

Determination of RHD zygosity using real-time quantitative PCR

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Summary

At present RHD incompatibility is still an obstetric problem despite prophylactic treatment. A very welcome recent technical advance has now made it possible to determine the foetal RHD status in a non-invasive risk-free manner using cell free foetal DNA in maternal plasma. In some cases, however, where there is a high risk that the foetus may be affected by HDN (haemolytic disease of the newborn), it may be of interest to determine whether the father is hetero- or homozygous for the RHD gene, since in the former instance there is only a 50% chance that the pregnancy is affected.

It has recently been shown that quantitative PCR assays, in particular real-time Taqman PCR, can be used to determine the RHD gene dosage, and also to determine foetal aneuploidies. We demonstrate that the same real-time Taqman PCR assay we had previously developed for non-invasive analysis of the foetal RHD gene and the foetal Y chromosome from maternal plasma can be used to determine the paternal RHD genotype.

Key words: RHD; paternal genotype; prenatal diagnosis; real-time PCR

The determination of RHD zygosity has until recently been a tedious procedure and is usually inferred from the serotype, family history and/or complex PCR-based RFLP assays [1-3]. To address this issue, two recent publications have shown that quantitative PCR assays can be used to accurately genotype the predominant Caucasian RHD locus. In the first instance, Chiu and colleagues determined RHD zygosity using a real-time Taqman PCR assay in which the dosage of the RHD gene was compared with a control locus, namely the albumin gene [4]. In the second approach, described by Pertl and colleagues, a quantitative fluorescent PCR assay compared the dosage of the RHD gene to that of the related RhCE gene locus [5]. A further development of the real-time PCR approach by our group has shown that this technology is sufficiently sensitive to detect even smaller differences in gene dosage, namely those which occur in foetal aneuploidies, in which instance only a 50% increase in gene copy number occurs and not 100% as is the case for the RHD gene [6].

Since it is only of interest to determine the RHD genotype of the male partner (the mother by definition being RHd), we were curious as to whether a real-time Taqman PCR assay we had previously developed for another purpose, non-invasive risk-free determination of foetal RHD sta-

tus and sex from maternal plasma [7], could be used for the determination of RHD zygosity.

The Taqman[®] real-time PCR assay centres upon the detection of a fluorescent signal generated from the cleavage of a target sequence specific probe by the Taq polymerase during each cycle of the PCR reaction [8]. As this signal is directly proportional to the PCR product being amplified, it permits very precise quantitation of the amount of initial input template. This is ascertained from the so-called threshold cycle, also termed the C_T value, the point where the exponential phase of the amplification curve crosses a defined threshold line. As this C_T value is a reflection of the number of PCR cycles required to reach this threshold, the lower the C_T value is, the higher is the concentration of input target template.

By using a real-time PCR assay in which two genetic loci are amplified simultaneously in a multiplex reaction, it is possible to determine the relative ratio of these two loci by subtracting their respective C_T -values, e.g.:

$$\Delta C_T = C_T(\text{target A}) - C_T(\text{target B}) = C_T(\text{RHD}) - C_T(\text{SRY})$$

Since 1 cycle entails a doubling of the PCR product, the ratio of RHD: SRY = $2^{-(\Delta C_T)}$

Hence, if both the RHD and SRY genes are present with the same gene dosage, e.g. 1 copy (heterozygous RHD/RHd), the difference in

threshold cycle number (ΔC_T) will be 0 cycles, whereas if 2 copies of the RHD gene are present (homozygous RHD/RHD) the difference in threshold cycle number (ΔC_T) will be 1 cycle. It should be noted that these are theoretical values and that slight differences are bound to occur due to minor variations in the PCR assay, especially if one reaction proceeds with slightly greater efficiency than the other.

These minor deviations are bound to occur even if considerable care has been taken to optimise the paired PCR reactions in such a manner that no significant difference is discernible between their efficiencies, as measured by the slope of the PCR assays [4, 6].

Precautions which need to be taken in order to obtain a correct result include the use of multiplex PCR reactions whereby both target gene sequences are analysed simultaneously in the same reaction vessel, as well as the inclusion of genotypically defined samples in each analysis [4, 6, 9].

By using such precautions it has previously been shown by Chiu and colleagues [4] that real-time PCR can be used to determine the paternal RHD genotype. More recently we have shown that real-time PCR can also be used to detect more subtle increments in gene dosage (only 50%), such as those occurring in foetal trisomies [6].

