Molecular Genetics of β-thalassemia in the Eastern Province of Saudi Arabia

A.K. Al-Ali, M. Al-Maden✲ and F. Al-Qaw

Departments of Biochemistry and Paediatrics✲

College of Medicine

King Faisal University

Dammam

Final Report

2001–2002 (1422–1523)

King Faisal University

Research Grant 2059

Contents

Summary

Hemoglobinopathies are the most commonly inherited genetic disorders encountered among the population of the Eastern Province of Saudi Arabia. The thalassemia disease has two major forms known as α and β-thalassemia, and are characterized by quantitative deficiency of either α-globin or β-globin respectively. The high frequency and heterogenicity of β-thalassemia constitutes a major health problem. The objective of this project was to establish the molecular genetics of β-thalassemias in the Eastern Province. Genome DNA was isolated from the blood of 25 patients suspected of being carriers of β-thalassemia. Multiplex PCR for amplification of 4 regions in β-globin gene was undertaken.

The data show that IVS-2 nt 1 and IVS-1 nt 110 mutations are the most common in the population. Codon 39 mutation was also found with relatively high incidence. One of the subjects had a compound mutation which included IVS-2 nt 1 and IVS-1 nt 110 mutations. Six of the subjects with high Hbg A_2 showed none of the five common mutations. The results demonstrate the feasibility of using this cost effective technique to screen for the mutation as a pre-marriage requirement.

الملخص

أمراض الدم الوراثية و التي تتعلق بالصبغة الدموية هي من اكثر الأمراض انتشار بين سكان المنطقة الشرقية من المملكة العربية السعودية. أحد هذه الأمراض هو الثلاسيميا بنوعيها البيتا و الألفا و التي تتصف بنقصان أحد سلاسل الهيمغلوبين. أن هذه الأمراض لها التأثير الكبير على صحة المصابين. من أهداف هذا البحث هو تعيين الأنماط الجزيئية لمرض البيتا ثلاسيميا في المنطقة الشرقية. تم استخلاص و تنقية الصبغة الوراثية (DNA) من الحاملين لهذا المرض و من ثم تعيين نوع الطفرات الوراثية في الصبغة و مدى انتشارها. أن النتائج تدل على أن الطفرات التالية IVS-2 nt 1 &IVS-1 nt 110 هي أكثر شيوعا بين الحاملين للبيتا ثلاسيميا. كم أن طفرة Codon 39 موجودة ولكن يحملها نسبيا عدد أقل من الطفرتين السبق ذكرهما. كما تم تشخيص وجود طفرتين في جينة الهيمغلوبين (IVS-1 nt110&IVS-2 nt 1) لأحد المصابين. هذا بالإضافة إلى أنه لم نتمكن من تعيين نوع الطفرات في ستة أشخاص من ذوي Hbg A2 المرتفع.

أن النتائج تدل على الطريقة التي تم استخدامها في هذا البحث هي طريقة ذات جدوى اقتصادية يمكن استعمالها لتشخيص الطفرات الوراثية في العديد من الأمراض و يمكن أن تقدم فائدة كبرى في فحوصات التشخيص قبل الزواج .

Literature Review

The thalassemias are a heterogeneous group of inherited diseases characterized by hypochromic anaemia due to deficient synthesis of one or more of the polypeptide chains of human hemoglobin (1). In β and α thalassemia, the primary difficulty is a quantitative deficiency of either β-globin, leading to β-thalassemia, or α-globin, leading to α-thalassemia (2). The name was applied because of the relatively high frequency of these disorders in individuals living around the Mediterranean Sea. In fact, the thalassemias are common not only in the Mediterranean area but also in parts of Africa and Southeast Asia (3). The distribution of thalassemias coincides with the frequency of malaria. The high frequency of thalassemia alleles in these areas is thought to be a reflection of the advantage that a heterozygote has for one of these conditions when infected with the malarial parasite (4).

