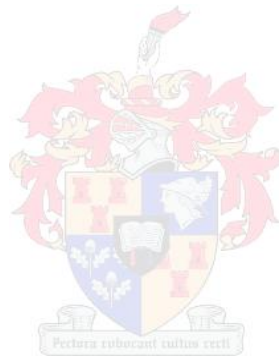# INVESTIGATION OF THE GENETIC AETIOLOGY OF AMINOGLYCOSIDE-INDUCED HEARING LOSS IN SOUTH AFRICAN POPULATIONS

Hanniquè Human

Dissertation presented in partial fulfilment of the requirements for the degree of Master of Science in Human Genetics at the Faculty of Health Sciences, Stellenbosch University

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Co-supervisor: Professor Greetje de Jong

December 2009

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2009

I

Abstract

South Africa is currently facing a major multidrug-resistant tuberculosis (MDR-TB) epidemic and has one of the highest incidences in the world. Aminoglycoside antibiotics are commonly used in this country as a treatment against MDR-TB. A well known side-effect of aminoglycosides is permanent hearing loss and this is thought to have a significant genetic component. To date, at least six mutations in the mitochondrial genome are known to confer susceptibility to aminoglycoside-induced hearing loss. It is imperative that we investigate the frequency of these mutations in our populations and determine whether certain sub-groups are at increased risk. The aim of the present study was therefore to investigate the genetic aetiology of aminoglycoside-induced hearing loss in the South African population.

A multiplex method using the ABI Prism$^{®}$ SNaPshot^{TM} Multiplex system was optimised to screen for six mutations in the *MT-RNR1*: A1555G, C1494T, T1095C, 961delT+C$_{(n)}$, A827G and T1291C. A total of 115 MDR-TB patients from the Brooklyn Chest Hospital in Cape Town who were receiving high doses of either streptomycin, kanamycin or capreomycin were recruited for this study. Furthermore, 439 control samples, comprising of 93 Afrikaner, 104 Caucasian, 112 Black and 130 Mixed Ancestry individuals were recruited and screened for the presence of the six mutations. Identification of novel variants in the *MT-RNR1* and the entire mitochondrial genome was performed using High Resolution Melt analysis (HRM) and whole mitochondrial DNA sequencing, respectively. A total of 97 family members from a South African family known to harbour the A1555G mutation were recruited and genotyped using SNaPshot analysis. In addition, mitochondrial functioning in the presence of different streptomycin drug concentrations, in transformed lymphoblasts of an individual harbouring the A1555G, was assessed by means of the MTT colorimetric assay. Detection of heteroplasmic mutations was performed using PCR-Restriction Fragment Length Polymorphism (RFLP) analysis and UN-SCAN-IT software.

We successfully developed a robust and cost-effective method that detects the presence of all six mutations simultaneously. The method worked equally well on both blood (from adults) and buccal swabs (from children). The C1494T, T1095C and T1291C mutations were not detected in any of the MDR-TB or control groups. Alarmingly, the A1555G mutation was detected in 0.9% of the Black control samples and in 1.1% of the Afrikaner controls (in one sample in the heteroplasmic state 25%). The A827G mutation was present at a frequency of 0.9% in the MDR-TB patients and in 1.1% of the Afrikaner controls. The 961delT + insC$_{(n)}$ mutation was found in relatively high frequencies in both the MDR-TB patients (3.5%) and control groups (1.1% of the Afrikaner, 1.5% of the Mixed Ancestry and 7.1% of the Black samples). Similarly, the T961G mutation was

detected at high frequencies in the Caucasian (2.9%) and Afrikaner (3.2%) controls. Screening for novel variants in *MT-RNR1* in MDR-TB patients experiencing ototoxicity revealed two novel variants (G719A and T1040C). However, G719A and T1040C are not likely to be pathogenic since they were detected in ethnic-matched controls: Mixed Ancestry (20.7%) and Black (1.8%) controls. Furthermore, a total of 50 novel variants were identified within the mitochondrial genome of eight MDR-TB patients with ototoxicity. Only five of the 50 variants (one in the *MT-TH, ND3, COX3* and two in the *CYTB* gene) were shown to reside at positions that are evolutionarily conserved across five species from human to frog, and the four variants in the protein coding genes resulted in missense changes. A total of 76 of the 97 family members recruited were found to be A1555G-positive (on mitochondrial haplogroup L0d) and are therefore at risk of developing irreversible hearing loss. Genes and variants known to act as genetic modifiers: $tRNA^{Ser(UCN)}$, homozygous A10S in *TRMU* and 35delG in *GJB2* were not present in this family. For the MTT assay, decreased mitochondrial functioning of cells harbouring the A1555G mutation in the presence of streptomycin were (compared to wild type) observed but this was not statistically significant (p-value: 0.615-0.999).

The high frequency of the A1555G mutation (0.9%) in the Black population in South Africa is of concern given the high incidence of MDR-TB in this particular ethnic group. However, future studies with larger numbers of samples are warranted to determine the true frequencies of the aminoglycoside deafness mutations in the general South African population. Our data suggests that the 961delT + $insC_{(n)}$ and T961G variants are common non-pathogenic polymorphisms due to the high frequencies observed in controls (>1%). The identification of the first novel variants within protein coding genes that could possibly be associated with aminoglycoside-induced hearing loss holds great possibilities with regards to the identification of a second gene involved in drug induced hearing loss. Future studies where the possible effect of these variants on the normal functioning of these genes could be assessed would contribute greatly to this field of research. All 76 A1555G-positive members of the family were given genetic reports and counseled about their risk and that of their children for developing hearing loss due to aminoglycoside use.

The development of a rapid and cost-effective genetic method facilitates the identification of individuals at high risk of developing hearing loss prior to the start of aminoglycoside therapy. This is of critical important in a low-resource country like South Africa where, despite their adverse side-effects, aminoglycosides will be continue to be used routinely and are accompanied with very limited or no audiological monitoring. Future studies and greater public awareness is therefore needed to address this serious problem.

Opsomming

Suid Afrika beleef tans 'n grootskaalse tuberculose epidemie (veral weerstandige vorme van tuberculose) (MDR-TB), met een van die hoogste voorkomssyfers in die wêreld. Aminoglikosied antibiotikums word baie algemeen gebruik in Suid Afrika vir die behandeling van MDR-TB. 'n Bekende newe effek van die middels is permanente gehoor verlies en dit is van mening dat dit gekoppel is aan 'n genetiese component. Daar is tans ses mutasies in die mitochondriale genoom wat vatbaarheid tot aminoglikosied-geinduseerde gehoor verlies veroorsaak. Daarom is dit van uiterse belang dat die frekwensie van die mutasies in ons populasies bepaal word sodat daar vasgestel kan word watter groepe 'n hoë risiko het om gehoor verlies te kan ontwikkel.

Die ABI Prism® SNaPshot™ Multipleks sisteem is gebruik en geoptimiseer om te toets vir die ses mutasies in die *MT-RNR1*: C1494T, T1095C, 961delT+$C_{(n)}$, A827G and T1291C. 'n Totaal van 115 MDR-TB pasiente van die Brooklyn Chest Hospital in Kaap Stad is gewerf vir die studie. Hierdie pasiente ontvang daaglikse hoë dosese van een van die volgende aminoglikosiede: streptomycin, kanamycin of capreomycin. Verder is 'n totaal van 439 kontrole DNA monsters gewerf vanuit die volgende etniese groepe: 93 Afrikaner, 104 Blank, 112 Swart and 130 Kleurling. Hierdie monsters is ook getoets vir die ses mutatsies. Hoë Resolusie Smelt analise (HRS) is gebruik om nuwe DNS volgorde veranderinge in die *MT-RNR* geen te identifiseer. Die hele mitochondriale genoom is blootgestel aan DNA volgorde bepaling in 'n poging om nuwe DNS volgorde verandering in die genoom te identifiseer wat moontlik betrokke kan wees by aminoglikosied-geinduseerde gehoor verlies. 'n Total van 97 lede van 'n Suid Afrikaanse familie waar die A1555G mutasie teenwoordig is, is deur middle van die SNaPshot metode gegenotipeer. Verder is die normale funcitoneering van die mitochondrion in getransformeerde witbloed selle, getoets in die teenwoordigheid van verskillende konsentrasies streptomycin met behulp van die MTT kleurmetrie toets. Deteksie van heteroplasmiese mutasies is gedoen deur middle van die PCR-RFLP tegniek en alle analises is gedoen op die UN-SCAN-IT program.

Ons was suksesvol in die ontwikkeling van 'n vinnige, koste effektiewe en kragtige tegniek wat al ses die mutasies in *MT-RNR1* in een reaksie kan optel. Hierdie tegniek het goed gewerk met DNA monsters van bloed en van selle verkry vanuit die wangholte (geneem van kinders jonger as 12 jaar). Die C1494T, T1095C en T1291C mutasies is glad nie waargeneem in enige van ons MDR-TB patiente of kontroles nie. Skrikwekkend is die hoë frekwensie (0.9%) waarby die A1555G mutasie in die Swart kontrole groep waargeneem is. Hierdie mutasie is ook in 1.1% van die Afrikaner kontrole groep opgemerk in heteroplasmie van 25%. Die A827G mutasie was teenwoordig in 0.9% en 1.1% van die MDR-TB patiente en Afrikaner kontrole monsters, onerskeidelik. Die 961delT + insC$_{(n)}$ mutasie is opgemerk in baie hoë frekwensies in beide die MDR-TB (3.5%) en kontrole groepe (1.1% van die Afrikaner, 1.5% van die Kleurling en 7.1% van die Swart monsters). Die T961G mutasie is ook in hoë frekwensies in slegs die Blanke (2.9%) en die Afrikaner (3.2%)

kontrole groepe waargeneem. Nuwe DNS volgorde veranderinge in *MT-RNR1* is gesoek in 'n groep MDR-TB patiente wat gehoor verlies ondervind. Slegs twee nuwe verandering is ontdek (G719A en T1040C). Dit is onwaarskynlik dat hierdie veranderinge patogenies is siende dat hulle teen frekwensies van 20.7% en 1.8% waargeneem is in die Kleurling en Swart kontrole groepe onderskeidelik. Tydens die soeke na nuwe DNS volgorde veranderinge wat moontlik geassosieer is met aminoglikosied-geinduseerde gehoor verlies in die mitochondriale genoom is 50 onbekende veranderinge ontdek (een in die *MT-TH, ND3, COX3* en twee in die *CYTB* gene). Die veranderinge is verder ondersoek vir evolusionêre konservasie op beide die nukliotied en amino suur vlak van mens to padda. Dit is bevind dat 76 uit die 97 familie lede positief is vir die A1555G mutasie en het dus 'n hoë risiko om aminoglikosied-geinduseerde gehoor verlies te ontwikkel as hul bloot gestel word aan hierdie antibiotikums. Verder is gevind dat hierdie familie op die L0d mitochondriale haplogroep lê. Geen van die sogenaamde genetiese modifiseerde gene of DNS volgorde veranderinge in hierdie gene (tRNA$^{Ser(UCN)}$, A10S in *TRMU* in homosigotiese vorm en die 35delG in *GJB2)* is gevind in die familie nie. Die MTT toets het 'n afname in die mitochondriale funksioneering van selle waar die A1555G mutasie teenwoordig was getoon, alhoewel die verskil tussen selle wat nie die A1555G mutasie het nie, nie statisties betekenisvol was nie (p-waarde: 0.615-0.999).

Die hoë frekwensie van die A1555G mutasie (0.9%) in die Swart populasie van Suid Afrika is skrikwekkend siende dat die voorkomssyfer van MDR-TB in hierdie groep baie hoog is. Toekomstige studies met grooter getalle is nodig om die ware frekwensie van die mutasies geassosieer met aminoglikosied-geinduseerde gehoor verlies in die algemende Suid Afrikaanse populasie te bepaal. Ons data dui aan dat die 961delT + insC$_{(n)}$ en die T961G mutasies slegs algemene nie-patogeniese polimorphismis is siende dat dit in sulke hoë frekwensies (>1%) in kontroles opgemerk is. Die identifiseering van die eerste DNS volgorde veranderinge in proteïen kodeerende gene wat moontlik geassosieer is met aminoglikosied-geinduseerde gehoor verlies hou groot en belowende moontlikehede in, interme van die identifiseering van 'n tweede geen. Toekomstige studies waarin die effek van hierdie veranderinge op die normale funktioneering van hierdie gene ondersoek word sal 'n besondere groot bydrae lewer tot hierdie veld van navorsing. Al 76 van die A1555G positiewe familie lede is voorsien van genetiese verslae en het berading ontvang in verband met hul risiko en die risiko van hul kinders om aminoglikosied-geinduseerde gehoor verlies te ontwikkel.

Die ontwikkeling van 'n kragtige, vinnige en koste-effektiewe genetiese metode vergemaklik die vinnige identifiseering van hoë risiko individue vir die ontwikkeling van gehoor verlies voordat hulle met hul aminoglikosiede behandeling begin. Dit is veral noodsaaklik in 'n derde wêreld land soos Suid Afrika waar, ten spyte van hul gevaarlike newe effekte, aminoglikosied antibiotikums steeds gebruik sal word. Daarom is grooter publieke bewusmaking nodig om hierdie problem te probeer oplos en te verhoed.

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Publications and Presentations arising from this study

Publications

- H.Human, D.Lombard, G.de Jong, S.Bardien, A South African family with the mitochondrial A1555G mutation on haplogroup L0d **Biochem.Biophys.Res.Commun. 382, (2009) 390-394**.

- S.Bardien, H.Human, T.Harris, G.Hefke, R.Veikondis, H.S.Schaaf, L.Van der Merwe, J.H.Greinwald, J.Fagan, G.de Jong, A rapid method for detection of five known mutations associated with aminoglycoside-induced deafness **BMC.Med.Genet. 10, (2009) 2**.

Presentations

- Oral presentation in the category Basic Sciences at the 52nd Annual Academic Year Day (2008), Stellenbosch University
- Poster presentation at the South African Society of Human Genetic (SASHG) congress 2009
- Poster presentation in the category Basic Sciences at the 53th Annual Academic Year Day (2009), Stellenbosch University - Winner of best poster in this category

List of abbreviations

%GC   Percentage Guanine and Cytosine

µg   Micrograms

µl   Microlitre

µM   Micromolar

A

ABR   Auditory Brainstem Response

AcCoA   Acetyl Coenzyme A

ADP   Adenosine diphosphate

AgNO3   Silver Nitrate

APS   Ammonium-Perox-Disulfate

A-site   Aminoacyl-tRNA binding site

ATP   Adenosine triphosphate

B

BAK   Bcl-2 antagonist killer

BAX   Bcl-2 associated X protein

BID   BH3 interacting domain death agonist

bp   Base pair

C

°C   Degrees Celsius

cDNA   Complementary DNA

cm   Centimetre

CO_2 Carbon dioxide

CPO   Chronic progressive opthalmoplegia

CYTB	Cytochrome b gene

D

dATP	Deoxy-adenosine triphosphate
dB	Decibels
dCTP	Deoxy-cytidine triphosphate
DFN	X-linked deafness locus
DFNA	Autosomal dominant deafness locus
DFNB	Autosomal recessive deafness locus
dGTP	Deoxy-guanosine triphosphate
D-Met	D-methionine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
DPOAE	Distortion product atoacoustic emissions
dTTP	Deoxy-thymidine triphosphate

E

E.coli	*Escherichia coli*
EDTA	Ethylene-diamine-tetra-acetic acid
EXO1	Exonuclease I

F

f_1	Lower frequency of the pair of eliciting stimuli
f_2	Higher frequency primary
FAD^+	Flavin Adenine Dinucleotide
FADH	Reduced Flavin Adenine Dinucleotide
Fwd	Forward

G

GJB2   Gap junction β gene

H

H$_2$O   Water
H$^+$ Hydrogen
HPLC   High-Performance Liquid Chromatography
HRM   High Resolution Melt
Hz   Hertz

I

IPTG   Isopropyl-beta-D-thiogalactopyranoside

K

K$^+$ Potassium
kb   Kilo bases

L

L   Litre
L strand   Light strand
LB   Luria-Bertani
LHON   Leber's Hereditary Optic Neuropathy
LDH   Lactate dehydrogenase

M

MDR-TB   Multi-drug resistant tuberculosis
MELAS   Encephalomyopathy with lactic acidosis and stroke-like episodes
MgCl$_2$ Magnesium Chloride
MIHL   Maternally Inherited Hearing Loss
Min   Minutes
mm   millimetre

mRNA	Messenger Ribonucleic acid
MT	Mutant
MT-ATP6	ATP synthase F0 subunit 6 gene
MT-ATP8	ATP synthase F0 subunit 8 gene
MTb	Mycobacterium tuberculosis
MT-CO1	Mitochondrially encoded cytochrome c oxidase I gene
MT-CO2	Mitochondrially encoded cytochrome c oxidase II gene
MT-CO3	Mitochondrially encoded cytochrome c oxidase III gene
MT-ND1	NADH dehydrogenase 1 gene
MT-ND2	NADH dehydrogenase 2 gene
MT-ND4	NADH dehydrogenase 4 gene
MT-ND5	NADH dehydrogenase 5 gene
MT-ND6	NADH dehydrogenase 6 gene
MT-RNR1	Mitochondrially encoded 12S RNA gene
MT-RNR2	Mitochondrially encoded 16S RNA gene
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MT-TA	Mitochondrially encoded tRNA alanine gene
MT-TC	Mitochondrially encoded tRNA cysteine gene
MT-TD	Mitochondrially encoded tRNA aspartic acid gene
MT-TH	Mitochondrially encoded tRNA histadine gene
MT-TP	Mitochondrially encoded tRNA proline gene
MT-TS1	Mitochondrially encoded tRNA serine 1 (UCN) gene
MT-TY	Mitochondrially encoded tRNA tyrosine gene

N

N	Number of samples
Na	Sodium
Na_2B_4O	Di-sodium tetraborate decahydrate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NAD^+	Nicotinamide Adenine Dinucleotide

NADH	Reduced nicotinamide adenine dinucleotide
ND3	NADH dehydrogenase 3 gene
ND4	NADH dehydrogenase 4 gene
ND4L	NADH dehydrogenase subunit 4L gene
ND5	NADH dehydrogenase 5 gene
ng	Nanograms
NH_4	Ammonium
NTC	Non-Template Control

O

O_2	Superoxide radical anion
O_H	Non-coding control region
O_L	Origin of replication
OMIM	Online Mendelian Inheritance in Man
OXPHOS	Oxidative phosphorylation

P

P_i	Inorganic phosphate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pmol	Picomole
POLG	Mitochondrial RNA polymerase

R

Rev	Reverse
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic acid

S

SAP	Shrimp Alkaline Phosphatase
SB	Sodium Borate
SDS	Sodium Dodecyl Sulfate
Sec	Seconds
$AgNO_3$	Silver Nitrate
SNHL	Sensorineural hearing loss
SNPs	Single Nucleotide Polymorphisms
SOD	Superoxide dismutase

T

T_a	Annealing temperature
T_m	Melting point temperature
TB	Tuberculosis
TBE	Tris-boric acid and EDTA
TEMED	Tetra-methyl-ethylene-diamine
TFAM	Mitochondrial transcription factor A
TRMU	tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase gene
tRNA	Transfer Ribonucleic Acid
$tRNA^{Gln}$	tRNA glutamine
$tRNA^{Glu}$	tRNA glutamic acid
$tRNA^{Lys}$	tRNA lycine

U

U	Unit
UV	Ultra violet

V

V	Volts

W

| WHO | World Health Organisation |
| WT | Wild Type |

X

| XDR-TB | Extensively-drug resistant tuberculosis |
| XGAL | 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside |

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Chapter 1

INTRODUCTION

This thesis deals with a specific genetic form of hearing loss caused by mutations in the mitochondrial genome. Therefore, in this chapter information regarding the auditory system and statistics on hearing loss is provided. A detailed overview on the mitochondria and mitochondrial genetics as well as the genes associated with non-syndromic form of hearing loss will be discussed in this chapter. Furthermore, a description of aminoglycoside antibiotics and aminoglycoside-induced hearing loss (the focus of this thesis) as well as the aim of this study will be provided.