To test the possibility that the real-time PCR assay we had previously developed for analysis of the foetal RHD and SRY genes in maternal plasma [7] could be used to determine the paternal RHD

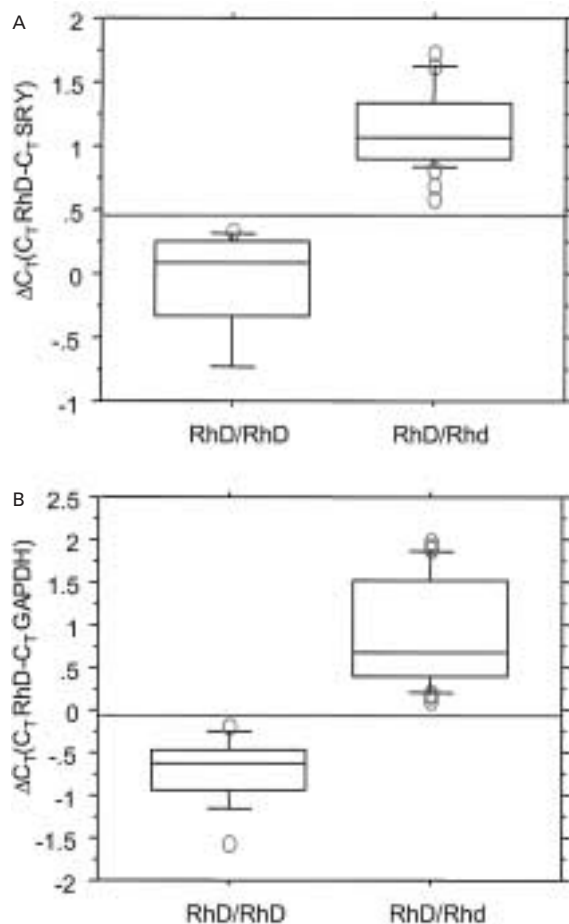
genotype, we examined 39 DNA samples obtained from males who had been serologically typed to be RHD (Swiss Red Cross Blood Bank, Basel, Switzerland). To confirm the accuracy of our assay we also determined RHD zygosity in these samples using a modification of the real-time PCR described by Chiu and colleagues [4]. In our investigation the dosage of the RHD gene was compared to another control locus, which in our case was the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene and not the albumin gene. The reason for this modification was that we have previously described the use of the Taqman real-time PCR assay for this GAPDH control locus in a number of studies, using either cell free DNA [7, 10] or genomic DNA [6]. In our analysis, all samples were run in triplicate.

The results of our analysis indicated that the samples in our investigation clustered into two specific groups, one having a median of almost 0 and the other of approximately 1 (see Figure 1A).

To ascertain the genotype of these two groups we also tested our samples using a modification of the assay described by Chiu and colleagues [4], whereby we compared the dosage of the RhD gene with that of the GAPDH gene [6]. Here we would expect an opposite pattern, as the GAPDH gene is normally present in 2 copies, i.e. the RHD/RHD genotype should yield a ΔC_T value of 0, whereas the RHD/Rhd genotype should differ by 1 cycle from the C_T value for the GAPDH reference. In this analysis we again found that the samples

Figure 1

Box-plot showing the discernment of RHD zygosity by real-time PCR. In Figure 1A, the RHD gene has been examined in comparison with the SRY gene. In Figure 1B, the RHD gene has been examined in comparison with the GAPDH gene. The lines inside the boxes indicate the median value, whereas the upper and lower limits of the boxes represent the 75th and 25th percentiles respectively. The upper and lower horizontal bars indicate the 90th and 10th percentiles respectively. Outliers are indicated by open circles. The X axis indicates the RHD genotype determined, whereas the Y axis indicates the pertinent ΔC_T values. No overlap was observed between the two genotypic groups. The cut-off values are indicated by stippled lines.



clearly clustered into two groups, one with a median ΔC_T value of approximately -0.5 and the other a median ΔC_T value of approximately 0.5 (see figure 1B). It is of interest that a 100% concordance was observed between the 2 groups of samples in both assays.

Interpretation of the results, however, is not as straightforward as it would seem, due to a significant deviation from the expected ΔC_T values. In this manner, even though the expected ΔC_T values in the first SRY:RHD assay would be -1 for the RHD/RHD genotype and 0 for the RhD/Rhd genotype, it is apparent that the PCR reaction for the SRY gene has proceeded with slightly better efficiency than that for the RHD gene. Consequently the expected RHD:SRY ΔC_T value in the case of the RHD/RHD genotype has shifted up from the theoretical value of -1 to almost 0 , whilst that for the RHD/Rhd genotype has similarly also been shifted up by 1 cycle from the theoretical value of 0 to almost 1 .

In an analogous manner the GAPDH PCR reaction has proceeded slightly more efficiently than that for the RHD gene, resulting in a shift in the expected RHD:GAPDH ΔC_T value. In this case the ΔC_T value for the homozygous RHD/RHD genotype has shifted down by almost half a cycle from the theoretical value of 0 to almost -0.5 . Equally, that for the heterozygous RHD/Rhd genotype has been shifted down by 0.5 cycles from the theoretical value of 1 to almost 0.5 .