β-Thalassemia

In β-thalassemia, it is the β-globin chains that are deficient. Hence, α-globin chains are in excess, which forms, α-tetramers. These tetramers are insoluble and precipitate within red blood cells leading to their premature destruction in the bone marrow and marked trapping in the spleen (5). Red cells from individuals with β-thalassemia are reduced in

size as well as in number (6). Since there is only one β-globin gene per chromosome 11, the potential for unequal crossing over is reduced (7). The genetics of β-thalassemia are complicated by the large number of mutations that can result in decreased or absent function of a β-globin gene (8). The disease is inherited in an autosomal recessive fashion.

The most severe form is β-thalassemia major, which is characterized by the dysfunction of both β-globin genes and hence no β-globin chains are produced. However, if one or both of the mutations still allows the production of small amounts of β-globin, then this is denoted as β+-thalassemia. Heterozygotes for β-thalassemia (thalassemia minor) carry one normal β-globin gene and are asymptomatic. It is usually impossible to tell by looking at the level of β-globin in such an individual whether the thalassemia allele carried is a β+ or a β^o mutation because the normal chromosome is producing the vast majority of β-globin in both situations.

Intense work over the past decade has demonstrated the presence of over 100 different mutations that have been shown to cause β-thalassemia (9, 10). Hence this disorder is quite heterogenic because of the wide diversity of mutations. The mutations in the β-globin gene are scattered throughout the length of the gene. These mutations include point mutations in the promoter region, which interfere with transcription

of mRNA, while others interfere with the efficient translation of the β-globin in mRNA into protein (11). Still others may occur in the splice region as they alter the invariant GT sequence at the beginning of an intron or the AG at the end (12, 13). These mutations usually lead to β-thalassemia.

The most common cause of β-thalassemia in the Mediterranean region is a mutation in intron 1. This is a simple point mutation of a G to an A at position 110 of intron 1 and occurs 21 nucleotides upstream of the normal splice acceptor site (14). The result of this mutation is the creation of an AG sequence. Therefore, the abnormally spliced RNA does not give rise to useful protein, and only 10% of the RNA that is normally spliced is useful.

β-thalassemia is common among the population of the Eastern Province (14). In addition, a large number of sickle cell anaemia patients in this area also carry the gene of β-thalassemia.

Objectives

General Objective

The major goal of this study is to establish the molecular basis of β-thalassemia in the population of the Eastern Province of Saudi Arabia.

Specific Objectives

1. To screen 40 samples genetically with β-thalassemia trait as diagnosed by phenotype characterization.
2. To determine the type of mutations common in this area.
3. To establish and present a feasible protocol for molecular diagnosis of β-thalassemia.

Project Design

Subjects

a- Criteria for selection

- Subjects with β- thalassemia trait
- Healthy adult unrelated Saudi subjects

b- Selection and sample size

Approximately 75 subjects will be randomly selected and classified in the following groups:

1. normal subjects (35)
2. subjects with β- thalassemia trait (40)

Laboratory Investigation

After informed consent, blood was obtained by routine venipuncture in EDTA coated tubes and was immediately analysed.

Complete Blood Count (CBC) and Morphology

Hematological indices were determined using a Hematology Analyzer (Coulter Counter MD II). Reticulocyte count was also

determined by staining techniques using methylene blue to exclude active hemolysis.

Sickle Cell Anemia Screening

This test was used as a screening test for the presence of sickle hemoglobin . It is based on the relative insolubility of deoxygenated sickle hemoglobin in solutions of high molarity. The test was performed by the addition of 50µl of heparinized blood to a test tube containing phosphate buffers, lysing and reducing agents. A turbid suspension was formed in the presence of sickle hemoglobin.

Glucose-6-Phosphate Dehydrogenase Deficiency

All samples were screened for G6PD deficiency by fluorescent spot technique using commercially available kits from Boehringer & Mannheim. The procedure required 10µl of whole blood added to 100µl of a solution containing G6P, NADP+, GSSG and saponin in tris. 10 µl were transferred to filter paper, air dried and viewed under UV light.

Determination of HbA_2

Hbg A_2 was determined by use of Helena Sickle-Thal Quik column method (15). This method is based on anion exchange chromatography. The negatively charged hemoglobins bind the

positively charged resin in the column. Following binding, each hemoglobin type was removed selectively from the resin by altering the pH or ionic strength of the elution buffer. Hbg A_2 was then eluted with specific buffer and compared to total hemoglobin by determining the absorbance of each fraction at 415nm.