1.   The Auditory System

1.1 The anatomy of the auditory system

Hearing forms part of the five senses that enable us to perceive the world around us. It is defined as the neural perception of sound energy. Hearing involves two components which includes the identification as well as the localization of sounds.

The human ear consists of three divisions: the *external* and *middle* parts are essential in transmitting sound waves whereas the *internal* ear, housing two different sensory systems, are important in the conversion of sound waves into nerve impulses and also necessary in producing a sense of equilibrium.

The external ear consists of the pinna **(Figure 1.1)** which is the visible portion of the ear that protrudes from the side of the head. The pinna collects the sound waves and channels them down the external ear canal towards the tympanic membrane (*ear drum*). The tympanic membrane is stretched across the entrance to the middle ear and vibrates when it is struck by the travelling sound waves. The middle ear is made up of a collection of three bones called the ossicles (malleus, incus and stapes) which extend across the middle ear. These bones are responsible for the conversion of the tympanic membrane vibrations into fluid movements (movement of the fluid in the cochlea due to the in and outward bending of the oval window) in the inner ear.
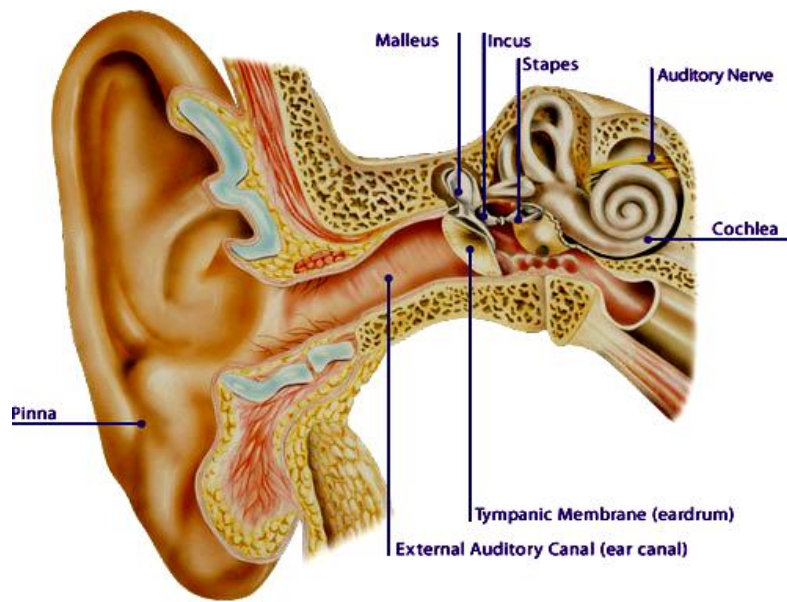
Figure 1.1: Anatomy of the human ear (www.audigygroup.com/info/mechanics.html).

The inner ear **(Figure 1.2)** is a fluid-filled system that is made up of a series of cavities located in the temporal bone. The inner ear has two functional units. The first unit is the vestibular apparatus which consists of semicircular canals and the otolith organs. The vestibular apparatus **(Figure 1.2)** is important in providing information essential for the sense of equilibrium and for coordinating movements of the head with the eyes. The second functional unit of the inner ear is the cochlea **(Figure 1.2)** which is a snail like structure and contains the sensory organ of hearing.
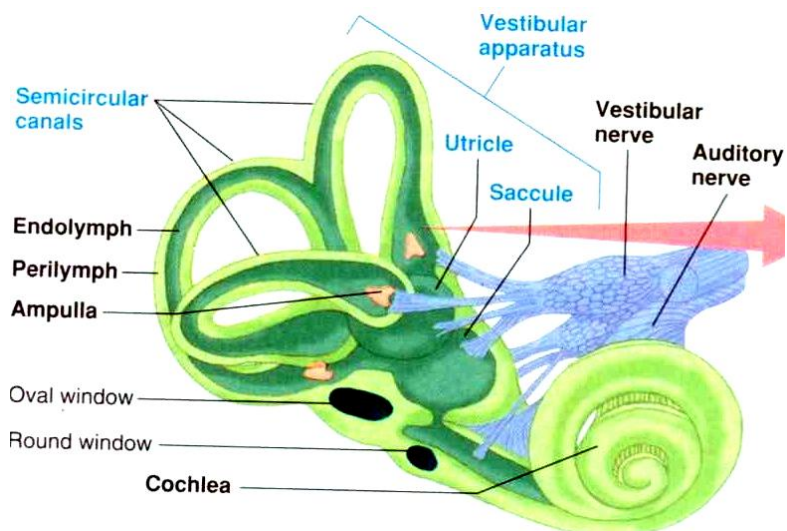
Figure 1.2: Anatomical structure of the inner ear (Taken from Sherwood, 2004b).

The cochlea is made up of three fluid-filled longitudinal compartments **(Figure 1.3a)**. The middle compartment (Scala media) tunnels through the centre of the cochlea and is filled with a fluid known as endolymph. The upper and lower compartments are known as the Scala vestibuli and Scala tympani, respectively and are both filled with a fluid known as perilymph. The endolymph and perilymph fluid have different ionic concentrations and are responsible for the generation of the endochochlear potential that is needed for the transduction of sound signals.

The cochlea houses the organ of Corti **(Figure 1.3b and c)** which is known as the sense organ of hearing. The organ of Corti is positioned on the basilar membrane in the Scala media and is made up of approximately 16 000 hair cells known as the receptors of sound. The hair cells found on the basilar membrane is divided into one row of inner hair cells and three rows of outer hair cells **(Figure 1.3c)**.
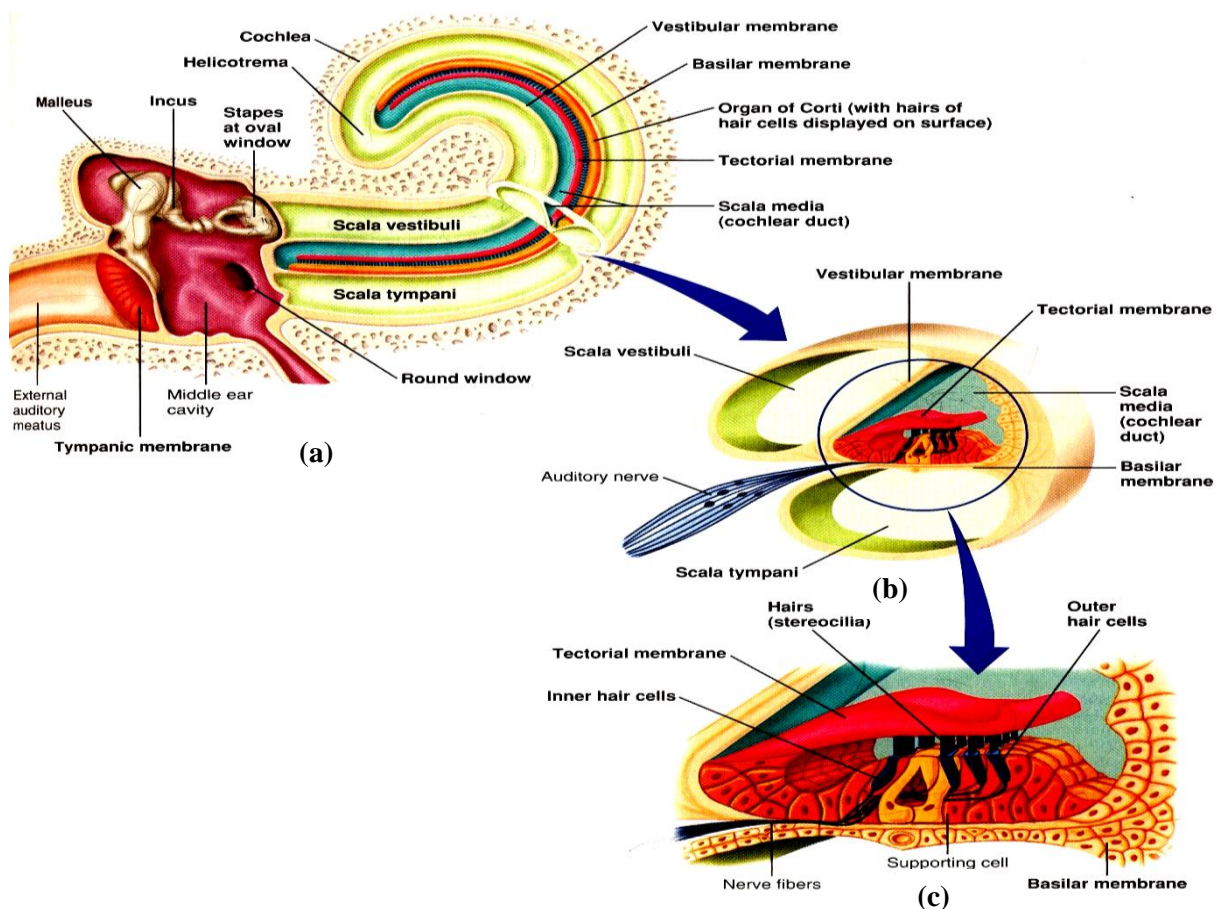
Figure 1.3: Structural view of the middle ear and the cochlea. (a) Anatomy of the middle ear and the "unrolled" cochlea. (b) Cross section of the cochlea. (c) Organ of Corti (Taken from Sherwood, 2004a).

There are approximately 100 hairs (stereocilia) protruding from each of the 16 000 hair cells, responsible for the generation of neural signals in response to mechanical distortion which is associated with the fluid movements in the inner ear **(Figure 1.4)**. The neural signals are conducted to the brain where they are converted into a sound perception.
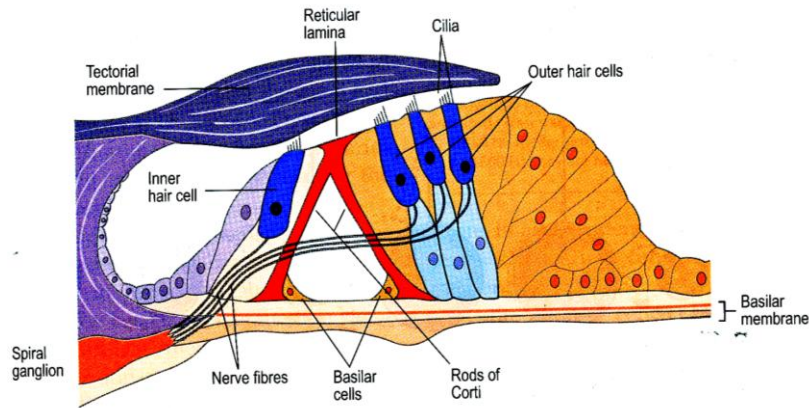
Figure 1.4: Bending of the hair cells on the deflection of the basilar membrane opens the ion channels leading to ion movements that result in a receptor potential (Taken from: Davies et al., 2001).

The inner and outer hair cells have different functions within the organ of Corti. The inner hair cells are responsible for the transformation of the mechanical forces, caused by the fluid movement in the cochlea, into an electrical impulse of hearing. The movement of the inner hair cells results in the opening and closing of mechanically gated ion channels, located in the hair cell, resulting in the generation of a receptor potential – at the same frequency as the original sound stimulus. The outer hair cells rapidly elongate in response to the changes in the membrane potential amplifying the motion of the basilar membrane thus enhancing the response of the inner hair cells (Silverthorn 1998).

1.2   Physiology of hearing

When a sound is produced, sound waves are captured by the pinna of the external ear and travel through the ear canal until they reach the tympanic membrane which then vibrates as it is struck by the sound waves **(Figure 1.5)**. Louder sounds leads to greater deflection of the membrane whereas

high frequency sounds cause the membrane to vibrate faster relating to a more high pitched sound. The vibration is transferred to the middle ear bones with the malleus connected to the tympanic membrane and the stapes connected to the oval window in the inner ear. Vibration of the stapes against the oval window creates pressure waves in the perilymph of the Scala vestibuli which is then transmitted to the endolymph in the Scala media. This results in the movement of the basilar membrane causing the organ of Corti to move against the tectorial membrane bending the hairs on the inner hair cells, The movement of the stereocilia (protruding into the endolymph with a high concentration of K^+ ions) opens the gated ion channels causing a influx of K^+ ions into the body of the hair cells, which is bathed in perilymph with a low concentration of K^+ ions. The sudden influx of K^+ ions depolarises Calcium (Ca^{2+}) channels which releases neurotransmitters into the afferent nerve fibres connected to the hair cells (Davies, Blakeley, and Kidd 2001).
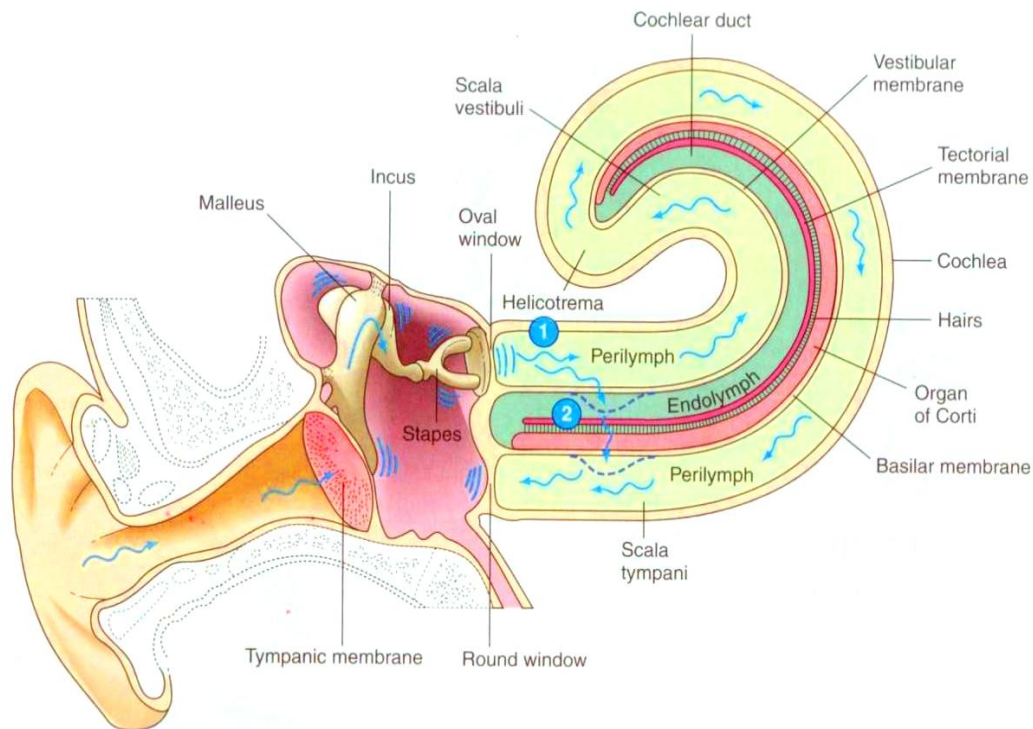
Figure 1.5: The path sound waves travel through the ear to the fluid-filled cochlea (Taken from Sherwood, 2004b).