We have previously indicated that to counter these unwanted shifts it is possible to use ΔC_T values, whereby the C_T value of the sample being analysed is compared to a mean C_T value comprised of the analysis of a large number of samples of known genotype [6, 9]. Furthermore, these drifts in ΔC_T values stress the importance of including samples of known genotype in each analysis, to counter inter-run variations [4, 6, 9]. It is also imperative to analyse the samples in a multiplex manner whereby both target genes are analysed simultaneously in the same reaction vessel, and not to attempt this type of analysis by comparing the assessed gene dosage against a standard curve [4, 6, 9].

An important feature that is evident from our analysis is that the two groups can be segregated with 100% accuracy by the use of particular cut-off values. With regard to the RHD/SRY assay, we determined that a cut-off ΔC_T (C_T RHD $- C_T$ SRY) value of 0.5 could be used to distinguish between the heterozygous (RHD/Rhd) and homozygous (RHD/RHD) genotypes, in that the values for the RHD/Rhd genotype had ΔC_T values which clustered around 1 , while RHD/RHD genotype had ΔC_T values which clustered around 0 (Figure 1A). No overlap between the two groups was found to occur.

Similarly, for the RHD/GAPDH assay, a ΔC_T (C_T RHD $- C_T$ GAPDH) cut-off value of 0.0 could

be used to distinguish the homozygous RHD/RHD genotype from the heterozygous RHD/Rhd one. In this test the heterozygous (RHD/Rhd) genotypes have ΔC_T values above 0.0 , while the homozygous (RHD/RHD) genotype had ΔC_T values below 0.0 (Figure 1B). Once again, no overlap between the two groups was found.

As explained previously, the reason for the difference in the ΔC_T cut-off values between these two assays is that for the RHD/GAPDH assay the RHD gene is compared with both alleles of the GAPDH gene on chromosome 12, whereas for the RHD/SRY assay, the dosage of the RHD gene is compared to the single SRY allele on the Y chromosome.

The validity of our analysis is underscored by our subsequent examination of 3 samples known to be from RHD heterozygous males, in which case we were able to determine the correct genotype in a blinded manner (data not shown).

In the sample cohort of 39 samples we determined that 26 (66%) were heterozygous for the RHD gene (RHD/Rhd) and 13 (34%) were homozygous (RHD/RHD). Once again there was complete concordance between the 2 assays. Although the expected frequency of RHD heterozygosity would be 56% [1], it is probable that our results are slightly skewed by the small number of cases examined.

Although our data obtained by the use of two independent real-time PCR assays do serve to indicate that this technology can potentially be used for the determination of RHD zygosity, we have also shown that the employment assays and their subsequent analysis require considerable experience if a correct diagnostic outcome is to be achieved. Furthermore, the data also emphasise the importance of running genotypically well defined control samples in parallel with the sample being analysed, as the theoretically expected ΔC_T values cannot be used. Our study also indicates the usefulness of running two independent analyses in parallel as a potential safeguard against erroneous results, a feature we have also observed previously when attempting to discern foetal trisomies by the use of real-time PCR [6, 9].

Despite these promising results we caution against the premature clinical use of these assays, since their efficacy has not yet been determined in large scale studies, nor is it yet known how they may be influenced by the RHD polymorphisms [1–3] prevalent in many ethnic populations.

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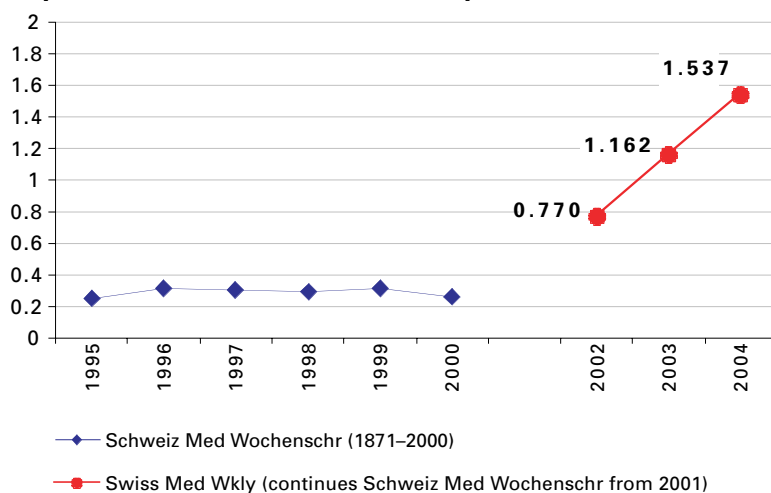
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