Iron / Total Iron Binding Capacity

The iron in serum was dissociated from its Fe III – transferring complex by the addition of an acidic buffer containing hydroxylamine which reduces the Fe III to Fe II. The chromogenic agent, PDTS, forms a highly coloured Fe II complex that is measured photometrically at 565 nm.

The unsaturated iron binding capacity (UIBC) was determined by adding Fe 11 ions to serum. The excess Fe 11 ions reacts with PDTS to form the coloured complex which is again measured photometrically at 565 nm. The TIBC was determined by adding the serum iron value to the UIBC value.

DNA Extraction

The protocol for DNA isolation included the following steps.

1. 300 µl whole blood was added to 5 ml tube containing 900 µl RBC lysis solution. The solution was mixed, incubated for 10 mins at room temperature and centrifuged for 20s at 13000-16000 g.
2. Supernatant was removed leaving behind visible white cell pellet and 10 – 20 µl of residual liquid. The tube was vortexed vigorously to resuspend the white blood cells.
3. 300 µl cell lysis solution was then added and mixed. 1.5 µl Rnase A solution was added, mixed thoroughly and incubated for 20 mins at 37C.
4. Sample was cooled to room temperature ,100 µl protein precipitating solution was added, vortexed and centrifuged as before.
5. The supernatant containing the DNA was poured into a tube containing 300 µl 100 % isopropanol. It was then mixed, centrifuged and the visible DNA was washed three times with 70% ethanol.

Multiplex Amplification Refractory Mutation System (MARMS)

This is a method for direct detection of normal and mutant β-globin genes in both homozygous and heterozygous (16). The strategy

involves multiplex PCR of four of the five regions within the β-globin gene in a single reaction containing Taq polymerase, deoxynucleotide triphosphate buffer, genomic DNA, water, and either the normal or mutant primers. These primers correspond to IVS-1 nucleotide 1(IVS-1 nt 1) or IVS-1 nucleotide 6(IVS-1 nt 6), IVS-1 nucleotide 110(IVS-1 nt 110), codon 39, and IVS-2 nucleotide 1(IVS-2 nt 1) regions. Primers were chosen so that the sizes of the four PCR products differed. This allowed the separation and thus detection of amplified fragments on agarose gel electrophoresis. PCR was carried in 50 µl volume as described elsewhere. Amplification included 35 cycles at 3 different temperatures.

Results

Subjects

Over a period of one year 25 blood samples were randomly collected from patients attending King Fahad Teaching Hospital, Al-Khobar and from volunteers suspected of being carriers of the β-thalassemia mutation in Al-Qatif area in the Eastern Province of Saudi Arabia. In addition 35 normal samples, used as controls, were collected from volunteers from the same area (Table 1). A written consent was taken from each subject prior to obtaining a blood sample.

Hematological analysis was carried out on all control samples to identify those with low hemoglobin and MCV to exclude other genetic abnormality. The majority of suspected subjects had low hemoglobin and low MCV values which might have indicated the presence of the β-thalassemia mutation.

All samples were screened for the presence of the sickle cell anemia and G6PD deficiency mutations. The results indicate that all but one of the subjects suspected of having β-thalassemia gene carried either one or both of the above mutations (Table 2).

Hbg A_2 measurements aid in the differential diagnosis of sickle cell anemia from sickle- β-thalassemia. Hence, the level of both Hbg A_2 and Hbg S were determined for all suspected β-thalassemia samples. All these samples had high Hbg A_2 (Range 2.0 – 5.6 %) and the majority of the subjects carry the sickle gene in heterozygous manner. Our results show that Hbg A_2 level in Hbg S containing samples partially overlap with those expected from β-thalassemia carriers. These results agree with previous reports of association of β-thalassemia with sickle cell anemia (15).