1.3 Hearing loss

Hearing loss is one of the most common sensory disabilities that affect millions of people around the world (Morton 1991). It is estimated that approximately 1/1000 individuals are affected by some form of hearing loss, severe or profound, at birth or during early childhood development (prelingual deafness). Prelingual deafness has a dramatic effect on the speech acquisition and literacy of the child. While late onset deafness may impact on an individual's quality of life, in many cases leading to social isolation of these individuals (Petit, Levilliers, and Hardelin 2001).

1.3.1 Classification

Hearing impairment is a broad term that is generally used to describe the loss of hearing in one or both ears. There is a difference between hearing impairment and deafness. Hearing impairment refers to either complete or partial loss of the ability to hear in one or both ears. The impairment level can range from mild loss to severe or profound. Deafness refers to the complete loss of the ability to hear from one or both ears (World Health Organization, 2006).

Hearing impairment is classified into two types *conductive hearing impairment* and *sensorineural hearing impairment* depending on the part of the hearing mechanism that fails to function correctly. Conductive hearing loss arises when sound waves are not properly conducted through the external and middle portions of the ear due to anatomical abnormalities. In sensorineural deafness the sound waves are transmitted to the inner ear but they are not translated into nerve signals which are interpreted into sound sensations in the brain and this is caused mostly by inner ear defects (Sherwood, 2004b;World Health Organization, 2006).

Hearing impairment can be caused by a number of factors such as infections, head injuries, excessive noise, blockage due to wax build up or foreign bodies, old age, use of ototoxic drugs or inherited risk.

Hearing impairment can further be classified by other criteria which include severity, cause, association with other clinical manifestations and age of onset (Willems 2000). The severity of the hearing loss can be divided into four categories. Mild: with a loss of 21-40 Decibels (dB), moderate: loss of 41-60dB, severe: loss of 81-100dB and profound with a loss of more than 100dB

(Parving and Newton 1995). As mentioned previously, hearing loss may be present at birth (congenital), develop during early childhood (prelingual) or thereafter (postlingual) (Willems 2000). In most prelingual cases the hearing loss is more likely to be more severe but stable, while postlingual hearing loss is more moderate but progressive. Postlingual hearing loss affect about 10% of the population by the age of 60 years and 50% by the age of 80 years (Davies 1989; Petit 1996).

In hereditary forms of hearing loss a clear distinction can be made between *syndromic* hearing loss and *non-syndromic* hearing loss. Syndromic hearing loss is usually associated with other abnormalities that are part of another syndrome or disorder. Non-syndromic hearing loss have no additional abnormalities and may occur due to sporadic mutational events but in 80% of the cases the hearing loss is inherited in an autosomal recessive manner (Cohen and Gorlin 1995; Morton 1991).

1.3.2 South African statistics on hearing loss

The World Health Organisation (WHO) estimates that there were 278 million people worldwide with moderate to profound hearing loss in both ears in the year 2005 (World Health Organization, 2006). A total of 80% of the world's hearing impaired population live in low and middle income countries (World Health Organization, 2006). In 2004 it was estimated that approximately 26 574 to 53 148 children are born yearly with congenital hearing impairment in developed countries (UNICEF, 2006). In contrast, the incidence of congenital hearing impairment in developing countries is probably much higher due to poorer health and socioeconomic conditions (UNICEF, 2006). It is estimated that yearly there are around 718 000 infants born with, or acquire early onset permanent hearing impairment in developing countries (UNICEF, 2006). Implementing quality management strategies, prevention techniques and early diagnostic services could aid in avoiding up to 50% of all hearing loss cases (World Health Organization, 2006). There are no reports on any statistics regarding hearing impairment in South Africa or any other country in Africa. South Africa is one of the very few sub-Saharan countries with a well developed infrastructure and reasonably developed health care system. The greater percentage (85%) of South Africa's population relies on the public health sector with the remaining 15% having access to the private health care sector

(National Treasury Department 2005; Swanepoel, Storbeck, and Friedland 2009). With the exception of a single study reporting the estimated prevalence of infant hearing loss in both the private and public health sectors, no other study has been conducted on the prevalence of infant hearing loss in South Africa (Swanepoel et al. 2007). The estimated prevalence of congenital and early onset infant hearing loss for South Africa is shown in **Table 1.1**.

Table 1.1: Estimated percentages for congenital and early onset infant hearing loss in South Africa.

Health sector	Prevalence	Annual rate	Daily rate
Private (15%)[a]	3/1000	496	1.5/day
Public (85%)[a]	6/1000	5620	15.5/day
National (100%)	5.5/1000	6116	17/day

Annual rate refers to the total infants born per year and daily rate refers to the total infants born per day with congenital and early onset infant hearing loss in South Africa (Swanepoel et al. 2007).

These values are based on the UNICEF national birth rates and estimated prevalence for public and private health sectors (Swanepoel et al. 2007;UNICEF, 2009).

Hearing impairment is a disability that imposes heavy social and economic burdens on the individual, families, communities and countries. This burden disproportionally falls on the poor, as it is these people that are unable to afford the preventative and routine care that is necessary to avoid the development of hearing loss, or to afford hearing aids in order to make the disability manageable (World Health Organization, 2006).

1.3.3 Clinical evaluation

Audiologists rely on a wide range of tests to evaluate hearing impairment. These tests are designed to determine the exact type and extent of the hearing loss. Some of these tests require the subject to co-operate when the hearing is tested (subjective tests) and other tests measure the hearing without any participation of the subject (objective tests). Audiometry is the most basic test used to test for

hearing loss. Other tests are used to determine to what extent the hearing loss affects the person's ability to understand speech and to determine whether the hearing loss is sensorineural, conductive or mixed.

Audiometry

Audiometric tests are used to determine a person's ability to understand speech and detect pure tones of various frequencies. A pure-tone audiometry measures how well the person can hear sounds of a different pitch (frequency) and volume. Frequency is measured in Hertz (Hz) and volume measured in decibels (dB). Most speech sounds are in the range of 500-4000Hz and ranges from 10 and 20dB. The results of an audiometry are recorded on a graph known as an audiogram **(Figure 1.6)**.
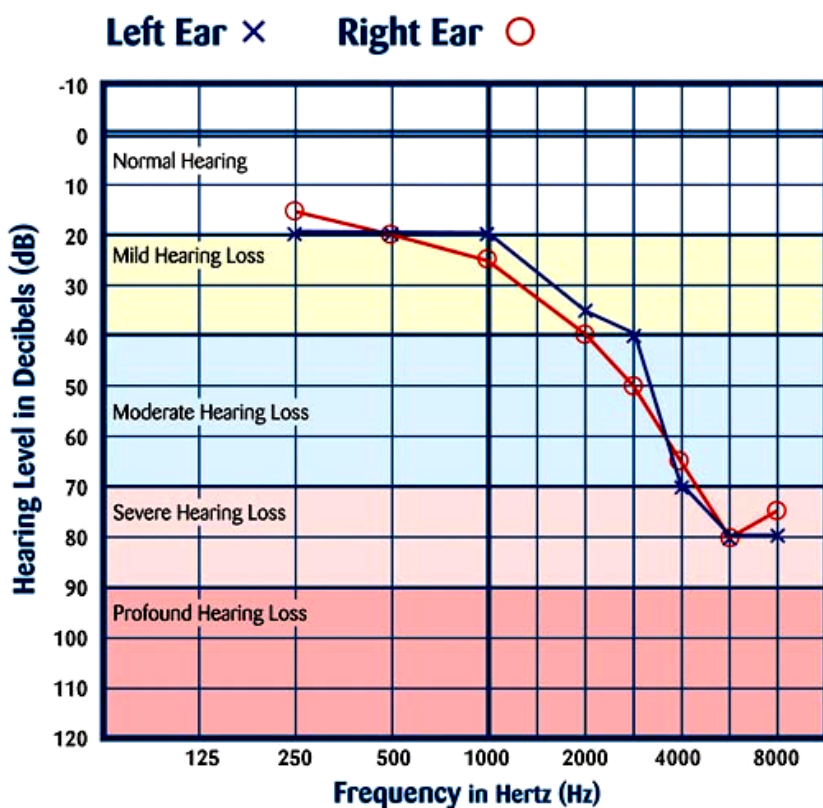
Figure 1.6: An audiogram illustrating the range of hearing across different frequencies and volumes. Both ears are plotted separately on one graph. Normal hearing is considered to be between 250 and 8000 Hz at volumes of 20dB or less (http://baasnotes.com)

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1.3.4. Causes of hearing loss

The causes of hearing loss can be multifaceted due to the complex process of hearing itself and can be caused by environmental factors, genetic defects or a combination of both.

Most, if not all, forms of syndromic deafness have a genetic origin with the exception of embryopathies due to rubella, toxoplasmosis or viral infections that can lead to polymalformations including hearing loss (Gorlin 1995). A number of different syndromes and various other disorders have hearing loss as one of the clinical phenotypes. These include disorders of the cardiovascular, nervous, endocrine and digestive systems (Gorlin 1995).

Non-syndromic hearing loss can be caused by either environmental or genetic factors. Hereditary forms of non-syndromic hearing loss was first reported in 1621 by Johannes Schenck (Stephans 1985). In developed countries, where prelingual non-syndromic hearing loss is mostly present as sporadic cases, a third of the cases have a genetic origin (Denoyelle et al. 1999) whereas environmental factors such as prematurity, neonatal hypoxia, pre or postnatal infections, trauma, tumours and exposure to ototoxic drugs are responsible for another third of all non-syndromic forms of hearing loss. However for the remaining third the aetiology remains unknown, though increasing evidence show that non-syndromic forms of hearing loss may be influenced by certain genetic predispositions or susceptibilities (Bitner-Glindzicz 2002; Tekin, Arnos, and Pandya 2001).

1.4 Genetics causes of hearing loss

Given the functional specificity of the auditory system and its sensitivity it is not surprising that it is estimated that approximately 1% of all human genes (approximately 300 genes) are involved in the hearing process. It is therefore these genes that are the potential candidates to bring about hearing impairment when mutated (Friedman and Griffith 2003).

There are several hundred forms of syndromic deafness for which the underlying genetic defect has been identified (Gorlin 1995; van Camp and Smith 2008). However, the vast majority of genetic hearing loss is non-syndromic (Morton 2002). Non-syndromic forms of hearing loss have been mapped to over 105 loci and have further been characterised into their mode of inheritance: a)

autosomal recessive – 77% of cases; b) autosomal dominant - 22% of cases; c) X-linked – 1%; d) mitochondrial in less than 1% of the cases (Morton 1991).

Generally, patients with autosomal recessive hearing impairment have prelingual, congenital hearing loss, whereas patients with autosomal dominant hearing impairment have postlingual, progressive hearing loss. This may possibly be explained by the fact that there is a complete absence of functional protein in recessive disorders, while with autosomal dominant disorders the dominant mutation may be consistent with an initial functioning protein with subsequent hearing impairment due to increasing progressive degeneration of the protein (Morton 2002).

1.4.1 Connexin 26 and inherited non-syndromic hearing loss

In the last eleven years many forms of non-syndromic deafness have been localized to the human nuclear genome by genetic linkage studies. The different loci have been named depending on the pattern of inheritance of the deafness, DFNA (autosomal dominant), DFNB (autosomal recessive) and DFN (X-linked). All these loci have been numbered chronologically in order of discovery and all forms of hereditary deafness have been summarized on the Hereditary Hearing Loss Homepage (Connexin-deafness homepage http://davinci.crg.es/deafness, 2009). The different genes and loci identified for non-syndromic hearing loss are indicated in **Table 1.2**.

Table 1.2: Nuclear loci and genes responsible for non-syndromic hearing loss.

AUTOSOMAL DOMINANT LOCI			AUTOSOMAL RECESSIVE LOCI		
Locus name	**Location**	**Gene**	**Locus name**	**Location**	**Gene**
DFNA1	5q31	DIAPH1	DFNB1	13q12	GJB2
DFNA2	1p34	GJB3 / KCNQ4	DFNB2	11q13.5	MYO7A
DFNA3	13q12	GJB2 / GJB6	DFNB3	17p11.2	MYO15A
DFNA4	19q13	MYH14	DFNB4	7q31	SLC26A4
DFNA5	7p15	DFNA5	DFNB5	14q12	ni
DFNA6/DFNA14	4p16.3	WFS1	DFNB6	3p14-p21	TMIE
DFNA7	1q21-q23	ni	DFNB7/DFNB11	9q13-q21	TMC1
DFNA8/DFNA12	11q22-24	TECTA	DFNB8/DFNB10	21q22	TMPRSS3
DFNA9	14q12-q13	COCH	DFNB9	2p22-p23	OTOF
DFNA10	6q22-q23	EYA4	DFNB12	10q21-q22	CDH23
DFNA11	11q12.3-q21	MYO7A	DFNB13	7q34-36	ni
DFNA13	6p21	COL11A2	DFNB14	7q31	ni
DFNA15	5q31	POU4F3	DFNB16	15q21-q22	STRC
DFNA16	2q24	ni	DFNB17	7q31	ni
DFNA17	22q	MYH9	DFNB18	11p14-15.1	USH1C
DFNA18	3q22	ni	DFNB19	18p11	ni
DFNA19	10 (pericentr.)	ni	DFNB20	11q25-qter	ni
DFNA20/DFNA26	17q25	ACTG1	DFNB21	11q	TECTA
DFNA21	6p21	ni	DFNB22	16p12.2	OTOA
DFNA22	6q13	MYO6	DFNB23	10p11.2-q21	PCDH15
DFNA23	14q21-q22	ni	DFNB24	11q23	ni
DFNA24	4q	ni	DFNB25	4p15.3-q12	ni
DFNA25	12q21-24	ni	DFNB26	4q31	ni
DFNA27	4q12	ni	DFNB27	2q23-q31	ni
DFNA28	8q22	TFCP2L3	DFNB28	22q13	TRIOBP
DFNA30	15q25-26	ni	DFNB29	21q22	CLDN14
DFNA31	6p21.3	ni	DFNB30	10p12.1	MYO3A
DFNA32	11p15	ni	DFNB31	9q32-q34	WHRN
DFNA34	1q44	ni	DFNB32	1p13.3-22.1	ni
DFNA36	9q13-q21	TMC1	DFNB33	9q34.3	ni
DFNA37	1p21	ni	DFNB35	14q24.1-24.3	ni
DFNA38	3q22	ni	DFNB36	1p36.3	ESPN
DFNA39	4q21.3	DSPP	DFNB37	6q13	MYO6
DFNA40	16p12	ni	DFNB38	6q26-q27	ni
DFNA41	12q24-qter	ni	DFNB39	7q11.22-q21.12	ni
DFNA42	5q31.1-q32	ni	DFNB40	22q	ni
DFNA43	2p12	ni	DFNB42	3q13.31-q22.3	ni
DFNA44	3q28-29	ni	DFNB44	7p14.1-q11.22	ni
DFNA47	9p21-22	ni	DFNB46	18p11.32-p11.31	ni
DFNA48	12q13-q14	MYO1A	DFNB47	2p25.1-p24.3	ni
DFNA49	1q21-q23	ni	DFNB48	15q23-q25.1	ni
DFNA50	7q32	ni	DFNB49	5q12.3-q14.1.	ni
DFNA51	9q21	ni	DFNB50	12q23	ni
DFNA52	4q28	ni	DFNB51	11p13-p12	ni
DFNA53	14q11-q12	ni	DFNB53	6p21.3	COL11A2
DFNA54	5q31	ni	DFNB55	4q12-q13.2	ni
			DFNB57	10q23.1-q26.11	ni
X-LINKED LOCI			DFNB58	2q14.1-q21.2	ni
Locus name	**Location**	**Gene**	DFNB59	2q31.1-q31.3	PJVK
DFN1	Xq22	TIMM8A	DFNB60	5q22-q31	ni
DFN2	Xq22	ni	DFNB62	12p13.2-p11.23	ni
DFN3	Xq21.1	POU3F4	DFNB65	20q13.2-q13.32	ni
DFN4	Xp21.2	ni	DFNB66	6p21.2-22.3	LHFPL5
DFN6	Xp22	ni			

ni; non identified

(Taken from: van Camp and Smith, 2008)

Many of the types of hearing impairment listed in **Table 1.2** are rare with exception of DFNB1. DFNB1 is an autosomal recessive form of deafness and is caused by mutations in the Gap junction β 2 gene (*GJB2*) encoding Connexin 26 (Kelsell et al. 1997). This form of non-syndromic deafness is usually prelingual with the severity varying from mild to more profound that may to some extent depend on the type of mutation (Denoyelle et al. 1999).

