

Antenatal Screening

John Old

Introduction

The application of molecular biology techniques to the study of genetic disease has provided a wealth of information about their molecular basis. The haemoglobinopathies were the first genetic disorders to be studied extensively at the molecular level, and probably all the common mutations plus more than 95% of the rare ones are now known. This article presents an educational review of the techniques currently in general use for the antenatal screening of these globin gene mutations. The defects are regionally specific with each local population having its own combination of structural haemoglobin variants and thalassaemia mutations.⁽¹⁾ Therefore, knowledge of the ethnic origin of a patient is often essential to enable the quick identification of the globin gene defect by molecular biology techniques based on amplification of DNA by the polymerase chain reaction (PCR).

Mutation identification is achieved by screening first for the expected known mutations, using one or more PCR-based techniques such as gel electrophoresis, restriction endonuclease analysis, allele-specific probe hybridisation and allele-specific primer amplification. In the few cases where these techniques fail to reveal the genetic defect, characterisation may be achieved by the application of non-specific detection methods such as denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, single-stranded conformational polymorphism analysis (SSCP) and direct sequencing of amplified DNA. Some laboratories are using DGGE as their first approach to mutation detection because of their need to identify a large number of different mutations arising from a variety of ethnic groups in their at-risk population.

Haematological Evaluation

Most populations have a complex mix of different traits, and thus individuals should be screened for both thalassaemias and abnormal haemoglobins. The precise identification of the underlying molecular defect(s) is dependent upon an accurate haematological evaluation of the individual to be studied. Screening normally consists of measurement of the red cell indices, haemoglobin electrophoresis, quantitation of Hb A₂, Hb F and HbH, and determination of iron status, following guidelines and a flow chart for the establishment of a diagnosis.⁽²⁾ New expensive technology such as high performance liquid chromatography (HPLC) allows rapid direct measurement of both abnormal haemoglobins and Hb A₂ on large numbers of samples. Isoelectric focusing has also proved useful for screening large numbers of samples for abnormal haemoglobins, providing better resolution and sharper bands than ordinary electrophoresis.

Prospective carrier identification can be achieved by antenatal screening, neonatal screening or by testing during childhood. In the UK universal antenatal screening (the offer of carrier diagnosis to all pregnant women) is done in districts where the at-risk ethnic groups make up more than 15% of the population. In districts with a lower

prevalence of genetic disease, screening is carried out on a selective basis, which is arguably more cost effective but will miss carriers in low-risk ethnic groups. DNA analysis to determine the genotype is required if prenatal diagnosis is requested or a diagnostic problem fails to be resolved.

β -Thalassaemia

The β -thalassaemias are characterised by reduced (β^+) or absent (β^0) β -globin chain synthesis leading to an imbalance of excess α -globin chains inside the red cell. It is an autosomal recessive single gene disorder; 170 different mutations have been identified.⁽³⁾ The majority of these defects are single nucleotide substitutions affecting the β -globin gene or its promoter region. Only 13 defects result from gene deletions of larger than 25 nucleotides. All except the largest of these gene deletions can be detected by PCR-based methods.

The strategy for characterising β -thalassaemia mutations in most laboratories depends upon knowing the most prevalent mutations likely to be encountered in the ethnic group of the individual being screened. These are then tested by using one of the many methods now available:

(i) *Allele-specific oligonucleotides*. The hybridisation of allele-specific oligonucleotide probes (ASOs) to amplified DNA bound to nylon membrane in the form of dots was the first method to be developed and is still widely used.⁽⁴⁾ The method is based on the use of two oligonucleotide probes for each mutation, one complementary to the mutant DNA sequence and the other complementary to the normal β -gene sequence at that position. The probes can be labelled with either ^{32}P , biotin or horseradish peroxidase, but the method is limited by the need for separate hybridisations to test for multiple mutations.

To overcome this problem, the method of reverse dot blotting has been developed in which the roles of the oligonucleotide probe and target amplified DNA are reversed.⁽⁵⁾ Probe pairs, complementary to mutant and normal DNA sequences, are bound to nylon membrane in the form of dots or slots. Then the amplified DNA, labelled by either using end labelled primers or the internal incorporation of biotinylated dUTP, is hybridised to the filter. This procedure allows multiple mutations to be tested for in one hybridisation reaction. It has been applied recently to the detection of β -thalassaemia mutations in Mediterraneans,⁽⁶⁾ African-Americans⁽⁷⁾ and Thais,⁽⁸⁾ using a two-step procedure with one strip for the common mutations and the other for the less common ones.

