennie J, Palmer CR, Maciver C, Hadfield R *et al.* The natural history of fetomaternal alloimmunization to the platelet-specific antigen HPA-1a (PlA1, Zwa) as determined by antenatal screening. Blood. 1998 Oct 1;92(7):2280-7.
(18) Jaegtvik S, Husebekk A, Aune B, Oian P, Dahl LB, Skogen B. Neonatal alloimmune thrombocytopenia due to anti-HPA 1a antibodies; the level of maternal antibodies predicts the severity of thrombocytopenia in the newborn. BJOG. 2000 May;107(5):691-4.
(19) Kjeldsen-Kragh J, Killie MK, Skogen B, Husebekk A. A screening and intervention program that significantly reduces mortality and serious morbidity associated with grave neonatal alloimmune thrombocytopenia. Vox Sanguinis (2006), 91 (Suppl. 2), 15.
(20) Berkowitz RL, Kolb EA, McFarland JG, Wissert M, Primani A, Lesser M, Bussel JB. Parallel randomized trials of risk-based therapy for fetal alloimmune thrombocytopenia. Obstet Gynecol. 2006 Jan;107(1):91-6.
(21) Rayment R, Brunskill SJ, Stanworth S, Soothill PW, Roberts DJ, Murphy MF. Antenatal interventions for fetomaternal alloimmune thrombocytopenia. Cochrane Database Syst Rev. 2005 Jan 25;(1)
(22) Durand-Zaleski I, Schlegel N, Blum-Boisgard C, Uzan S, Dreyfus M, Kaplan C. Screening primiparous women and newborns for fetal/neonatal alloimmune thrombocytopenia: a prospective comparison of effectiveness and costs. Immune Thrombocytopenia Working Group. Am J Perinatol. 1996 Oct;13(7):423-31.
(23) Killie MK, Husebekk A, Kjeldsen-Kragh J, Skogen B. Significance of antibody quantification. Vox Sanguinis (2006), 91 (Suppl. 2), 15.
(24) P. Raven and G. Johnson: Biology, fifth edition, McGraw-Hill, 1999
(25) Latif S, Bauer-Sardina I, Ranade K, Livak KJ, Kwok PY. Fluorescence Polarization in Homogeneous Nucleic Acid Analysis II: 5'-Nuclease Assay. Genome Research, 2001 Mar;11(3):436-40.
(26) http://www.ncbi.nlm.nih.gov/BLAST/
(27) Ficko T, Galvani V, Rupreht R, Dovc T, Rožman P. Real-time PCR genotyping of human platelet alloantigens HPA-1, HPA-2, HPA-3 and HPA-5 is superior to the standard PCR-SSP method. Transfus Med. 2004 Dec;14(6):425-32.

(28) Kjaer KM, Jaegtvik S, Husebekk A, Skogen B. Human platelet antigen 1 (HPA 1) genotyping with 5' nuclease assay and sequence-specific primers reveals a single nucleotide deletion in intron 2 of the HPA 1a allele of platelet glycoprotein IIIa. Br J Haematology, 2002 May; 117(2): 405-8.
(29) Higgins M, Hughes A, Buzzacott N, Lown J. High-throughput genotyping of human platelet antigens using the 5'-nuclease assay and minor groove binder probe technology. Vox Sanguinis (2004) 87, 114-117.
(30) Bertrand G, Jallu V, Gouet M, Kjaer KM, Lambin P, Husebekk A, Kaplan C. Quantification of human platelet antigen-1a antibodies with the monoclonal antibody immobilization of platelet antigens procedure. Transfusion 2005 Aug;45(8):1319-23.
(31) Campbell-Lee SA, DeSantis-Parsons D, Shirey RS, Kickler TS. Neonatal alloimmune thrombocytopenia due to anti-HPA 5b (Bra). Immunohematol. 2003;19(4):127-31.