The *GJB2* gene was shown to be the major contributor to autosomal recessive forms of deafness (Kelsell et al. 1997). *GJB2* is found on chromosome 13q11 and is approximately 5.5kb in length. It consist of 2 exons of which only exon 2 is transcribed (Denoyelle et al. 1999; Kelsell et al. 1997). *GJB2* codes for the Connexin 26 gap junction protein that belongs to a family of membrane proteins (Guilford et al. 1994). Each protein consists of four transmembrane domains (Kumar and Gilula 1996). A cluster of six connexins together embedded in the cell membrane forms a complex called a connexon (a hexamer). Two connexons of adjacent cells form a structure called a gap-junction (cell-to-cell channel), which is responsible for ion movement in and out of the cell as well as between cells **(Figure 1.7)** (Bruzzone, White, and Paul 1996; Kikuchi et al. 1994).
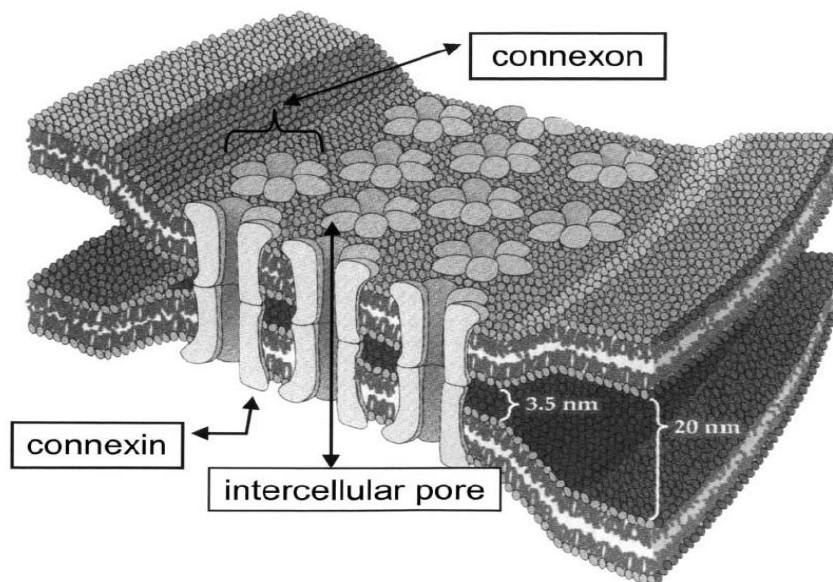
Figure 1.7: Schematic representation of a typical gap junction. Six connexins forms a connexon with two connexons of adjacent cells forming a gap junction allowing intercellular transport of ions (Taken from: Kemperman et al., 2002).

Connexin 26 is found mainly in the cochlea of the ear, where it has been hypothesized to play a role in Potassium (K^+) recycling between the hair cells and the supporting cells and is thus ultimately important for the regeneration of neural signals to the brain **(Figure 1.8)** (Kikuchi et al. 1994).

Expression profile of various proteins

(grey)	TECTA and COL11A2
(blue)	MYO7A, POU4F3, and KCNQ4
(red)	ATP6B1
(green)	PDS
(purple)	KVLQT1 and KCNE1
(orange)	OTOF and KCNQ4
(pink)	GJB2 and GJB3
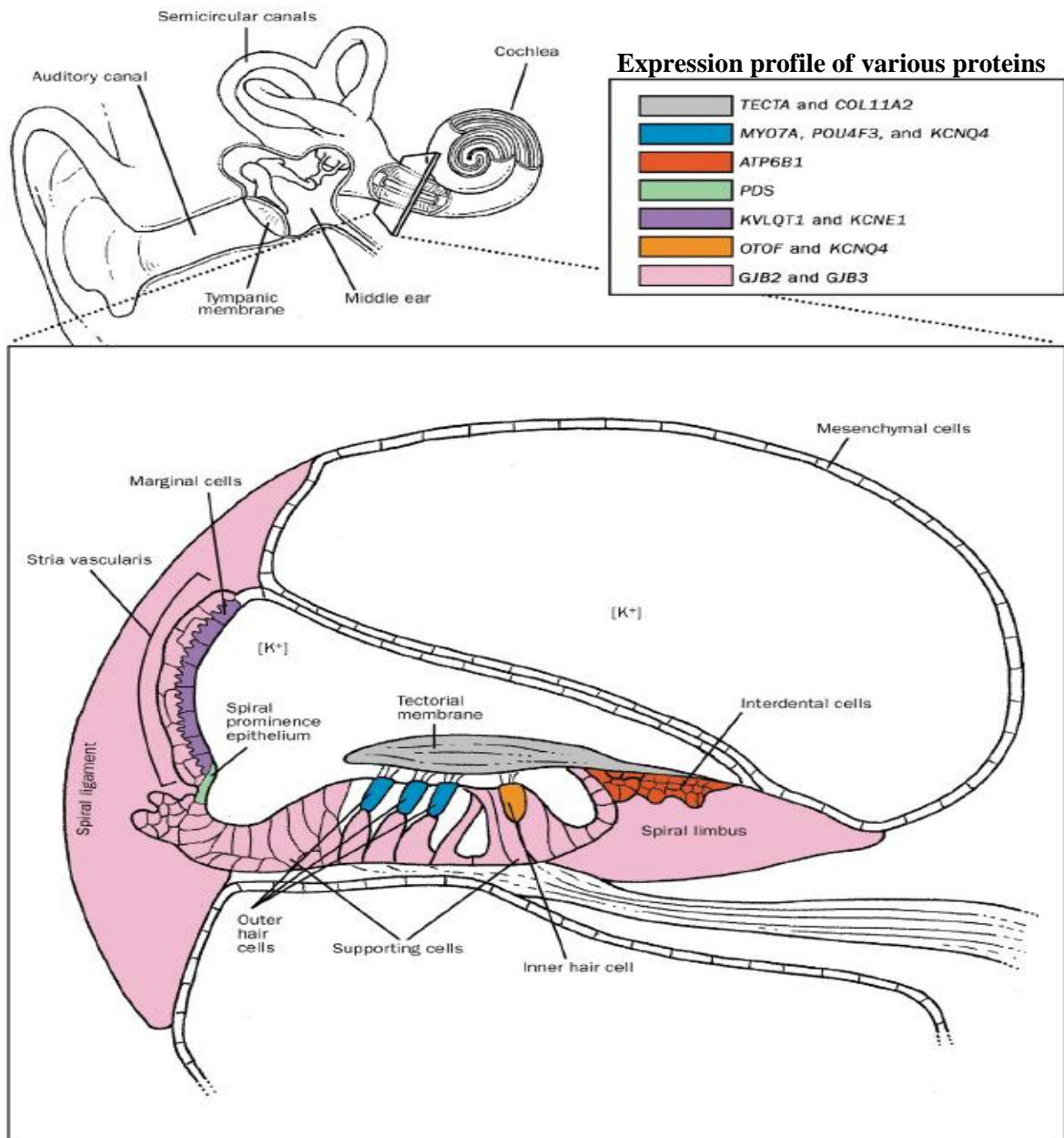
Figure 1.8: Schematic representation of the K^+ recycling pathway and expression of connexin 26 (*GJB2*) in a cross section of the human cochlea. Parts of the cochlea where connexin 26 are expressed are shown in pink (Taken from: Tekin et al., 2001).

Mutations in *GJB2* result in an altered functioning of the gap junctions thereby leading to hearing loss by reducing the cycling of the K^+ ions in the hair cells that will influence the generation of neural signals to the brain (Kemperman, Hoefsloot, and Cremers 2002). There have been several pathogenic mutations and polymorphisms identified in *GJB2* (Connexin-deafness homepage http://davinci.crg.es/deafness, 2009). One of the most common mutations found is the 35delG mutation which is a deletion of a guanine residue in an area of six G's at position 35 of the cDNA (Carrasquillo et al. 1997; Morell et al. 1998). This deletion causes a premature stop codon which results in a severely truncated protein, from 226 amino acids to only 13 amino acids in length. As a result the shortened protein will not be inserted into the cell membrane and consequently no gap-junctions between cells will be produced (Lee et al. 1992; Zelante et al. 1997). 35delG is found in very high frequencies in the Caucasian population and accounts for up to 75-80% of all autosomal recessive deafness cases related to *GJB2* (Gasparini et al. 2000; Morell et al. 1998). The 35delG mutation can cause mild to profound hearing loss (Murgia et al. 1999). Phenotype-genotype correlation studies showed that individuals homozygous for the 35delG mutation had more severe hearing loss than in the compound heterozygous individuals (Cryns et al. 2004).

Besides the 35delG mutation in connexin 26, as many as 60 more mutations have been identified in this gene alone. The 167delT has been detected at a frequency of 4% in the Ashkenazi Jewish population (Morell et al. 1998). The 235delC mutation has only been detected in the Oriental populations in relatively high frequencies (Abe et al. 2000).

Taking into consideration the high frequency of the 35delG mutation of up to 40% in familial and sporadic patients (Estivill et al. 1998a) there is a need for a rapid screening method that is easy, cost effective and high throughput, so that it may be used in diagnostic setting to detect this mutation. Presently there is a selection of different methods used in diagnostic settings to detect the 35delG mutation of which most are expensive, labour intensive or not suitable for high-throughput diagnostic screening such as restriction fragment length polymorphism (Baris, Koksal, and Etlik 2004), multiplex PCR followed by direct sequencing (Wu et al. 2003), allele specific oligo-nucleotide hybridization (Dong et al. 2001), high-performance liquid chromatography (HPLC) (Lin et al. 2001) and PCR-mediated site-directed mutagenesis (Storm et al. 1999).

To identify the cause of deafness in infants soon after birth, or the identification of carrier parents would assist families in the modification of the child's educational process as well as the

development of better communication skills to accommodate the child's specific needs (Estivill et al. 1998a).

2.   The Mitochondria and Mitochondrial Disease

The first disease caused by mitochondrial dysfunction was described by Luft in 1962 (Luft et al., 1962).   Since then mitochondrial dysfunction has become an important area in human pathology including **hearing loss** as it has been shown to play a pivotal role in the process of hearing. In 1988, the first mutations in mitochondrial DNA were discovered and since then understanding the role of mitochondrial DNA in certain diseases has developed rapidly (Holt, Harding, and Morgan-Hughes 1988; Wallace et al. 1988). To date, more than a 100 mitochondrial point mutations and 200 deletions and rearrangements have been identified in a variety of diseases that affect the skeletal muscles, brain, liver, heart, and the cochlea (Brown and Wallace 1994; Wallace 1992).   The discovery of these mutations stimulated researchers to understand the functions, and different components of the mitochondria, which in turn have provided new and better insights into the way mitochondrial DNA alterations influence the functioning of the different mitochondrial components and processes resulting in pathological or age-related phenotypes.

2.1   Mitochondrial structure and function

The mitochondrion is an intracellular organelle and is present in all eukaryotic cells with exception of erythrocytes. They are responsible for the generation of more than 80% of the energy (adenosine triphosphate: ATP) required by the cell through a process known as oxidative phosphorylation (OXPHOS). However, in addition to generating cellular energy, the mitochondria also carry out other vital cellular functions which are all essential for cell survival. These include: respiration, synthesis of heme, lipids, amino acids, nucleotides, maintaining intracellular homeostasis of inorganic ions, calcium homeostasis and the initiation of apoptosis (Kroemer, Zamzami, and Susin 1997; Schatz 1995). A total of 15% of the protein in the cell is housed in the mitochondria (approximately 1000 proteins) of which only 13 of these proteins are synthesised by the mitochondria itself. The 13 mitochondrial proteins are essential components of the electron

transport chain. The remaining proteins are all encoded on the nuclear genome and transported into the mitochondrion.   These proteins are also important in the normal functioning of the mitochondrion (Schatz 1995).

2.1.1. The structure of the mitochondria

Mitochondria are small cylindrical structures of approximately 0.5-1µm in diameter (Riva et al. 2003), but the size, shape and number per cell varies greatly depending on the specific function and energy requirements of the cell (Sherwood 2004a). The basic structure of the mitochondria consists of four compartments: (1) outer membrane, (2) inner membrane, (3) intermembrane space and the (4) matrix **(Figure 1.9)** (Sherwood 2004a).
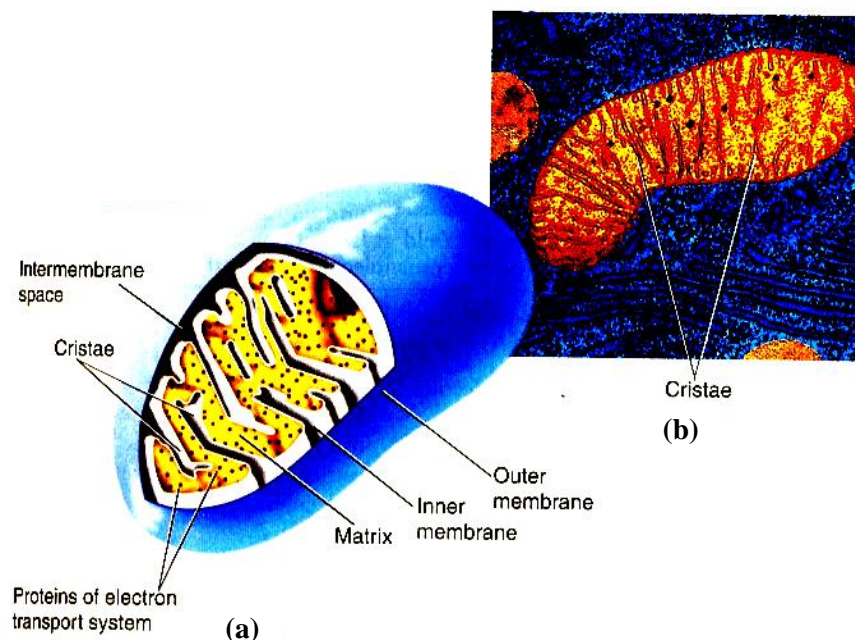
Figure 1.9:   The mitochondrion (a) Schematic representation of the structure of the mitochondria. (b) Electron micrograph of a mitochondria (Taken from: Sherwood, 2004a).

The **outer membrane** has the same composition as that of the plasma membrane and is permeable to metabolites and other small molecules due to the presence of porins (Strachan and Read 1999).

The **inner membrane** is very protein rich and contains the anionic phospholipid *cardiolipin*, which is responsible for the impermeable nature of the inner membrane to ions (Lewin 1998). The inner membrane is highly folded into structures known as *cristae*. The cristae increases the surface area of the inner membrane for better functioning of the mitochondria in the generation of cellular energy (Sherwood 2004a). Embedded within the inner membrane are the four respiratory chain complexes and the ATP synthase that are the key components of the oxidative phosphorylation system. There are also other membrane proteins embedded within the inner membrane such as metabolites and ion carriers that link the metabolism of the cytoplasm with the inner mitochondrial space (Sherwood 2004a).

Between the inner and outer membrane there is a very narrow **intermembrane space**. This is the location for enzymes such as cytochrome C that plays an important role in the initiation of apoptosis (Finsterer 2004).

The **matrix** is located inside the inner membrane and has been described as a gel-like solution as a result of the presence of various macromolecules and high concentration of proteins. The matrix is also home to the various enzymes involved in the Krebs cycle, urea cycle, fatty acid oxidation as well as mRNAs, tRNAs and the mitochondrial genome (Lewin 1998).

2.1.2. *The function of the mitochondria*

Mitochondria have many different functions of which energy production is the most important. The other functions of the mitochondria include: (a) mediation of apoptosis, (b) heat production and (c) regulation of calcium homeostasis. Only ATP production and apoptosis which is relevant for this study and will be discussed very briefly in this section. A more detailed review of the different functions of mitochondria can be found in a review by Taylor and Turnbull (2005) (Taylor and Turnbull 2005).

ATP production

As mentioned previously the main function of the mitochondria is the production of energy in the form of ATP by means of oxidative phosphorylation. Oxidative phosphorylation is the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i). The process is driven by the

free energy formed from the oxidation of food by molecular oxygen. Before the main oxidative reactions of the oxidative phosphorylation can begin, a large number of enzymatic reactions of the intermediary metabolism need to take place for the later production of ATP. All the enzymatic reactions take place within the matrix space of the mitochondria (Wikström 2003).

Metabolites from amino acids, pyruvate and fatty acids are transferred from the cytosol, where they have been metabolised by glycolysis, to the mitochondria where they are further metabolised and form acetic acid. The acetic acid combines with coenzyme A to form a compound, acetyl coenzyme A (AcCoA). AcCoA is then further metabolised through the Krebs cycle leading to the reduction of both oxidized nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD^+). After a number of cycles of the Krebs cycle, a number of hydrogen ions are transferred to the mitochondrial inner membrane by NADH (reduced nicotinamide adenine dinucleotide) and FADH (reduced flavin adenine dinucleotide) carrier molecules. The NADH and FADH molecules release their hydrogen ions within the inner membrane and are then transferred back to the matrix to acquire more hydrogen ions from the Krebs cycle. The released hydrogen ions then pass through the electron transport chain located in the inner membrane of the mitochondria. Here high energy electrons are extracted from the hydrogen. The energy is slowly released as the electrons fall to a lower energy level when they are moved through the electron transport chain. The released energy is then used to transport the hydrogen ions from the matrix to the inter membrane space. The accumulation of the hydrogen ions in the inter membrane space results in the flow of hydrogen ions from the inter membrane space to the matrix and in doing so activating ATP synthase. Activation of ATP synthase converts ADP+P_i to ATP which is then transported to the cytosol as the source of cellular energy **(Figure 1.10)** (Sherwood 2004a).
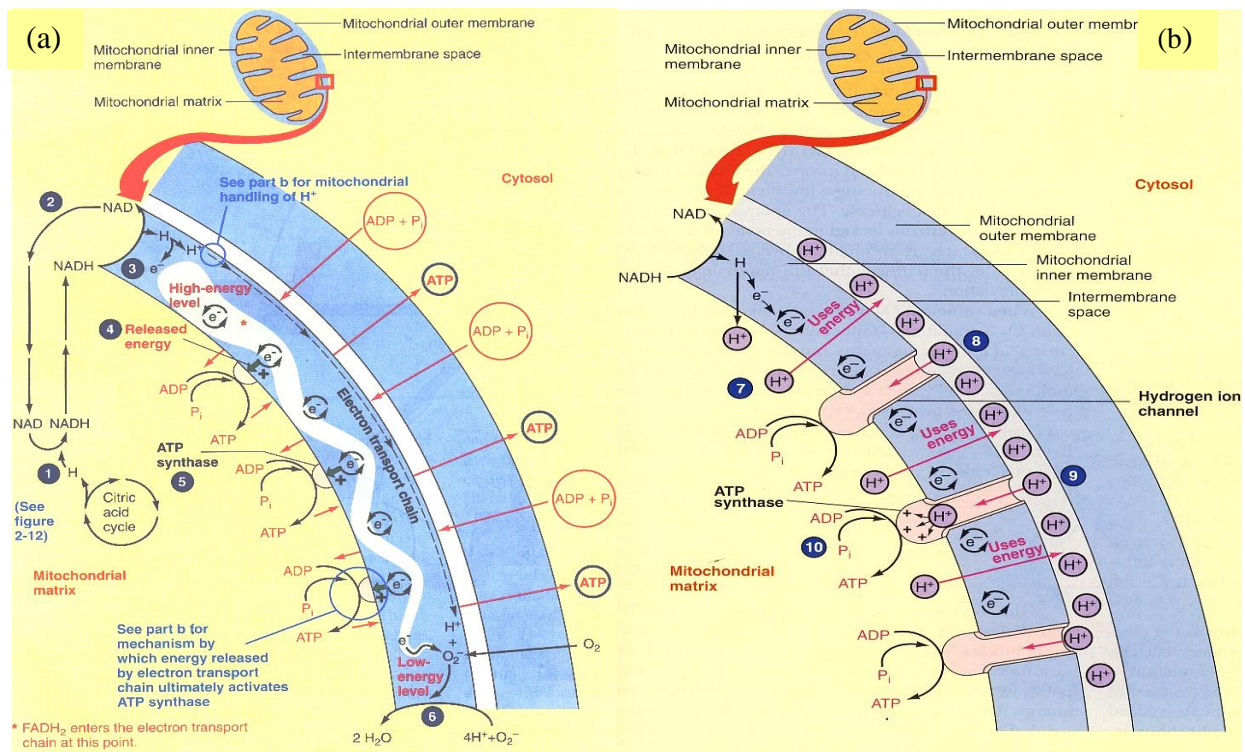
Figure 1.10: ATP synthesis by the mitochondrial inner membrane. (a) ATP synthesis as a result of the movement of high-energy electrons through the mitochondrial electron transport chain. (b) Activation of ATP synthase through the movement of H^+ from the inter membrane space to the mitochondrial matrix (Taken from: Sherwood, 2004a).