DNA Extraction

After several attempts, the isolation of DNA from all samples was successful. Some of the DNA samples extracted at the beginning of the project had to be discarded due to fragmentation of the DNA and absence of any PCR products. The concentrations of isolated DNA are presented in table 3. The concentrations obtained were in the expected range and agrees with the range reported by the manufacturers (Puregene DNA isolation Kit).

Mutation Detection

Primer solutions were made and their concentrations were determined (Table 4). Concentrations of all the primers were in the range required for the PCR amplification.

Screening for the five most common Mediterranean mutations involved four separate reactions. Reaction mixtures contained the common upstream primer and either four normal or four mutant primer pairs for the IVS-1 nt 1, IVS-1 nt 110 codon 39 and IVS-2 nt 1 sequences. Another separate mixture was prepared which contained the common upstream primer and either normal or mutant primers for detection of IVS-1 nt 6 sequence. Mutant PCR products were then identified by the presence or absence of the correctly sized band following electrophoresis on agarose. The results of mutation screening for some samples (different mutation) are presented in figures 1 to 5.

All the samples were heterozygotes for β-thalassemia. Figure 1 shows an example of electrophoretic bands when mutation IVS-1 nt 110 is present, whereas figure 2 shows an example of electrophoretic bands when mutation at codon 39 is present. Figure 3 shows an example of electrophoretic bands when mutation IVS-2 nt 1 is present. Figure 4 shows electrophoretic bands of

compound mutations for IVS-2 nt 1 and IVS-1 nt 110. Figure 5 shows normal electrophoretic bands when no mutations are present. The results of mutation identification for all samples are presented in table 5. There were samples with high Hbg A_2 but they did not show any of the five common Mediterranean mutations. These results show that normal primers can be multiplexed in the same tube, and that mutant primers will not prime normal DNA under these conditions.

Discussion

The β-thalassemias are widespread throughout the Mediterranean region and other parts of the world (10). Within each population at risk for β-thalassemia, a small number of common mutations are found, as well as rarer ones. A limited number of haplotypes are found in each population so that 80 percent of the mutations are associated with only 20 different haplotypes. There is evidence that the high frequency of β-thalassemia in certain regions reflects an advantage of heterozygotes against plasmodium falciparum malaria as has already been demonstrated in α-thalassemia (17, 18).

In the Mediterranean population in the Eastern Province a large number of homozygous and heterozygous sickle cell anemia patients also carry the gene for β-thalassemia. This association has an ameliorating effect on the clinical course of sickle cell disease (18). Our results have shown this clearly, since most of the carriers of sickle gene in the present investigation also carry the β-thalassemia gene. The present data also show that most subjects with high hemoglobin A_2 carry the β-thalassemia gene. In addition our data show that a large number of those who carry the β-thalassemia gene also carry the G6PD deficiency mutation. G6PD deficiency is very common among the population of the Eastern province of Saudi Arabia (19).

It has been reported that the most common cause of β-thalassemia in the Mediterranean region is a mutation in intron 1 (14). This is a simple point mutation of a G to an A at position 110 of intron 1 and occurs 21 nucleotides upstream of the normal splice acceptor site. The result of this mutation is the creation of an AG sequence. In the small number of samples in which we have been able to identify the mutations, mutation IVS-2 nt 1 and mutation IVS-1 nt 110 were the most common. This was followed by mutation at codon 39. Mutations corresponding to IVS-1 nt 6

and IVS-1 nt 1 have been reported among the five common Mediterranean mutations (16). However, in our small number of subjects these mutations were not present. Moreover, one sample was found to have compound mutations of IVS-2 nt 1 and IVS-1 nt 110. These results may indicate that both IVS-2 nt 1 and IVS-1 nt 110 are the most common in our area. However, one cannot be certain until a larger number of samples are investigated.

A number of samples which had high hemoglobin A_2, showed none of the five common mutations. This result could be due to either the existence of other β-thalassemia mutations not screened for by the present methodology, or alternatively the sample is normal. In this case, the only way to be certain if these samples carry a mutation or not is to sequence their β-globin gene. It is hoped that such techniques will soon be available in our institute to carry out such work. These results demonstrate selective multiplex amplification of normal and mutant alleles for the common mutations in Mediterranean populations.