(ii) *Primer-specific amplification*. A number of different methods have been developed based on the principle of primer-specific amplification, which is that a perfectly matched PCR primer is much more efficient in annealing and directing primer extension than one containing one or two mismatched bases. With the method known as the amplification refractory mutation system (ARMS),⁽⁹⁾ the target DNA is amplified using a common primer and either of two allele-specific primers, one complementary to the mutation to be detected (β -thalassaemia primer) and the other complementary to normal DNA at the same position (normal primer). A second pair of primers complementary to a different part of the β -globin gene are included in the PCR to amplify a fragment simultaneously in order to control the amplification step of the procedure.

ARMS primers have been made to detect all the common β -thalassaemia mutations in the four major ethnic groups, namely the Mediterraneans, the Asian Indians, the Chinese and Africans.⁽¹⁰⁾ This method provides a quick screening method which does not require any form of labelling as the amplified products are visualised simply by agarose gel electrophoresis and ethidium bromide staining. More than one mutation may be screened for at the same time in a single PCR reaction (multiplexing) provided the ARMS primers are coupled with the same common primer.⁽¹¹⁾ Fluorescence labelling of the common primer allows the sizing of the amplification products on an automated DNA fragment analyser.⁽¹²⁾

If the normal and mutant ARMS primers for a specific mutation are co-amplified in the same reaction, they compete with each other to amplify the target sequence. This technique is called competitive oligonucleotide priming (COP) and requires the two ARMS primers to be labelled differently. Fluorescent labels permit a diagnosis to be made by means of a colour complementation assay.⁽¹³⁾ A variation of this method is simply to use ARMS primers that differ in length, and thus a diagnosis can be made by analysis of the different product sizes. This technique, called mutagenetically separated polymerase chain reaction (MS-PCR), has been applied to the prenatal diagnosis of β -thalassaemia in Taiwan.⁽¹⁴⁾

(iii) *Restriction enzyme analysis.* Approximately forty β -thalassaemia mutations are known to create or abolish a restriction endonuclease site. The majority of these can be detected simply and quickly by restriction endonuclease analysis of amplified DNA. The presence or absence of the enzyme site is determined from the pattern of digested fragments after agarose or polyacrylamide gel electrophoresis.

Mutations which do not naturally create or abolish restriction sites may be detected by the technique of amplification created restriction sites (ACRS). This method uses primers that are designed to insert new bases adjacent to the mutation sequence and thus create a new restriction site, allowing known mutants to be detected by restriction analysis of the PCR product.⁽¹⁵⁾

(iv) *Gap PCR.* Deletion mutations in the β -globin gene sequence may be detected by PCR using two primers complementary to the sense and antisense strand in the DNA regions which flank the deletion. For large deletions, amplified product is obtained only from the deletion allele as the distance between the two primers is too great to amplify normal DNA. In such cases, the normal allele may be detected by amplifying a product spanning one of the breakpoints, using a primer complementary to the deleted sequence and one complementary to flanking DNA. As well as deletion β -thalassaemia, Hb Lepore and a number of $\delta\beta$ -thalassaemia and hereditary persistence of fetal haemoglobin (HPFH) deletion mutations can be diagnosed by this method.⁽¹⁶⁾

(v) *Other methods.* Known single-base changes may be detected by fluorescence-based DNA minisequence analysis. This technique takes advantage of fluorescently tagged dideoxynucleotide chain termination developed for automated DNA sequence analysis and has been applied for the detection of the common Mediterranean β -thalassaemia mutations using a multiplex-like strategy.⁽¹⁷⁾ Another method amenable to automation is the oligonucleotide ligation assay (OLA) in which two oligonucleotide probes complementary to adjacent sequences at the mutation site are joined by ligase only when they hybridise to the amplified target DNA to form perfectly matched duplexes.⁽¹⁸⁾

(vi) *Unknown mutations.* A number of techniques have been applied for the detection of β -thalassaemia mutations without prior knowledge of the molecular defect. The most widely used of these methods is DGGE, which allows the separation of DNA fragments differing by a single base change according to their melting properties.⁽¹⁹⁾ Another approach is by heteroduplex analysis by nondenaturing gel electrophoresis. Unique heteroduplex patterns can be generated for each mutation by annealing an amplified target DNA fragment with an amplified heteroduplex generator molecule, a synthetic oligonucleotide of about 130 bases in length containing deliberate sequence changes or identifiers at known mutation positions.⁽²⁰⁾ Other methods such as chemical mismatch cleavage (CMC), single-stranded conformational polymorphism analysis (SSCP), and the protein truncation test are also good methods of detecting unknown mutations, but they have not been applied to the haemoglobinopathies.