Apoptosis

The mitochondria play a pivotal role in activating the apoptotic pathway in response to cellular dysfunction. A number of key events in apoptosis take place within the mitochondria. There are 3 main mechanisms through which the mitochondria are able to control cell death: (1) disruption of the electron transport chain, oxidative phosphorylation and ATP production, (2) protein release that trigger the activation of the caspase family proteases and (3) alteration of cellular redox potential (Green and Reed 1998).

In the cell the caspases are synthesised as inactive zymogens or procaspases. The procaspase family of proteins can be divided into two classes, *proapoptotic caspases* or *initiator procaspases*-2, -8, -9, and -10 and the group of *executioner caspases* -3, -6, and -7. The caspases are the central initiators and executioners of apoptosis. There are two main pathways in which the caspases activates

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apoptosis; *extrinsic apoptosis pathway* involves the activation of the initiator procaspases-8 in response to the ligation of cell surface death receptors. The intrinsic apoptosis pathway involves the activation of the initiator procaspases-9 in response to signals originating from inside the cell.

Therefore, when the cell encounters a specific death inducing signal such as the ligation of a death receptor or exposure to cytotoxic drugs the apoptotic signalling pathway is activated (Wang 2001). This pathway is regulated by certain antiapoptotic molecules and repression of proapoptotic factors (Ameisen 2002; Raff et al. 1993). One of the most important proapoptotic factors is the Bcl-2 family of proteins which include the following group of proteins, Bcl-2 associated X protein (BAX), Bcl-2 antagonist killer (BAK), BH3 interacting domain death agonist (BID) to name a few (Cory and Adams 2002). The antiapoptotic Bcl-2 members inhibit the activation of the proapoptotic Bcl-2 members by binding to their BH3 domains (Reed 1998). Activation of certain proapoptotic Bcl-2 members (BID, BAX, BAK) increases the permeability of the mitochondrial outer membrane allowing the release of Cytochrome C into the cytosol (Cory and Adams 2002; Luo et al. 1998). Release of Cytochrome C activates the formation of the apoptosome and subsequent activation of procaspase-9 (Acehan et al. 2002; Slee et al. 1999). Activated caspases-9 initiates the caspase cascade that involves other downstream effector caspases such as caspases-3, -7, and -6, ultimately leading to apoptosis of the cell (Slee et al. 1999).

2.2. Mitochondrial genetics

The mitochondrion contains its own genome and is the only other location of extra-chromosomal DNA except for the chloroplasts (Taylor and Turnbull 2005). The mitochondrial genome is completely independent from the nuclear genome. It was first discovered in 1963 (Nass and Nass, 1963) and about 20 years later the complete mitochondrial genomes of mouse, bovine and human was sequenced (Anderson et al. 1981; Anderson et al. 1982; Bibb et al. 1981). These milestones led the way for the subsequent sequencing of the nuclear genome.

The mitochondrial genome is a small, circular double stranded molecule. A typical eukaryotic cell contains on average 500-2000 mitochondria with each mitochondrion having 2-10 mitochondrial chromosomes in their matrix. Therefore each cell contains at least 1000-4000 copies of the mitochondrial genome (Naviaux 2000).

2.2.1. *Mitochondrial genes*

The human mitochondrial genome consists of 16 569 base pairs arranged in circular double stranded DNA molecule. The 2 strands are known as the heavy (H) strand and the (L) strand based on the G+C base composition of each strand **(Figure 1.11)** (Andrews et al. 1999; Kokotas, Petersen, and Willems 2007). The method by which mitochondrial DNA replicate is still not clear. One mode of replication suggests that the replication takes place as a strand displacement model, where replication of the H strand occurs first initiating at a specific site in the non-coding control region known as the O_H site. When the replication on the leading strand is two thirds of the way the origin of replication (O_L) on the L strand is exposed, L strand replication can begin in the opposite direction **(Figure 1.11)** (Clayton 1982). This mode of replication have been heavily debated, and other modes of replication have been suggested in which the leading-lagging strand replication are a coordinated process (Bowmaker et al. 2003; Holt, Lorimer, and Jacobs 2000; Yang et al. 2002).

Mitochondrial DNA transcription is much better understood than the replication process. Transcriptional initiation can occur on both strands of human mitochondrial DNA. During transcription the mitochondrial promoters produce a polycistronic precursor RNA that is processed to produce tRNA and mRNA molecules (Clayton 1991; Ojala, Montoya, and Attardi 1981). Transcription is initiated by the mitochondrial RNA polymerase (POLG), this polymerase requires mitochondrial transcription factor A (TFAM) and either mitochondrial transcription factor B_1 or B_2 (Falkenberg et al. 2002; Fernandez-Silva, Enriquez, and Montoya 2003).
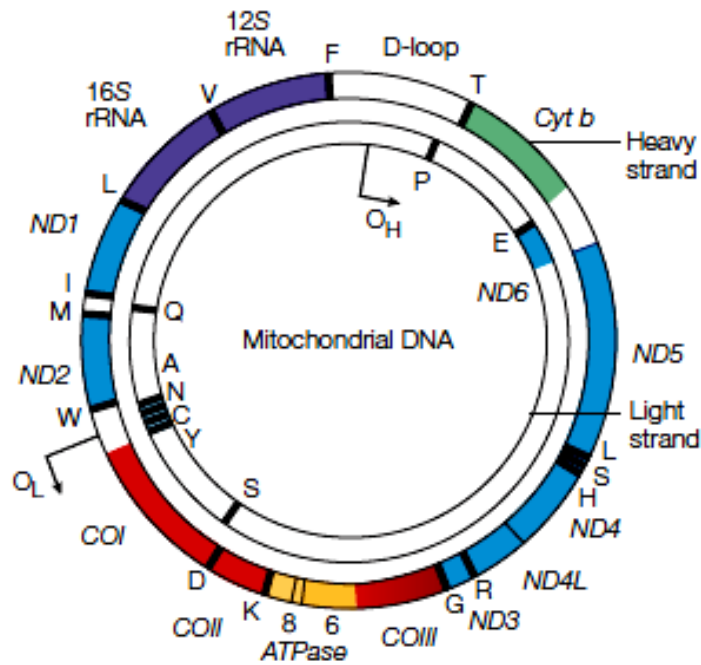
Figure 1.11: A map of the human mitochondrial genome. The heavy strand is located on the outside of the circular genome with the light strand on the inside of the genome. The genes encoded by the mitochondrial genome are indicated in different colors. The two ribosomal RNAs (16S rRNA & 12S rRNA) are shown in purple and the 22 transfer RNAs are indicated by the black lines. The 13 genes involved in the oxidative phosphorylation system are indicated in the blue, red, green and yellow sections (Taken from: Taylor and Turnbull, 2005).

The mitochondrial genome codes for 37 genes: 13 polypeptides, 2 rRNAs and 22 tRNAs that are necessary for translation. The 13 proteins encoded by the mitochondrial genome are key components of the respiratory chain. All these genes are located close together and are intronless (Kokotas, Petersen, and Willems 2007). The D-loop region is the only non-coding region in the genome. This region contains promoters for transcription and initiation sites for replication (Clayton 1992). The D-loop region is the most polymorphic region in the mitochondrial genome (MITOMAP: A Human Genome Database http://www.mitomap.org).

2.2.2. Inheritance of mitochondrial DNA

Mitochondrial genetics is different from Mendelian genetics in most aspects **(Table 1.3)** (Strachan and Read 1999).

Table 1.3: Comparison between the mitochondrial genome and the human nuclear genome.

Characteristic	Nuclear genome	Mitochondrial genome
Size	~3.3 x 10^9 bp	16,569 bp
Number of DNA molecules per cell	23 in haploid cells; 46 in diploid cells	Several thousand copies per cell (polyploidy)
Number of genes encoded	~20,000–30,000	37 (13 polypeptides, 22 tRNAs and 2 rRNAs)
Gene density	~1 per 40,000 bp	1 per 450 bp
Introns	Frequently found in most genes	Absent
Percentage of coding DNA	~3%	~93%
Codon usage	The universal genetic code	AUA codes for methionine; TGA codes for tryptophan; AGA and AGG specify stop codons
Associated proteins	Nucleosome-associated histone proteins and non-histone proteins	No histones; but associated with several proteins (for example, TFAM) that form nucleoids
Mode of inheritance	Mendelian inheritance for autosomes and the X chromosome; paternal inheritance for the Y chromosome	Exclusively maternal
Replication	Strand-coupled mechanism that uses DNA polymerases α and δ	Strand-coupled and strand-displacement models; only uses DNA polymerase γ
Transcription	Most genes are transcribed individually	All genes on both strands are transcribed as large polycistrons
Recombination	Each pair of homologues recombines during the prophase of meiosis	There is evidence that recombination occurs at a cellular level but little evidence that it occurs at a population level

(Taken from: Taylor and Turnbull, 2005)

Homoplasmy and Heteroplasmy

In the mammalian cell the sequences of most of the copies of the mitochondrial DNA are identical at a given nucleotide position. This is a condition known as homoplasmy. Occasionally a condition known as heteroplasmy arises where there is a mixture of both wild type and mutant DNA at a given nucleotide position present in a single cell. The terms **homoplasmy** and **heteroplasmy** is important when considering mitochondrial mutations that lead to disease. (Kokotas, Petersen, and Willems 2007). Heteroplasmy is a rare condition in normal individuals, and is often only associated with mitochondrial DNA disease. Heteroplasmy rates vary within and between cells and also between tissues and organs (Naviaux 2000). The rate of heteroplasmy formation is a product of random

segregation between wild type and mutant DNA occurring during early embryogenesis (Finsterer 2004).

Mitochondrial DNA inheritance is exclusively maternal with any copies of paternal mitochondrial DNA being eliminated upon entering the oocyte (Sutovsky et al. 1999). Therefore, mitochondrial DNA mutations can lead to disease equally in both sexes but can only be transmitted from the mother (Fischel-Ghodsian 1999). However there has been a very unique case reported in which a patient with a mitochondrial disease inherited the causative mutation from the paternal line. In this case 90% of the mitochondrial DNA in the skeletal muscle was paternally inherited but all the other tissue contained maternal mitochondrial DNA (Schwartz and Vissing 2002).

2.2.3. *Mitochondrial Disorders*

As stated in a previous section, mitochondria are essential components of all nucleated cells. As a result the genes affecting the mitochondria or mitochondrial function therefore play an important role in human health and disease. A mitochondrial disease can be due to mutations in one of the 37 mitochondrial genes or in one of the 1000 nuclear genes that encode mitochondrial proteins. Since mitochondrial-encoded proteins are involved in the OXPHOS system, all diseases caused by mutations in one of these genes will be characterized by a defective OXPHOS system (Kokotas, Petersen, and Willems 2007).

The spectrum of mitochondrial disorders caused by nuclear gene mutations is very broad. This is because the genes encoding mitochondrial proteins code for proteins involved in the regulation of the OXPHOS system, apoptosis, proteins involved in reactive oxygen species (ROS) formation and the integrity and replication of mitochondrial DNA (Kokotas, Petersen, and Willems 2007). Mitochondrial disease can either be caused by sporadic or spontaneous mutations in either mitochondrial or nuclear DNA (Finsterer 2004). These mutations can also be caused by other exogenous factors such as drugs, toxins and infections (Finsterer 2004). It has also been suggested that acquired mitochondrial DNA mutations and their role in mitochondrial dysfunction is involved in the aging process and age-related diseases (Mootha et al. 2003; Petersen et al. 2003). Inherited disorders affecting normal mitochondrial functioning lead to disease that present in childhood and

adulthood with varied clinical presentations. Mitochondrial diseases rarely affect a single organ and tend to mostly present as multisystem disorders (Finsterer 2004). Some of the most common diseases resulting from mitochondrial mutations include sensorineural hearing loss, diabetes, loss of vision, cardiomyopathy, stroke and headaches. It is usually the postmitotic and more metabolically active cells that are more affected by mitochondrial disease for example myocytes, neurons, cochlear hair cells and pancreatic cells (Hutchin and Cortopassi 2000).

Most pathogenic mutations are heteroplasmic with the exception of a few such as the homoplasmic A1555G mutation associated with non-syndromic deafness (Prezant et al. 1993). In the heteroplasmic state there is a *threshold* level of mutation (percentage of mutant versus wild type DNA) which is important in both the clinical expression of the associated disease and the biochemical defects. This threshold effect is routinely demonstrated by single cell and transmitochondrial cybrid cell studies showing the mutated form being functionally recessive to the wild type form and that the biochemical phenotype is associated with high levels of the mutation being above the crucial threshold (Sciacco et al. 1994). However for some mitochondrial diseases the phenotype is independent of the levels of mutant DNA, signifying the role of other factors in the phenotypic manifestations of the disease. These other factors can include age, environment, nuclear genes and other mitochondrial genes (Leonard and Schapira 2000).

2.2.4. Pathological mutations of mitochondrial DNA

To date, over a 100 point mutations and approximately 200 deletions and rearrangements in the mitochondrial genome have been associated with disease. These mitochondrial mutations are all archived in the human mitochondrial genome database MITOMAP (MITOMAP: A Human Genome Database http://www.mitomap.org). Mitochondrial mutations such as deletions, duplications, insertions or inversions usually involve several genes. Mutations in all of the 37 mitochondrial genes have been implicated in a number of mitochondrial diseases (Di Mauro and Schon 2001).

The mutation rate in the mitochondrial genome is up to 10 fold higher than in the nuclear genome with the virtual absence of any mitochondrial DNA repair system (Bachtrog 2003; Wallace et al. 1987). The mitochondrial genome is very susceptible to both environmental factors as well as

oxidative stress. Oxidative stress caused by excess reactive oxygen species (ROS) production, a byproduct of OXPHOS, leads to the accumulation of acquired mutations in the mitochondrial genome (Kokotas, Petersen, and Willems 2007).

As a result of the high mutation rate in the mitochondrial genome and the spontaneous occurrence of mutations, most changes are neutral polymorphisms without any clinical implications. A set of rules or guidelines have therefore been established to indicate the pathogenicity of a novel mitochondrial mutation (Di Mauro and Davidzon 2005).

(1) The mutation should not be detected in any normal individuals (controls) from the same ethnic background.

(2) The mutation should modify a mitochondrial DNA site that is evolutionary conserved indicating its functional importance.

(3) The mutation should cause deficiencies in either single or multiple OXPHOS enzymes.

(4) There should be a correlation between the level of heteroplasmy and the clinical severity of the disease.

This last criteria is debatable since the level of heteroplasmy varies between cells, between tissues and from person to person showing no obvious genotype-phenotype correlation for most diseases caused by mitochondrial DNA mutations (Di Mauro and Davidzon 2005).

There are three broad categories in which mitochondrial mutations can be classified: (1) point mutations affecting protein-coding genes, (2) point mutations affecting the protein synthesis apparatus and (3) large deletions (Jacobs 2003).

Point mutations affecting the respiratory chain and oxidative phosphorylation have been increasingly recognised. One of the most common missense mutations in the mitochondrial genome (G11778A) occurs in families with Leber's hereditary optic neuropathy (LHON). To date, several other mutations have been described and associated with LHON. These mutations occur in the *ND1, ND4* and *ND6* genes. These genes are all part of the NADH ubiquinone-oxidoreductase of complex I (McFarland, Taylor, and Turnbull 2002). Mutations in the other respiratory genes such as the ATPase genes and the *Cytochrome b* gene have also been identified and associated with a number of diseases such as Leigh syndrome, mitochondrial myopathy and mitochondrial encephalopathy (Ciafaloni et al. 1993; Legros et al. 2001).

The most common mutations in the tRNA genes have been associated with mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), diabetes mellitus, deafness and cardiomyopathy (Reardon et al. 1992; Santorelli et al. 1996). The mutations identified in one of the rRNA genes (*MT-RNR1*) have mainly been associated with non-syndromic hearing loss. These mutations will be discussed in further detail in Section 4.3 of this thesis.