Conclusion

With the limited resources available, it has been possible to carry out mutation detection for β-thalassemia in a small number of samples. Therefore this work shows promise for rapid, cost

effective assay for mutation screening. Its usefulness becomes apparent in pre-marriage testing in order to decrease the incidence of this disorder. Also, this will hopefully open the way for more intensive investigation and extend the work for the detection of other mutations. It has been shown that many people are susceptible toward development of hypertension and diabetes due to mutations in certain genes that can be identified prior to the onset of the disease.

References

1. Scriver CR, Beaudet AL, Sly WS, Valle D. The metabolic and molecular bases of inherited disease. 7th ed. NY. McGraw-Hill, 1995.
2. Weatherall DJ, Clegg JB. The thalassemia syndromes, ed 3, Oxford, 1981, Blackwell Scientific Publication.
3. Livingstone FB: Abnormal hemoglobin in human populations. Chicago, 196, Aldine Publishing Co.
4. Nagel RL, Roth EF, Jnr. (1989). Malaria and red cell genetics defects. Blood, 74, 1213.
5. Sturgeon P, Finch CA: (1957). Erythrokinetics in cooley's anemia. Blood 12: 64.
6. Harrison CR: Harmening DM (ed): Clinical Hematology and Fundamentals of Clinical Hemostasis, 2nd ed. Philadelphia: FA Davis, 1992.
7. Bunn HF, Foorget BH: Molecular, genetic and clinical aspects, Philadelphia 1986, WB Saunders Co.
8. Weatherall DJ, Clegg JB: The Thalassemia Syndromes, 3rd ed. Oxford: Blackwell Scientific, 1981.

9. Weatherall, DJ: The thalassemias. In Williams WJ; Beutler E, Erslev AJ, Lichtman MA (eds): Hematology, 4th ed. New York: McGraw-Hill, 1990.

10. Weatherall DJ: The thalassemia. In stamatoyannopoulos G, Nienhuis AW, Majerus TW, Varmus H, editors: The molecular basis of blood diseases, ed 2, Philadelphia, 1993, WB Saunders.

11. Kazazian HH, Jr: The thalassemia syndromes: molecular basis and prenatal diagnosis in 1990. Semin Hematol 1990; 27: 209.

12. Ramao L, Inacio A, Santos S, Avila M, Faustin P, Pacheco P, Lavinha J. (2000) Nonsense mutations in the human beta-globin gene lead to unexpected level of cytoplasmic mRNA accumulation. Blood 15; 2895.

13. Hasounah FH, Sejeny SA, Omer JA, Old JM, Oliver RW. Spectrum of beta-thalassemia mutations in the population of Saudi Arabia (1995). Hum Hered 45: 231.

14. El-Hazmi MA, Warsy AS, Al-Swailem AR. (1995). The frequency of 14-beta-thalassemia mutations in the Arab populations. Hemoglobin 19: 353.

15. Tadmouri GD, Yuksel L, Basak AN. (1998). HbS beta-del-thalassemia with high levels of hemoglobin A_2 and F in Turkish family. Am J Hematol 59: 83.

16. Fortina P, Dotti G, Conant R, et al. (1992). PCR methods and application.;2:163-6

17. Weatheral DJ. (1987). Common genetic disorders of the red cell and the malaria hypothesis. Ann Trop Md Parassitol 81:539-48.

18. Allen SJ, O'Donnell A, Alexander NDE, et al. (1997) α-thalassemia protects children against disease caused by other infections as well as malaria. Proc Narl Acad Sci USA. 94:14736-41

19. Al-Ali AK, Al-Mustafa Z, Al-Madan M, Qaw F, Al-Ateeq S. (2002) Molecular characterization of G6PD deficiency in the eastern province of Saudi Arabia. Clin Chem Lab Med 40:314-6

Table 1. Total number, gender, mean age and age range for various groups investigated.

	Gender	Number	Age	Age Range
Normal	female	18	23.0	16-36
	male	17	25.0	19-34
β–thalassemia	female	7	13.9	4-25
suspects	male	18	21.8	3-43
Total				
Normal		35		
suspected		25		

Table 2. Classification of suspected β–thalassemia subjects according to existence of other mutations.