The above techniques simply pinpoint the presence of a mutation or DNA polymorphism in the amplified target sequence. Sequencing of the amplified product is then required to identify the localised mutation. This can now be done very efficiently using an automated DNA-sequencing machine utilising fluorescence detection technology. The specialised equipment required for this technique is currently very expensive, but as the machines become more efficient the cost will come down, and direct DNA sequencing will become the primary method of mutation detection.

α -Thalassaemia

α -thalassaemia is classified into two types; α^+ -thalassaemia, in which one of the two α -globin genes per chromosome is deleted or inactive, and α^0 -thalassaemia in which both α -globin genes are deleted. Prenatal diagnosis is indicated for the homozygous state of α^0 -thalassaemia but not for the homozygous state of α^+ -thalassaemia (which has a similar phenotype to the carrier state of α^0 -thalassaemia) or usually for the more severe clinical state of HbH disease, which results from the doubly heterozygous combination of α^+ and α^0 -thalassaemia.

All of the common deletion α^+ and α^0 -thalassaemic genes can be detected easily by the classic method of Southern blot analysis. Even though many of the deletions can be diagnosed by PCR, Southern blot analysis still remains a useful technique for α -thalassaemia diagnosis, especially when a wide range of genotypes are required to be characterised. Analysis with an α -probe diagnoses the $-\alpha^{3.7}$, $-\alpha^{4.2}$ and triple α -gene alleles, ζ -probe analysis diagnoses the $--^{MED}$, $-(\alpha)^{20.5}$, $--^{SEA}$, $--^{BRIT}$ and $--^{SEA}$ alleles, and finally the LO probe is used to diagnose the $--^{THAI}$ and $--^{PHIL}$ alleles.⁽¹⁰⁾

The two most common α -thalassaemia alleles, $-\alpha^{3.7}$ and $-\alpha^{4.2}$, can be diagnosed by gap PCR.^(21,22) Three α^0 -thalassaemia deletion mutations, $--^{MED}$, $-(\alpha)^{20.5}$ and $--^{SEA}$, can also be diagnosed by PCR.⁽²³⁾ The α -2 and α -1 globin genes can be amplified selectively, enabling DNA sequence changes in either gene to be characterised by direct DNA sequencing.⁽²⁴⁾ The non-deletion α^+ -thalassaemia defects may be detected by this method or by selective amplification of the α -2 gene followed by restriction enzyme analysis for the ones that affect a restriction enzyme site, such as Hb Constant Spring.⁽²⁵⁾ Because of

the unusually high GC content of the α -globin genes, amplification is technically more difficult than that of the β -globin gene and requires more stringent conditions for success.

Hb Variants

The clinically important β -globin chain variants giving rise to Hb S, Hb E, Hb D Punjab and Hb O Arab can be diagnosed most easily and quickly by restriction endonuclease digestion of an amplified β -globin gene fragment.⁽¹⁰⁾ The Hb S mutation abolishes a Dde I or Mst II site, Hb E abolishes an Mnl I site and Hb D Punjab and Hb O Arab both abolish an EcoR I site. The Hb C mutation does not affect a restriction site and must be diagnosed by one of the methods for detecting known β -thalassaemia mutations, such as dot blot analysis or ARMS.

Fetal DNA Analysis

Chorionic villus sample DNA analysis in the first trimester is the method of choice for prenatal diagnosis of thalassaemia and sickle cell anaemia. Samples taken between 10 and 12 weeks gestation by transcervical or transabdominal aspiration yield sufficient DNA for PCR or Southern blot analysis. Amniocentesis yields sufficient cells for DNA analysis by PCR-based techniques in most cases, but back-up cell cultures should be grown for the few failures to obtain DNA, for providing larger quantities of DNA for Southern blot analysis, and for retesting when there is any suggestion of possible maternal cell contamination, such as bloody amniotic fluid.