Disorders associated with mitochondrial deletions have several clinical outcomes. The large mitochondrial deletions, ranging from a few hundred bp to >10kb, generally cause sporadic disorders such as Chronic progressive external opthalmoplegia (CPEO) and Kearns-Sayre syndrome (Moraes et al. 1989).

3.   Mitochondria and non-syndromic hearing loss

The first mitochondrial DNA variant causing non-syndromic hearing loss was identified in 1993 in a large Arab-Israeli family (Prezant et al. 1993). To date, a large number of mitochondrial mutations have been identified and associated with both syndromic and non-syndromic hearing loss and so defining a phenotype known as maternally inherited hearing loss (MIHL) (Guan 2004). The large number of mitochondrial mutations **(Table 1.4)** identified in patients with hearing loss, demonstrate the importance of the mitochondria in the normal functioning of the inner ear.

Table 1.4: Mitochondrial mutations associated with hearing loss.

Gene	RNA/Protein	Allele	Disease
MTRNR1	12S rRNA	A827G	Deafness
MTRNR1	12S rRNA	T961C	Deafness
MTRNR1	12S rRNA	961delT+C(n)ins	Deafness
MTRNR1	12S rRNA	T961insC	Deafness
MTRNR1	12S rRNA	T990C	Deafness
MTRNR1	12S rRNA	T1005C	Deafness
MTRNR1	12S rRNA	T1095C	Deafness
MTRNR1	12S rRNA	A1116G	Deafness
MTRNR1	12S rRNA	T1291C	Deafness
MTRNR1	12S rRNA	A1491C	Deafness
MTRNR1	12S rRNA	C1494T	Deafness
MTRNR1	12S rRNA	A1517C	Deafness
MTRNR1	12S rRNA	A1555G	Deafness
MTTS1	tRNA Ser $^{(UCN)}$ precursor	A7445C	Deafness
MTTS1	tRNA Ser $^{(UCN)}$ precursor	A7445G	Deafness
MTTS1	tRNA Ser $^{(UCN)}$ precursor	A7445T	Deafness
MTTS1	tRNA Ser $^{(UCN)}$	7472insC	Deafness
MTTS1	tRNA Ser $^{(UCN)}$	G7444A	Deafness
MTTS1	tRNA Ser $^{(UCN)}$	T7510C	SNHL

Gene	RNA/Protein	Allele	Disease
MTTS1	tRNA Ser [(UCN)]	T7511C	SNHL
MTTA	tRNA Ala	T5655C	Deafness Enhancer
MTTC	tRNA Cys	G5780A	SNHL
MTTC	tRNA Cys	T5802C	Deafness A1555G increased penetrance
MTTP	tRNA Phe	A636G	Deafness
MTCO1	Cytochrome c oxidase I	A7443G	Deafness
MTCO1	Cytochrome c oxidase I	A7445C	Deafness
MTCO1	Cytochrome c oxidase I	A7445G	SNHL
MTCO2	Cytochrome c oxidase Π	A8108G	SNHL
MTND5	NADH dehydrogenase 5	T12338C	Deafness A1555G increased penetrance
MTND6	NADH dehydrogenase 6	C14340T	SNHL

- SNHL: Sensorineural Hearing Loss (MITOMAP: A Human Genome Database http://www.mitomap.org).
- tRNA Ser [(UCN)] precursor: Is a nucleotide sequence that precedes the start of the tRNA Ser [(UCN)] gene and is cleaved by enzymes resulting in the functional gene.
- Deafness enhancer (T5655C): is thought to modulate the phenotypic expression of the tRNA Ser [(UCN)] (T7511C) mutation (Li et al. 2004c).

The majority of the mutations associated with hearing loss are found in the rRNA and the tRNA genes (MITOMAP: A Human Genome Database http://www.mitomap.org). Interestingly, most of the mutations linked to non-syndromic deafness are found in the homoplasmic form.

3.1. Mitochondrial mutations and non-syndromic hearing loss

Non-syndromic hearing loss can occur as the only clinical phenotype of a mitochondrial disease, suggesting that hearing is strongly dependant on normal mitochondrial functioning. Mitochondrial inherited forms of hearing loss usually arise due to mutations in the genes involved in the protein-synthesis machinery: rRNAs and tRNAs (Guan 2004). A number of mutations have been identified in the *MT-RNR1* (12S rRNA) and *MT-TS1* (tRNA$^{(Ser)(UCN)}$) genes that cause progressive non-syndromic hearing loss. The mutations in these genes account for most cases of maternally inherited non-syndromic hearing loss (Guan 2004). Non-syndromic hearing loss mutations are often found in the homoplasmic state or at high levels of heteroplasmy, signifying a high threshold for pathogenicity (Guan 2004). The phenotypic expression of the mutations in these genes varies in severity from one patient to another. This indicates that other factors such as nuclear modifying genes, environmental factors or mitochondrial haplotypes are required for the expression of these mutations. The hearing loss due to these mitochondrial mutations is generally of late childhood or early adulthood onset and progressively worsens with age (Guan 2004).

3.1.1. *Mutations in the tRNA genes*

Mitochondrial tRNA genes are one of the major hot spots for mutations associated with maternally inherited hearing loss. The *MT-TS1* encoding (tRNA$^{(Ser)(UCN)}$) gene is the major tRNA gene involved in non-syndromic hearing loss cases (MITOMAP: A Human Genome Database http://www.mitomap.org). In *MT-TS1* at least five mutations have been identified and associated with non-syndromic hearing loss: A7445G (Reid, Vernham, and Jacobs 1994), 7472insC (Verhoeven et al. 1999), T7510C (Hutchin et al. 2000), T7511C (Sue et al. 1999) and G7444A (Pandya et al. 1999).

The A7445G mutation was first identified in a Scottish family in 1994 (Reid, Vernham, and Jacobs 1994). It has also been identified in two unrelated families from New Zealand and Japan (Fischel-Ghodsian et al. 1995; Sevior et al. 1998). This mutation requires the contribution of other genetic of environmental factors to cause hearing loss in individuals harbouring this mutation.

The 7472insC mutation has been identified in both a Sicilian and a Dutch family (Tiranti et al. 1995; Verhoeven et al. 1999). This mutation is found in the heteroplasmic state even though most

individuals have been found to harbour this mutation in very high levels of up to 90% in some tissues. This mutation is sufficient to produce the deafness phenotype and when present in very high levels may lead to neurological dysfunction (Verhoeven et al. 1999).

The T7510C mutation is hypothesised to disrupt the base pairing at the acceptor stem of the tRNA causing mitochondrial dysfunction. This mutation has been identified in two families of British and Spanish descent (del Castillo et al. 2002; Hutchin et al. 2000).

The T7511C mutation has been found in families of different ethnic groups. This mutation often exists in the homoplasmic state but has been detected at high levels of heteroplasmy. It has been found that the levels of heteroplasmy do not correlate with the severity of the patient's hearing loss suggesting that other modifying factors are needed for the phenotypic expression of this mutation (Friedman et al. 1999; Ishikawa et al. 2002; Sue et al. 1999).

The G7444A mutation has until recently only been identified to coexist with the A1555G mutation in *MT-RNR1* in two different Asian pedigrees (Pandya et al. 1999). Therefore the pathogenic role of this mutation was unclear until it was identified in the homoplasmic state in two Chinese families with aminoglycoside-induced and non-syndromic hearing loss (Zhu et al. 2006).

3.1.2. Mutations in the rRNA genes

Several mutations have been identified in *MT-RNR1*, encoding the 12S rRNA, of the mitochondrial genome and associated with non-syndromic hearing loss as well as aminoglycoside-induced hearing loss. To date, no hearing loss associated mutations have been identified in the *MT-RNR2* gene encoding the 16S rRNA (MITOMAP: A Human Genome Database http://www.mitomap.org).

The different mutations in *MT-RNR1* associated with non-syndromic and aminoglycoside induced hearing loss will be discussed in greater detail in Section 4.3 of this thesis.

3.1.3. Pathophysiology of hearing loss due to mitochondrial mutations

The fact that mitochondrial mutations so frequently lead to hearing loss suggests that the cells of the inner ear are particularly dependent on mitochondrial function. What remains unclear is why some

homoplasmic mutations only affect hearing despite being present in all other tissues of the human body. Histological studies show that advanced cochlear degeneration include the organ of Corti, stria vascularis and supporting cells (Lindsay and Hinojosa 1976).

The mechanism by which mitochondrial mutations induce hearing loss is currently unclear. The cells that are most likely to be affected by a mitochondrial defect are the cells of the stria vascularis and the hair cells in the cochlea. The main pathogenic factor involved in the degeneration of these cells might be a deficiency in mitochondrial oxidative phosphorylation. Other factors may also include increased ROS production as well as altered apoptotic signalling (McKenzie, Liolitsa, and Hanna 2004). There are many different hypothesis to explain the manner in which mitochondrial mutations induce sensorineural hearing loss. One hypothesis involves the energy dependence of the organ of Corti and the stria vascularis on mitochondrial oxidative phosphorylation. The accumulation of the mutant DNA reduces the oxidative phosphorylation capacity of the cell with an increase in age. Thus, there would be a reduction in the amount of energy produced by the mitochondria and so influencing the release of neurotransmitters in the cochlea. Another possibility is a disruption in the ion transport in the hair cells, leading to a reduction in the efficiency of transduction of acoustic signals to the brain. The stria vascularis is the most metabolically active site in the cochlea with its primary function to maintain the ionic environment of the hair cells (Steel and Kros 2001). Ion homeostasis in the inner ear requires the secretion of ions, particularly K^+, into the endolymph against the ionic gradient and in the process making use of ATP-dependant pumps. As a result, the most likely effect of a decrease in the ATP production caused by mitochondrial dysfunction is a slowing of the ATP-dependant pumps. The decreased functioning of these pumps disrupts the ionic balance in the inner ear, reducing the inner ear capacity to detect and transmit sound waves entering the ear (Steel and Kros 2001).

The exact mechanism regarding the pathophysiological pathways of mitochondrial induced hearing loss still remains unclear and there are still some questions that need to be answered. Why does a homoplasmic mutation that affects the oxidative phosphorylation system lead to a clinical defect that is solely restricted to the cochlea instead of affecting every tissue in the body? The second question is why the same mutation can cause more severe hearing loss in one individual from a family than in others? The answers might lie within one of three possible factors that modulate the phenotypic expression of mitochondrial mutations associated with hearing loss: nuclear modifier genes,

mitochondrial haplotypes and certain environmental factors such as aminoglycoside antibiotics (Hutchin and Cortopassi 2000).

4. Aminoglycoside-induced hearing loss

4.1. History of the aminoglycosides

Mutations in mitochondrial genes have been associated with susceptibility to hearing loss in individuals who receive aminoglycoside therapy for treatment of bacterial infections.

The aminoglycosides are a group of antibiotics used to treat bacterial infections such as *Escherichia coli* (E.coli) and *Mycobacterium tuberculosis* (Mtb). These antibiotics are produced by both *Streptomyces* spp and *Micromonospora* spp (Noone 1978). The first aminoglycoside to be discovered, stimulated by the discovery of penicillin, was streptomycin isolated from *Streptomyces griseus* in 1944. The most commonly known aminoglycosides are streptomycin, kanamycin, gentamicin, tobramycin and amikacin which are all isolated from different bacterial species. Aminoglycosides are made up of two or more amino sugars (aminoglycosides) connected to an aminocyclitol nucleus **(Figure 1.12)** (Sande and Mandell 1990).
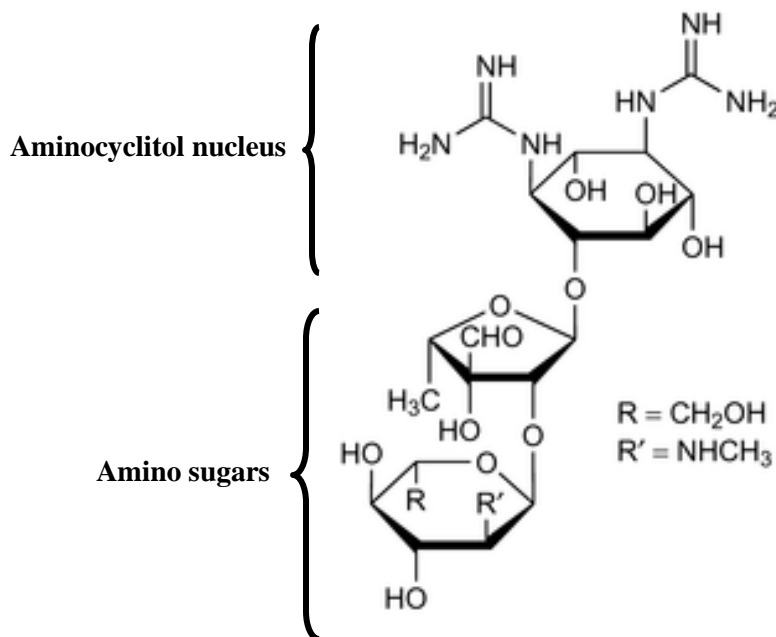
Figure 1.12: Chemical structure of the aminoglycoside streptomycin (Taken from: Sande and Mandell, 1990).

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Aminoglycosides enter the bacterial organism through the aqueous channels formed by the porin proteins in the outer membrane of the gram negative bacteria. These drugs are subsequently transported across the cytoplasmic membrane (Bryan and Kwan 1981), followed by the binding of the aminoglycosides to polysomes (a cluster of ribosomes that is bound to a mRNA molecule) and inhibition of protein synthesis of the bacteria (Bryan 1989). The primary intracellular site of action of aminoglycosides in bacteria is the 30S ribosomal subunit which is made up of 21 proteins and a single 16S molecule of RNA (Mitsuhashi 1975).

Aminoglycosides are very poorly absorbed from the gastrointestinal tract due to their highly polar cationic properties. Following oral or rectal administration of the drugs, less than 1% of the administered dose is absorbed. As a result, all aminoglycosides are usually administered by means of an intramuscular injection with plasma concentrations peaking within 30 to 90 minutes after administration (Breen et al. 1972; Cox 1970). Due to their high polarity aminoglycosides are mainly excluded from secretions and tissues and the only sites where high concentrations of the drug has been observed are in the renal cortex and the endolymph and perilymph of the inner ear (Davies et al. 1984). Excretion of these drugs takes place entirely through glomerular filtration (Sande and Mandell 1990).

4.2. Toxicity in humans

Aminoglycosides exert their antibacterial effects by binding directly to the 16S rRNA (ribosomal ribonucleic acid) ribosome of the bacteria where their main target site is the Aminoacyl-tRNA binding site (the A site). Binding of the aminoglycosides to the bacterial 16S rRNA ribosome leads to premature termination of protein synthesis or mistranslation of proteins. Human *mitochondrial* ribosomes have striking structural similarities to *bacterial* ribosomes **(Figure 1.13)** and it is proposed that the 12S rRNA of the human mitochondrial ribosomes are the main target sites for the aminoglycosides (Xing et al. 2006b). Binding of the aminoglycosides to the human 12S rRNA will lead to disruption of mitochondrial protein synthesis in the cell. All proteins synthesized in the mitochondria are involved in oxidative phosphorylation (Section 2.2.1), therefore disruption of mitochondrial protein synthesis will ultimately lead to death of the cell.

Human mt 12S rRNA   *E. coli* **16S rRNA**
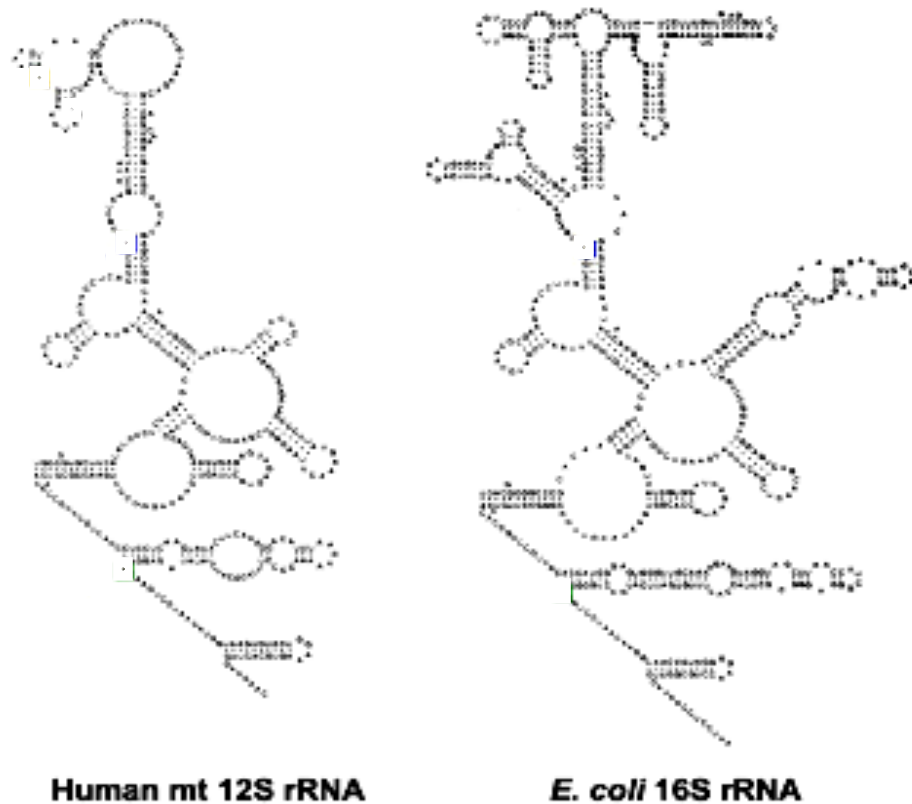
Figure 1.13: Secondary structures of the human mitochondrial 12s rRNA and the bacterial 16S rRNA showing similarities between the two species (Taken from: Ballana et al. 2006b).