Total No. of Subjects	Sickle Mutation	G6PD Deficiency	Range of AgA_2
1	Normal	Normal	5.3
15	Positive	Normal	1.9 – 4.2
6	Normal	Deficient	2.9 – 3.9
3	Positive	Deficient	2.6 – 5.6

Table 3. Yields of DNA extraction using puregene DNA isolation kit produced by Gentra system.

Subjects	Range of Optical Density	Yields (mg/ml)
Normal	0.016-0.047	160-470
Suspected B-thalassemic carriers	0.004-0.047	40-470

Table 4. Determined concentrations of all normal and mutant primers used in multiplex procedures.

Primer Name	Total amount (nmoles)
IVS -1 nt 1-N	68.48
IVS –1 nt 1-M	68.17
IVS –1 nt 6-N	73.46
IVS –1 nt 6-M	74.38
IVS –1 nt 110-N	64.3
IVS –1 nt 110-M	64.02
Codon 39 –N	65.26
Codon 39- M	64.56
IVS-2 nt 1-N	62.3
IVS-2 nt 1-M	62.04
CRP 1	221.67

Table 5. Number of different mutations identified in 25 subjects with high $HbgA_2$.

Mutation	Total Number	Percentage
IVS-2 nt 1	7	28
IVS-1 nt 110	7	28
Codon 39	4	16
IVS-2 nt 1 + IVS-1 nt 110	1	4
Unknown	6	24

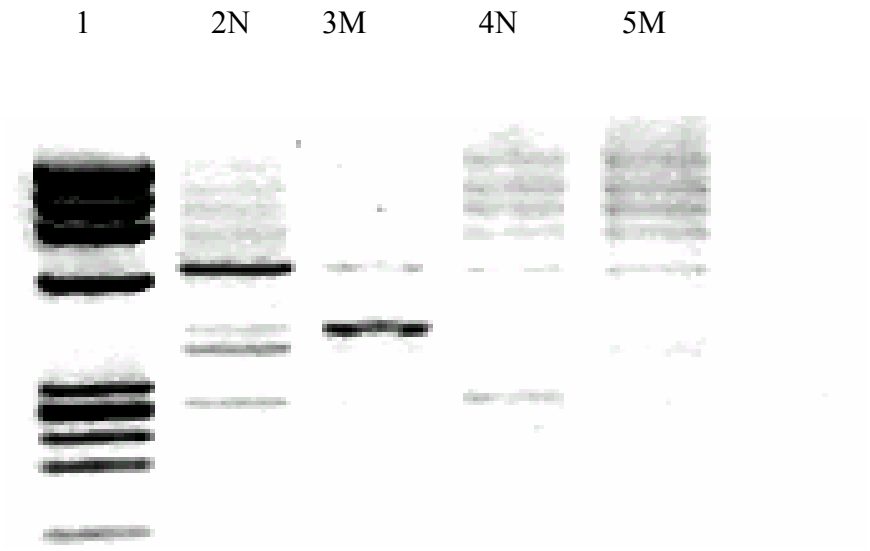

Figure 2. Multiplex amplification using separate reactions containing a mixture of either the normal (N) or corresponding mutant (M) primer sets. DNA from a heterozygote for codon 39 (lane 3M). PCR products are sized relative to markers generated from φX174 Rf DNA (lane 1).