Diagnostic Pitfalls

A problem with all PCR-based techniques used for fetal DNA diagnosis is the co-amplification of maternal sequences. With chorionic villus samples this event can be avoided by the careful dissection of maternal decidua from the fetal trophoblast and by reducing the number of amplification cycles to 25, as shown by the experience of the Italian groups who have reported no misdiagnoses in 457 first-trimester fetal DNA analyses.⁽²⁶⁾ Uncultured amniotic fluid samples are more of a problem. A recent study found the presence of maternal cell contamination in 21% of samples compared with 0.2% in cultured fluid⁽²⁷⁾; thus, all such cases in which the fetal diagnosis is identical to the maternal genotype should be retested using a back-up culture to avoid the possibility of misdiagnosis.⁽²⁸⁾ The presence of co-amplified maternal DNA sequences may also be revealed by the amplification of informative polymorphic sequences such as the apolipoprotein B gene variable number tandem repeat sequence.

Another potential source of error is the failure to amplify one of the target DNA alleles. In my own experience, this occurred once in a prenatal diagnosis of α -thalassaemia, for which the PCR-based result was different to that obtained by Southern blot analysis because of a failure of amplification of the fetal normal α -globin gene allele. Amplification failure may also result when the hybridisation of a primer or probe is compromised by an unexpected change in the target DNA sequence.⁽²⁹⁾

Summary

Mutation identification by the application of molecular biology techniques is essential for accurate genetic counselling and prenatal diagnosis. The visualisation of amplified products by gel electrophoresis after ARMS analysis, gap PCR and restriction enzyme analysis provides a simple, quick and cheap approach to the direct detection of mutations, enabling developing countries such as India, Pakistan, Turkey and Iran to introduce prenatal diagnosis services for the haemoglobin disorders.⁽³⁰⁾ Dot blot analysis is popular for detecting small numbers of mutations, whereas reverse dot blotting offers the potential to screen large numbers of mutations simultaneously through the use of commercially available kits of reagents, such as the one recently marketed by Vienna Lab for the diagnosis of seven Mediterranean mutations. Fluorescence-based automatic DNA sequencing will become the method of choice for characterisation of unknown mutations and even diagnosis as cheaper, second-generation machines are developed using capillary electrophoresis to separate amplified DNA fragments.

References

1. Flint J, Harding RM, Boyce AJ, Clegg JB: The population genetics of the haemoglobinopathies. In: Higgs DR, Weatherall DJ (eds): Baillière's Clinical Haematology; Haemoglobinopathies, London, Baillière Tindall and W.B. Saunders, 1993, p 215.
2. Bhavnani M, Brozovic M, Old JM, Stephens AD: Guidelines for investigation of the a and b thalassaemia traits. *J Clin Pathol* 47:289, 1994.
3. Baysal E: The b- and d-thalassaemia repository. *Hemoglobin* 19:213, 1995.
4. Ristaldi MS, Pirastu M, Rosatelli C, Cao A: Prenatal diagnosis of b-thalassaemia in Mediterranean populations by dot blot analysis with DNA amplification and allele specific oligonucleotide probes. *Prenat Diag* 9:629, 1989.
5. Saiki RK, Walsh PS, Levenson CH, Erlich HA: Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proc Natl Acad Sci USA* 86:6230, 1989.
6. Maggio A, Giambona A, Cai SP, Wall J, Kan YW, Chehab FF: Rapid and simultaneous typing of hemoglobin S, hemoglobin C and seven Mediterranean b-thalassaemia mutations by covalent reverse dot-blot analysis: application to prenatal diagnosis in Sicily. *Blood* 81:239, 1993.
7. Sutcharitchan P, Saiki R, Huisman THJ, Kutlar A, McKie V, Embury SH: Reverse dot-blot detection of the African-American β -thalassaemia mutations. *Blood* 86:1580, 1995.
8. Sutcharitchan P, Saiki R, Fucharoen S, Winichagoon P, Erlich H, Embury SH: Reverse dot-blot detection of Thai β -thalassaemia mutation. *Br J Haemat* 90:809, 1995.
9. Newton CR, Graham A, Heptinstall LE: Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucl Acids Res* 17:2503, 1989.