In the developed countries aminoglycosides are only used to treat patients with cystic fibrosis or tuberculosis (TB), while in the developing countries these drugs are used more routinely to treat many types of minor infections as well as the more serious infections (Sande and Mandell 1990).

Regardless of the effectiveness of aminoglycosides against bacterial infections, serious toxicity is a major limitation in the usefulness of these drugs. The same spectrum of toxicity is shared by all members of the aminoglycoside family of which the most notable is ototoxicity and nephrotoxicity (Sande and Mandell 1990). Nephrotoxicity is usually a reversible side effect with symptoms that may include the following: excess urea in the blood (azotemia), anaemia, excess fluids in the body (overhydration), increased hydrogen ion concentration in the blood (acidosis), high blood pressure and decrease in urinary output. In some cases blood or pus may be present in the urine, as may uric acid crystals.

Ototoxicity causes permanent hearing loss in patients hypersensitive to these drugs (Higashi 1989; Noone 1978). The ototoxicity is the result of damage to the outer hair cells in the cochlea and vestibular labyrinth (Braveman et al. 1996). Both human and animal studies have documented the progressive accumulation of aminoglycosides in the perilymph and endolymph of the inner ear. Accumulation of the drugs occur when the concentration in the plasma is high and the diffusion back into the bloodstream is slow (Huy et al. 1983). It has been noted that aminoglycosides persists in the inner ear tissue for more than 6 months or longer after administration which may explain the increased susceptibility of patients to ototoxicity after a previous history of aminoglycoside therapy (Dulon et al. 1993). Consequently, there is a progressive destruction of vestibular and cochlear sensory cells, which are very sensitive to damage by aminoglycosides (Brummett and Fox 1982). Animal studies on guinea pigs showed that there was a marked amount of degradation of the type-1 sensory hair cells in the vestibular organ following exposure to large amounts of gentamicin (Wersall et al. 1973). Once the sensory hair cells are lost, regeneration does not occur (Lietman and Smith 1983). Aminoglycoside ototoxicity is recognized by a distinct pattern of hearing loss starting in the high frequency range with lower frequencies only affected later **(Figure 1.14)**. This is due to the fact that the degradation of the hair cells starts at the base of the cochlea where the outer hair cells are situated and high frequency sounds are detected. Therefore, with prolonged exposure and increase in dosage, the degradation moves to the apex of the cochlea, the site of the inner hair cells. The hair cells in the apex are necessary for the perception of low frequency sounds.
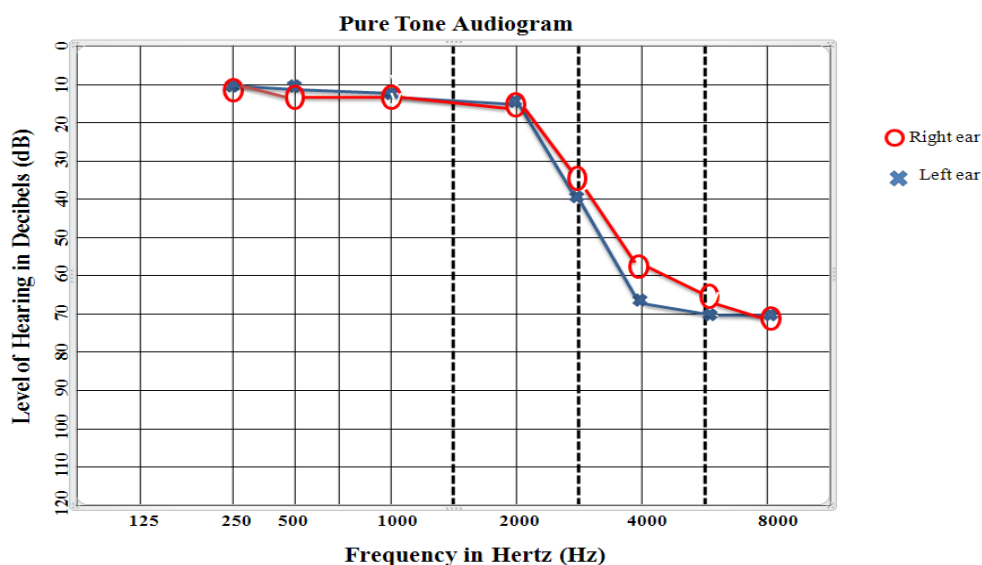
Figure 1.14: Audiogram of a patient with aminoglycoside induced hearing loss. The data shows the typical downward slope in the high frequency range (4000 – 8000 Hz).

The toxicity of aminoglycosides not only affects the patient receiving the therapy but can also cause hearing loss in an unborn child. Streptomycin has been administered to pregnant women for more than 30 years. Administration of aminoglycosides to women late in their pregnancy results in the accumulation of these antibiotics in the fetal plasma and in the amniotic fluid. A study done in South Africa on a group of 87 women who had received tuberculosis treatment during the late stages of their pregnancy showed that, if given in acceptable doses, streptomycin may possibly cause a minor degree of hearing loss (found in 2/33 children tested) in the unborn child (Donald and Sellars 1981).

4.3. Mitochondrial 12S rRNA mutations and aminoglycoside-induced hearing loss

Hearing loss due to aminoglycoside exposure has been identified in a number of families from all over the world. To date, a total of six mutations (A1555G, C1494T, T1095C, 961delT + insC$_{(n)}$, A827G, T1291C) have been identified in the mitochondrial *MT-RNR1* gene that encodes for the 12S rRNA ribosomal subunit. These mutations have been identified within a number of different families and associated with specifically aminoglycoside-induced hearing loss.

A1555G

The first mutation found to be associated with non-syndromic hearing loss was A1555G (Prezant et al. 1993). This mutation is located in the highly conserved A site of *MT-RNR1* of the (Hutchin et al. 1993). The A1555G mutation has been detected in a number of families of different ethnic backgrounds (Asian, Spanish, American, Caucasian, Hispanic, Arab-Israeli, Brazilian, Italian and South African) around the world, mostly in the homoplasmic form (del Castillo et al. 2003; Fischel-Ghodsian et al. 1997; Li et al. 2004b; Prezant et al. 1993). The A1555G mutation causes a hypersensitivity to aminoglycosides in individuals that harbor this mutation (Estivill et al. 1998b; Fischel-Ghodsian et al. 1993; Fischel-Ghodsian 1998; Hutchin et al. 1993). The hypersensitivity is likely due to the fact that the A1555G mutation modifies the structure of the human 12S rRNA ribosome subunit, by replacing Adenine with Guanine, making it more structurally similar to the 16S rRNA ribosome of bacteria **(Figure 1.15)**.
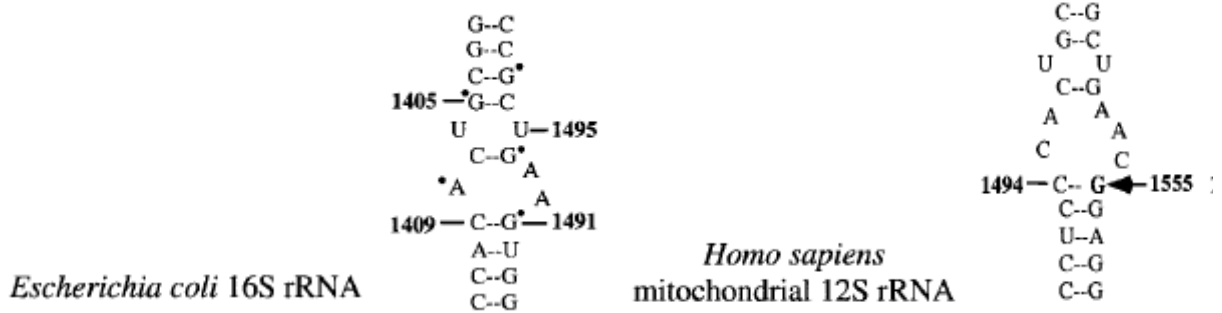
Figure 1.15: Secondary structures of the human mitochondrial 12S rRNA with the A1555G mutation and bacterial 16S RNA (Taken from: Zhao et al., 2004).

The A1555G mutation most likely **increases** the binding of aminoglycosides to the human mitochondrial ribosome disrupting the protein synthesis of the cell and so reducing the ATP production (Inoue et al. 1996). Biochemical studies have shown that, in cells in which the A1555G mutation was present, both mitochondrial protein synthesis and mitochondrial oxygen consumption of the cells was reduced by up to 50% (Guan, Fischel-Ghodsian, and Attardi 1996) clearly demonstrating the deleterious effects of this mutation. It was then further demonstrated in a study by Inoue et al 1996 that cells harbouring the A1555G mutation showed decreased mitochondrial protein synthesis and growth rates in the presence of streptomycin (Inoue et al. 1996). Furthermore, the results show that the A1555G mutation by itself can be harmful even in the absence of aminoglycosides (Estivill et al. 1998b; Guan, Fischel-Ghodsian, and Attardi 1996; Hutchin 1999; Inoue et al. 1996). It was also shown that the A1555G mutation decreases the accuracy of translation in hybrid *M*. *smegmatis* strains in which the bacterial decoding site sequence was replaced with its mitochondrial counterpart. The hybrid bacterial strains (containing the A1555G mutation) had a 7-fold increase in the mistranslation rate compared to the hybrids with the wild type allele at position 1555 (Hobbie et al. 2008b).

Since the discovery of the A1555G mutation, a number of other mutations, (C1494T, A827G, T1095C, 961delT, T1291C), all in *MT-RNR1* have been identified in multiple families and have been associated with both aminoglycoside and non-syndromic hearing loss.

C1494T

The C1494T mutation is also found in the highly conserved A site of *MT-RNR1*. To date, this mutation has only been reported in families of Spanish and Chinese descent. Similar to the A1555G mutation, the C1494T mutation has also been shown to be associated with maternally inherited hearing loss **without** any exposure to aminoglycosides but only with late onset and varying degrees of severity (Rodriguez-Ballesteros et al. 2006). The C1494T mutation was shown to be pathogenic in a study done by Zhao et al., 2005 where the lymphoblast cells of a number of individuals that harbor the mutation was cultured and compared to wild type cell lines. A significant reduction in the growth rate of the mutation positive cells in addition to a decrease in the total oxygen consumption rate of these cells where observed when compared to the wild type cell lines in the presence of aminoglycosides (Zhao et al. 2004; Zhao et al. 2005). However, due to the incomplete penetrance and the mild biochemical defects observed in cell lines from the above mentioned study it is clear that the C1494T mutation by itself is not usually sufficient to produce the hearing impaired phenotype. It is thus clear that other contributing factors such as aminoglycoside exposure and nuclear background modulates the phenotypic expression of the C1494T mutation (Zhao et al. 2005). Studies by Hobbie et al 2008 showed that there was an increase in the codon misreading rate of bacterial hybrids harbouring the C1494T mutation when compared to hybrids that harboured only the wild type allele at position 1494. Thereby suggesting that misreading of the genetic code is an important molecular mechanism in the disease pathogenesis of non-syndromic hearing loss.

A827G

The homoplasmic A827G mutation has, to date, only been found in two different population groups, Chinese and Argentinean (Chaig et al. 2008; Xing et al. 2006b; Xing et al. 2006a). In all families investigated there was incomplete penetrance of the mutation indicating that the A827G mutation on its own is not responsible for the clinical phenotype and the contribution of other factors like aminoglycosides, mitochondrial haplotypes or nuclear modifier genes is required. The fact that the A827G mutation is present in two genetically unrelated families from China with aminoglycoside-induced hearing loss strongly suggest that the A827G mutation is involved in the pathogenesis of both non-syndromic sensorineural hearing loss and aminoglycoside ototoxicity (Xing et al. 2006b; Xing et al. 2006a). This mutation is also located within the conserved A site of *MT-RNR1* indicating

its importance in the pathogenesis of hearing loss. However, no functional studies have been done on this mutation to determine its effect of this mutation on mitochondrial protein synthesis.

T1095C

Another mutation in *MT-RNR1* associated with hearing impairment is the T1095C mutation. This mutation has been found in homoplasmic form in families from Chinese and Italian descent as well as in a few Chinese sporadic cases (Wang et al. 2005). The T1095C mutation has not only been associated with aminoglycoside-induced and non-syndromic hearing loss but an association has also been found with neuropathy and parkinsonism in an Italian family (Thyagarajan et al. 2000). The pathogenesis of T1095C was investigated in a study by Thyagarajan *et al*., 2000 where lymphoblast cells were cultured in an individual known to have the T1095C mutation. Respiratory chain enzyme analysis was done on the cultured cells and it was found that there was a significant decrease in the cytochrome *c* oxidase activity in the cells that had the mutation. It is hypothesized that this mutation causes a disruption in a highly conserved loop in the small ribosomal RNA subunit, which is vital in the initiation of mitochondrial protein synthesis suggested by a variety of experimental studies on *E.coli* (Thyagarajan et al. 2000).

961delT+insC$_{(n)}$, 961insC and T961G

Several different mutations at position 961 in *MT-RNR1* have been detected. They include a (a) T deletion with the insertion of a variable number of Cs (961delT+insC$_n$) (Tang et al. 2002) or (b) C insertion (961insC) (Li et al. 2004b) or (c) a substitution of the T at position 961 with a G (T961G) (Tang et al. 2002). The 961delT+insC$_{(n)}$ mutation has been found in individuals of Japanese and Italian descent (Casano et al. 1999; Yoshida et al. 2002). The 961delT+insC$_{(n)}$ mutation is possibly associated with aminoglycoside-induced hearing loss but its actual role in the pathogenesis of the disease still remains unclear. The 961insC mutation has been found to co-segregate with the A1555G mutation (Li et al. 2004b). This mutation has been detected in control samples raising the question of whether or not the 961insC mutation actually causes aminoglycoside-induced hearing loss. There is however a possibility that the co-segregation of the 961insC mutation plays a role in

enhancing the penetrance of the A1555G mutation in subjects harboring both mutations (Li et al. 2004b). T961G has only been detected in Caucasian paediatric subjects (Li et al. 2004a). The mutation was found in homoplasmic form in these subjects with hearing loss that ranged from moderate to profound. This mutation was not detected in any of the control samples from this study. Initially, the mutations at position 961 were thought to be disease causing since these mutations were only found in deaf individuals in a number of studies. However, recently the mutations at position 961 have been detected in control populations (Kobayashi et al. 2005). The pathogenic mechanism of the mutations at this position is not understood and further functional studies on cell lines derived from affected individuals with either one of the mutations is needed (Li et al. 2004a). However, the 961 mutations are localized in a region of the *MT-RNR1* gene that is not evolutionarily conserved and for which the function is not known. Thus, it might be possible that the mutations described at position 961 may in fact not be involved in aminoglycoside induced hearing loss and are polymorphisms instead.

T1291C

The variant at position 1291 was discovered in 2006 in a Cuban family with non-syndromic hearing loss (Ballana et al. 2006b). It was observed that the 1291 variant causes a major structural change in the secondary structure of the 12S rRNA **(Figure 1.16 a and b)** indicating that it possibly might be pathogenic, however no functional studies have been conducted. Studies by Abreu-Silva *et al.,* 2006 implied that the T1291C mutation is only a rare African polymorphism since the mitochondrial haplogroup analysis of five deaf Brazilian subjects harbouring the T1291C variant all belonged to the L1/L2 haplogroups. These haplogroups indicate that their mitochondrial DNA is of African origin (Abreu-Silva et al. 2006a; Abreu-Silva et al. 2006b). Furthermore, the position of the 1291 variant is not conserved across species and the pathogenic mechanism of this variant therefore remains unclear. Ballana and co-workers (2006a) confirmed that their Cuban subjects also belonged to the African haplogroup L1 but further stated that the T1291C variant may very well be a susceptibility factor for hearing impairment, specifically in the Cuban family. They further state that the pathogenicity of the T1291C variant could not be excluded due to the fact that different clinical phenotypes are expressed in carriers which is similar to that found in carriers of the A1555G mutation (Ballana, Morales, and Estivill 2006a).

(a)   (b)
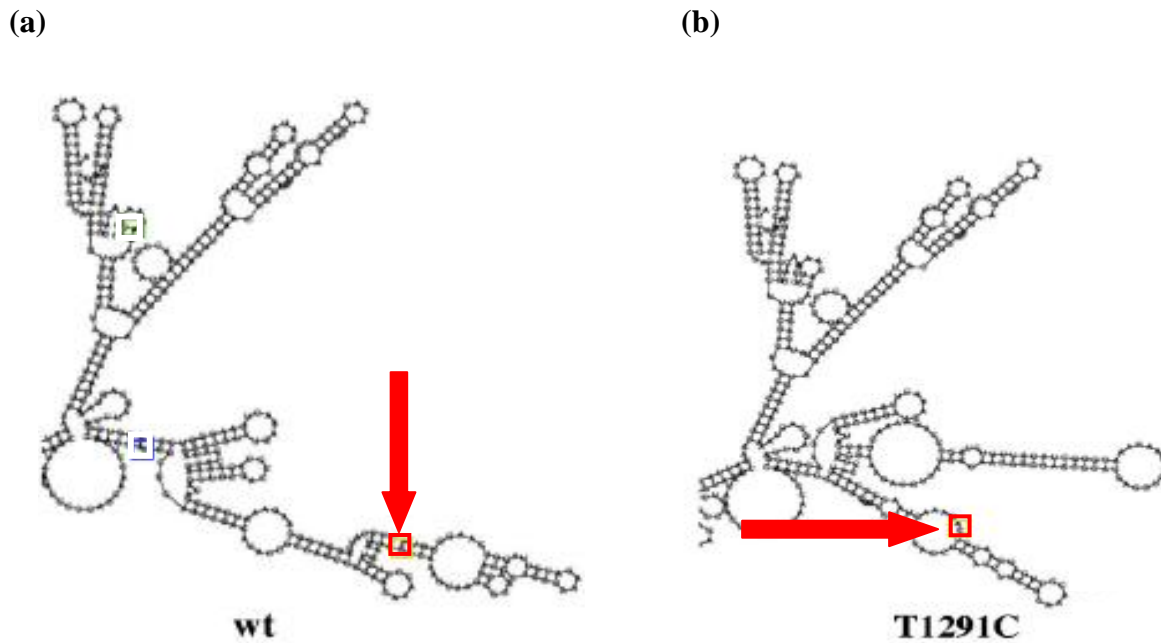
Figure 1.16: Secondary structures of the 12S rRNA. (a) Secondary structure of 12S rRNA with the wild type allele (T allele) at position 1291, indicated by the arrow. (b) Secondary structure of 12S rRNA with the mutant allele (C allele) at position 1291, indicated by the arrow (Taken from: Ballana et al., 2006b).