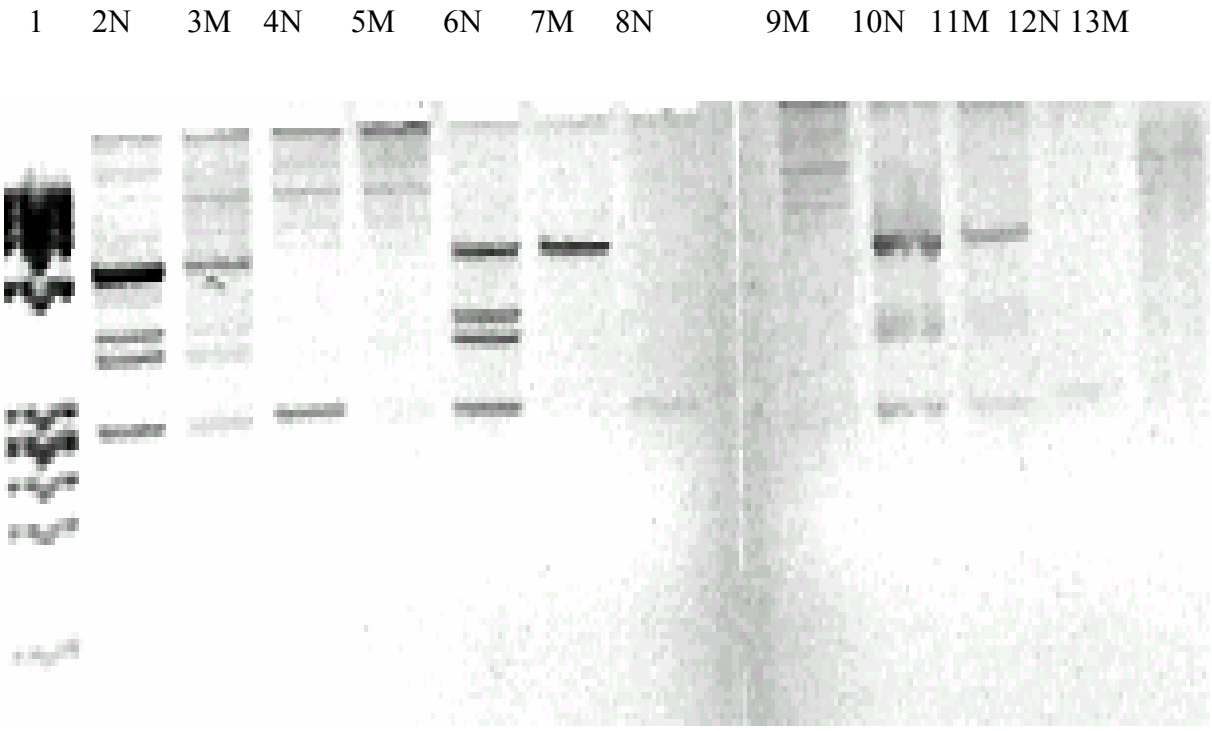

Figure 3. Multiplex amplification using separate reactions containing a mixture of either the normal (N) or corresponding mutant (M) primer sets. DNA from a heterozygote for IVS-2 nt 1 (lane 6N-9M).PCR products are sized relative to markers generated from φX174 Rf DNA (lane 1).(note. Lanes 2N-5M show normal amplification)