10. Old JM: Haemoglobinopathies. Community clues to mutation detection. In: Elles R (Ed): *Methods in Molecular Medicine, Molecular Diagnosis of Genetic Diseases*, Totowa, NJ, Humana Press Inc., 1996, in press.
11. Tan JAMA, Tay JSH, Lin LI, Kham SKY, Chia JN, Chin TM, Norkamov BT, Aziz AOB, Wong HB: The amplification refractory mutation system (ARMS): a rapid and direct prenatal diagnostic technique for β -thalassaemia in Singapore. *Prenat Diag* 14:1077, 1994.
12. Zschocke J, Graham CA: A fluorescent multiplex ARMS method for rapid mutation analysis. *Mol Cell Probes* 9:447, 1995.
13. Chehab FF, Kan YW: Detection of specific DNA sequence by fluorescence amplification: a colour complementation assay. *Proc Natl Acad Sci USA* 86:9178, 1989.
14. Chang JG, Lu JM, Huang JM, Chen JT, Liu HJ, Chang CP: Rapid diagnosis of β -thalassaemia by mutagenically separated polymerase chain reaction (MS-PCR) and its application to prenatal diagnosis. *Br J Haemat* 91:602, 1995.
15. Linderman R, Hu SP, Volpato F, Trent RJ: Polymerase chain reaction (PCR) mutagenesis enabling rapid non-radioactive detection of common β -thalassaemia mutations in Mediterraneans. *Br J Haemat* 78:100, 1991.
16. Craig JE, Barnetson RA, Prior J, Raven JL, Thein SL: Rapid detection of deletions causing $\delta\beta$ -thalassaemia and hereditary persistence of fetal hemoglobin by enzymatic amplification. *Blood* 83:1673, 1994.
17. Kobayashi M, Rappaport E, Blasband A, Semeraro A, Sartore M, Surrey S, Fortina P: Fluorescence-based DNA minisequence analysis for detection of known single-base changes in genomic DNA. *Mol Cell Probes* 9:175, 1995.
18. Barany F: Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proc Natl Acad Sci USA* 88:189, 1991.
19. Losekoot M, Fodde R, Harteveld CL, Van Heeren H, Giordano PC, Bernini LF: Denaturing gradient gel electrophoresis and direct sequencing of PCR amplified genomic DNA: a rapid and reliable diagnostic approach to beta thalassaemia. *Br J Haemat* 76:269, 1991.
20. Savage DA, Wood NAP, Bidwell JL, Fitches A, Old JM, Hui KM: Detection of β -thalassaemia mutations using DNA heteroduplex generator molecules. *Br J Haemat* 90:564, 1995.
21. Dode C, Krishnamoorthy R, Lamb J, Rochette J: Rapid analysis of $-\alpha^{3.7}$ -thalassaemia and α 3.7 triplication by enzymatic amplification analysis. *Br J Haemat* 82:105, 1992.
22. Baysal E, Huisman THJ: Detection of common deletional α -thalassaemia-2 determinants by PCR. *Am J Hematol* 46:208, 1994.
23. Bowden DK, Vickers MA, Higgs DR: A PCR-based strategy to detect the common severe determinants of α -thalassaemia. *Br J Haemat* 81:104, 1992.
24. Molchanova TP, Pobedimskaya DD, Postnikov YV: A simplified procedure for sequencing amplified DNA containing the α -2 or α -1 globin gene. *Hemoglobin* 18:251, 1994.

25. Ko TM, Tseng LH, Hsieh FJ, Lee TY: Prenatal diagnosis of HbH disease due to compound heterozygosity for south-east Asian deletion and Hb Constant Spring by polymerase chain reaction. *Prenat Diag* 13:143, 1993.
26. Rosatelli MC, Tuveri T, Scalas MT, Leoni GB, Sardu RS, Faa V, Meloni A, Pischedda MA, Demurtas M, Monni G, Cao A: Molecular screening and fetal diagnosis of β -thalassaemia in the Italian population. *Hum Genet* 89:585, 1992.
27. Winsor EJT, Silver MP, Theve R, Wright M, Ward BE: Maternal cell contamination in uncultured amniotic fluid. *Prenat Diag* 16:49, 1996.
28. Wang X, Seaman C, Paik M, Chen T, Bank A, Piomelli S: Experience with 500 prenatal diagnoses of sickle cell diseases: the effect of gestational age on affected pregnancy outcome. *Prenat Diag* 14:851, 1994.
29. Chan V, Chan TPT, Lau K, Todd D, Chan TK: False non-paternity in a family for prenatal diagnosis of β -thalassaemia. *Prenat Diag* 13:977, 1993.
30. Petrou M, Modell B: Prenatal screening for haemoglobin disorders. *Prenat Diag* 15:1275, 1995.