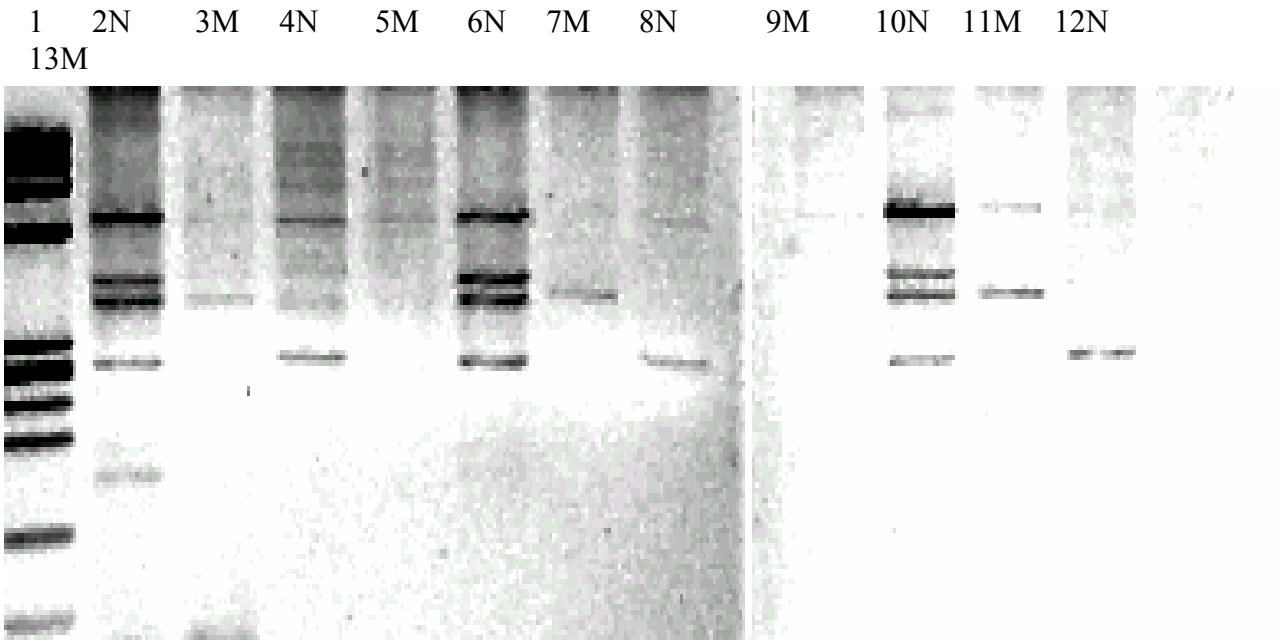

Figure 4. Multiplex amplification using separate reactions containing a mixture of either the normal (N) or corresponding mutant (M) primer sets. DNA from a compound heterozygote for IVS-2 nt 1 + IVS-1 nt 110 (lane 10N-13M). PCR products are sized relative to markers generated from φX174 Rf DNA (lane 1). (note. Lanes 2N-5M show IVS-1 nt 110 mutation).

1 2N 3M 4N 5M 6N 7M 8N 9M 10N 11M 12N 13M

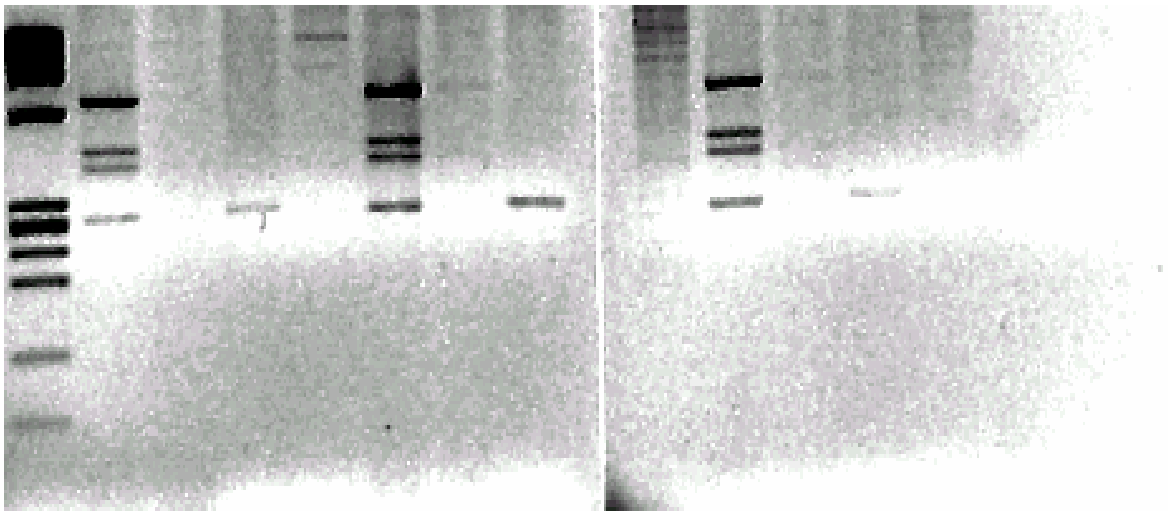

Figure 5. Multiplex amplification using separate reactions containing a mixture of either the normal (N) or corresponding mutant (M) primer sets. DNA from normal samples (lane 2N-5M, 6N-9M & 10N- 13M). PCR products are sized relative to markers generated from φX174 Rf DNA (lane 1).