To date, only these six mutations (A1555G, C1494T, T1095C, 961delT + insC$_{(n)}$, A827G and T1291C) have been demonstrated to be associated with aminoglycoside-induced deafness in a number of different populations groups. The remaining mutations, as listed in Table 1.4, have only been found in isolated families or individuals and their pathogenicity is equivocal. However, the existence of other mutations in *MT-RNR1* as well as other mitochondrial or nuclear genes is a real possibility.

4.4.   Aminoglycoside use in South Africa and the current Tuberculosis epidemic

4.4.1   Tuberculosis epidemic in South Africa

In South Africa, aminoglycosides are used widely to treat tuberculosis (TB) which is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb). Globally there are three million reported deaths each year as a result of TB infection and an estimated eight million new active cases of TB reported annually (Dye, Floyd, and Uplekar 2008). It is mostly the third world countries, like China, India and certain African countries that are hit the hardest by the burden of TB. Nine of the world's 22 high burden TB countries are in Africa, with South Africa having the 4th highest TB incidence in the world (Dye, Floyd, and Uplekar 2008). In the period (1996-2006) there was a threefold increase in the number of people reported with TB, in South Africa, from 269 cases/100 000 population to 720 cases/100 000 population (Department of Health Republic of South Africa 2007).

Up until 50 years ago there was no known cure for TB (Dye, Floyd, and Uplekar 2008). Currently, TB is treated with a number of so-called first-line drugs such as isoniazid, rifampacin, ethambutol and pyrazinamide. Patients with TB are required to stay on treatment for a minimum of six months. However, resistant strains of TB, multi-drug resistant tuberculosis (MDR-TB) and extensively-drug resistant tuberculosis (XDR-TB) have emerged in the last few years, as a result of the poor compliance of patients to their treatment as well as inadequate management and treatment regimes.

MDR-TB is defined as the resistance of Mtb bacillus to at least isoniazid and rifampacin, the two most powerful first-line drugs (Thaver and Ogunbanjo 2006). MDR-TB is thought to be a 'man made' problem. In the majority of the cases, MDR-TB emerges when a TB patient receives either inappropriate treatment which results in the naturally occurring resistant TB bacteria to survive and multiply, or poor compliance of the TB patient to their treatment. It is estimated that up to 2.9% of all TB cases are diagnosed as MDR-TB. A recent study by the South African Medical Research Council showed there are 6000 new MDR-TB cases in South Africa per year (Weyer, van der Walt, and Kantor 2006). In South Africa from the period January 2004 to April 2007 over 11 000 laboratory cases were confirmed to be MDR-TB cases in all provinces, with 36% of the cases being reported in the Western Cape alone (Dye, Floyd, and Uplekar 2008). The Western Cape and KwaZulu Natal have the highest percentage of MDR-TB cases in South Africa. There are a number of factors that contribute to the increased number of MDR-TB cases such as the lengthy treatment

(patients need to stay on the treatment for at least 2 years), increased toxicity and increased cost of the 2nd line drugs (ciprofloxacin, ofloxacin, sparfloxacin) (Duggal and Sarkar 2007). The cost for drugs per MDR-TB patient is projected to be about R30 000 compared with the cost for a drug susceptible TB patient being around R300 for the six month regiment (Weyer, van der Walt, and Kantor 2006).

4.4.2. Use of aminoglycosides in South Africa

In South Africa, according to WHO guidelines MDR-TB is treated with a number of second-line drugs including the quinolones (ciprofloxacin, ofloxacin and sparfloxacin) and aminoglycosides (streptomycin, kanamycin, amikacin and capreomycin) (Thaver and Ogunbanjo 2006). The second line drugs are much more costly, less effective and more toxic than the first line drugs (Gupta et al. 2001). As the incidence of MDR-TB increases in South Africa the use of the second line drugs, such as aminoglycosides, are also increasing. These aminoglycosides are given to all MDR-TB cases as a daily injection for four to six months while these patients are hospitalized (Weyer, van der Walt, and Kantor 2006).

4.5   Modifiers for aminoglycoside-induced hearing loss

Varying degrees of penetrance and severity of hearing loss has been observed in most families known to harbour one of the mutations associated with aminoglycoside-induced hearing loss. It is suggested that these differences are the result of certain modifying factors that influence the phenotypic expression of these mutations. Biochemical, clinical and genetic data have shown that aminoglycoside antibiotics (Prezant et al. 1993), mitochondrial haplotypes (Guan et al. 1998; Pandya et al. 1999) and nuclear modifier genes (Abe et al. 2001; Guan, Fischel-Ghodsian, and Attardi 2001; Yan et al. 2006) are the three major modifying factors for the phenotypic expression of the hearing loss associated mutations in *MT-RNR1*.

4.5.1. Environmental factors

Aminoglycoside antibiotics, as discussed in Section 4.1, bind to the human mitochondrial 12S rRNA subunit leading to mistranslation and premature termination of protein synthesis in the mitochondria. The binding of these antibiotics is enhanced by mutations (Section 4.3) that occur in *MT-RNR1*. Supported by both genetic and biochemical evidence (Guan 2004) it is hypothesized that the aminoglycosides accumulate in the mitochondria of the inner ear following administration, where protein synthesis is disrupted through binding to the 12S rRNA subunit. The resultant outcome of a disruption in protein synthesis is the decrease in ATP production of the cochlear hair cells. The decrease in ATP thus leads to a defect in the OXPHOS system increasing the ROS formation, ultimately leading to apoptosis of the hair cells resulting in hearing loss (Guan 2004). Functional studies by Guan *et al.,* 1996 showed that in lymphoblast cell lines derived from mutation positive individuals a marked reduction in the growth rate of cells was seen after treatment with certain aminoglycoside antibiotics thereby indicating that the main target site for aminoglycoside antibiotics is the 12S rRNA carrying the A1555G mutation (Guan, Fischel-Ghodsian, and Attardi 1996).

4.5.2. Nuclear genes

A few nuclear genes [including *TRMU (*tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase) and *GJB2*] have been implicated as being genetic modifiers (Guan, Fischel-Ghodsian, and Attardi 2001; Yan et al. 2006) for the phenotypic expression of the aminoglycoside-induced hearing loss mutations. These nuclear modifier gene (s), may functionally interact with the mutated 12S rRNA, enhance or suppress the effects of the mutations thereby influencing the phenotypic manifestations of these mutations (Yan et al. 2006).

A potential candidate as a nuclear modifier is the *TRMU* gene. The gene is located on chromosome 22q13, consists of 11 exons and spans a region of approximately 22kb (Yan et al. 2006). The function of *TRMU* is primarily 2-thiolytion of hypermodified nucleoside 5-methyl-aminomethy-2-thio-uridine. This nucleotide which is found in the wobble position of a number of bacterial and human mitochondrial tRNAs (tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}) has an critical role in the structure and function of tRNAs. This includes aminoacylation, structural stabilization and codon recognition at the decoding site of small mitochondrial tRNAs (Umeda et al. 2005). Studies on a large Arab-Israeli family, one Italian and 6 Spanish families who harboured either the A1555G or the C1494T

mutations revealed a missense mutation (A10S) in the evolutionary conserved N-terminal region of *TRMU*. All members of the families who carried either the A1555G or C1494T in combination with the homozygous A10S mutation presented with profound deafness (Guan et al. 2006). Functional studies on the A10S mutation revealed that this mutation (in the homozygous form) results in a defect in the 2-thio modification of mitochondrial tRNAs, which leads to a decrease in the steady state levels of mitochondrial tRNAs and followed by impairment of mitochondrial translation. Therefore, the impaired translation contributes to the impaired mitochondrial protein synthesis caused by mutations in the 12S rRNA, thus increasing the severity of the phenotypic manifestations of the deafness-associated 12S rRNA mutations (Guan et al. 2006).

GJB2 coding for connexin 26 (Section 1.4.1) has also been considered as being a possible modifier in the expression of the deafness associated mutations in *MT-RNR1* (Abe et al. 2001). Mutations in *GJB2* result in the altered functioning of the gap junctions thereby influencing the cycling of the K^+ ions in the hair cells that affects the generation of neural signals to the brain, leading to hearing loss (Connexin-deafness homepage http://davinci.crg.es/deafness, 2009). Mutations in *GJB2* account for up to 50% of all recessive forms of hearing loss and have been implicated in modulating the severity of hearing loss associated with the A1555G mutation (Abe et al. 2001; Morell et al. 1998). The modulating effect might be explained by the inability of cochlear cells to maintain normal turnover rates of gap junction proteins due to the reduction in ATP production caused by the A1555G mutation (Abe et al. 2001). In the study done by Abe *et al.,* 2001 it was found that individuals heterozygous for the *GJB2* 35delG mutation and harbouring the A1555G mutation had more profound hearing loss than individuals with only the A1555G mutation. These findings suggest that mutations in *GJB2* acts as an aggravating factor in the phenotypic expression of the A1555G mutation (Abe et al. 2001).

4.6   Drugs that protect against aminoglycoside-induced hearing loss

There are currently no clinically approved drugs available for the prevention of ototoxin-induced hearing loss. However, a number of studies have been done which have identified a few potential otoprotective agents but more research on these agents is needed.

Presently, aminoglycoside-induced hearing loss is more prevalent in developing countries as a result of their frequent use and affordability. There is also a concern in industrialized countries where aminoglycosides are used in emergencies such as in the case of renal dialysis or as a prophylactic antibacterial treatment for cystic fibrosis patients. The use of aminoglycosides has also increased worldwide due to the increase of MDR-TB, with these drugs being the recommended treatment by the WHO. Unfortunately, this increased use of these antibiotics may increase the incidence of aminoglycoside-induced hearing loss significantly.

In vitro animal models suggests that the formation of ROS participates in the etiology of aminoglycoside-induced auditory and vestibular complications (Forge and Schacht 2000). It is suggested that the formation of ROS is due to aminoglycosides forming a complex with iron. The antibiotics enhance the iron-catalyzed formation of ROS from unsaturated fatty acids as electron donors (Lesniak, Pecoraro, and Schacht 2005; Priuska and Schacht 1995; Sha and Schacht 1999a). As a result the redox-imbalance leads to the activation of apoptosis or necrosis of the cell (Jiang et al. 2006). Some of the most convincing evidence for a causal relationship between ROS and ototoxicity came from a study by Sha *et al*., 2001. In this study mice over expressing the enzyme superoxide dismutase (SOD) were protected from the ototoxic effect of kanamycin (Sha et al. 2001). Other studies showed that antioxidants attenuate the loss of hearing and balance in animal models (Garetz, Altschuler, and Schacht 1994; Lautermann, McLaren, and Schacht 1995; Song and Schacht 1996; Song, Sha, and Schacht 1998).

The development of protective treatments has mainly focused on antioxidants as potential otoprotective agents. Antioxidant therapy has been shown to be very effective in animals regardless of the aminoglycoside antibiotic administered with both the cochlear and vestibular side effects of streptomycin, kanamycin, gentamicin and amikacin being attenuated by the simultaneous administration of antioxidants (Conlon, Perry, and Smith 1998; Conlon et al. 1999; Song, Sha, and Schacht 1998). Some of the compounds that have emerged from these animal studies validate further clinical exploration since they are already approved medications or are available as over the counter drugs or nutritional supplements, like D-methionine (D-met) and salicylate (Campbell et al. 2007; Sha and Schacht 1999b).

The first report of D-met came in 1996 after it was shown that it significantly protected against hair cell and weight loss in rats with cisplatin-induced auditory brainstem response (ABR) (Campbell et

al. 1996). D-met only partially protected against aminoglycoside-induced ototoxicity as shown by the study from Sha *et al* 2000 where guinea pigs were administered twice daily injections of D-met 7 h apart. There animals were also administered daily doses of gentamicin for 19 days. The results showed a reduction in the ABR threshold shifts of the animals who had received D-met (Sha and Schacht 2000). The same form of otoprotection was found in guinea pigs treated with amikacin and D-met (Campbell et al. 2007). D-met is an optical isomer of the essential amino acid L-methionine and is commonly found in fermented proteins like cheese and yogurt, therefore is not foreign to the human body, making it a promising candidate as an otoprotective agent. However, further clinical trials are needed to clarify whether this compound is equally effective in humans as it has been in the animal studies (Campbell et al. 2007).

Salicylate is another promising drug for the treatment as an otoprotective agent due to the fact that it is relatively inexpensive and can be administered to patients in the form of aspirin. When aspirin (acetyl salicylate) is ingested it is hydrolysed and is then present in the serum as salicylate (Sha, Qiu, and Schacht 2006; Sha and Schacht 1999b). Salicylate as an antioxidant was shown to protect against ototoxicity in a study where a group of patients receiving gentamicin therapy for various infections were treated with salicylate (Sha, Qiu, and Schacht 2006). The group of patients that received one gram of aspirin at 8h intervals for 14 days experienced significantly less hearing loss compared to the placebo group. However, a number of patients experienced gastrointestinal side effects caused by the aspirin. The use of aspirin as an otoprotective agent may be very appealing to developing countries due to the low cost which will make this type of treatment affordable (Sha, Qiu, and Schacht 2006).

4.7. Do we still need aminoglycosides?

Since the first introduction of aminoglycoside antibiotics in 1940 to clinical practice, a number of other antimicrobial agents have been identified. These newer antibiotics are just as effective in the treatment of bacterial infections and are much safer to use. A number of studies have been done to demonstrate the superiority of aminoglycoside treatments regimes over other antibiotics and have failed to do so, raising doubts regarding the clinical efficacy of aminoglycosides. However, the recent emergence of drug resistance of gram-negative bacterial strains, have encouraged clinicians

to reconsider the use of these older antimicrobial agents. The revived interest in aminoglycoside use in infectious settings has reopened the debate regarding two major issues regarding these antibiotics, the spectrum of antimicrobial susceptibility and toxicity.

It has been consistently shown that aminoglycosides have very good post antibiotic effects. Demonstrated in both Gram-negative bacilli and *S. aureus*, growth was persistently suppressed even up to 7.5h after administration of aminoglycosides (Novelli et al. 1995). The biggest obstacle clinicians are facing presently with the treatment of bacterial infections is the development of resistance to present day antibiotics.

In contrast, aminoglycoside resistance is rather rare and commonly develops with regard to only one of the antibiotics rather than to all members of the class (Durante-Mangoni et al. 2009). Mechanisms of resistance to aminoglycosides may arise by either one of the following ways. Bacteria may express certain efflux systems that will reduce the amount of aminoglycosides present in the cell (Westbrock-Wadman et al. 1999). In addition, Methylation of the 16S rRNA located within the 30S subunit of the bacteria impairs aminoglycoside binding thereby impairing the antibacterial activity of these drugs (Doi and Arakawa 2007).

Even though the development of resistance against aminoglycosides is rare, long term treatment with these drugs is often discontinued to favour those antibiotics with less toxic side effects. Combined treatments regimes with aminoglycosides and other antibacterials such as β-lactum and ampicillin are being administered more frequently. These combined therapies have proven that synergism exists with ampicillin increasing the uptake of aminoglycosides by disrupting the peptidoglycan cell wall in order for the aminoglycosides to enter the cell. In the case of β-lactum another potential benefit may exist where the aminoglycosides may prevent the development of resistance to β-lactum (Bliziotis et al. 2005).

Toxicity is perhaps the most important factor that prevents clinicians from using aminoglycosides more extensively in the current clinical practice. In the last few years great efforts have been made to address this issue, with the most important being the implementation of dose dependent strategies. It has thus been demonstrated that once-daily administration of the total dose of aminoglycosides and monitoring of the blood levels of these antibiotics are better strategies in order to achieve the

optimal concentrations and to improve the efficacy and toxicity of these antibiotics in critically ill patients (Nicolau et al. 1995; Olsen et al. 2004).

Despite the issue of toxicity, aminoglycosides are still one of the few classes of antibacterials that maintain their activity against the majority of MDR gram-negative bacterial strains, making these antibiotics still of great value in present clinical practice.

4.8. Prevalence of aminoglycoside-induced hearing loss in South Africa

There is no known data on the incidence of aminoglycoside-induced hearing loss in South Africa. Our South African populations are a unique resource to study the contribution of aminoglycosides to hearing loss because of the high rate of aminoglycosides that are administered here due to the unfortunate increase in the prevalence of drug resistant TB infections. We are therefore in a very unique position to be able to study the short and long term effects of aminoglycosides on the auditory system and the genetic aetiology of aminoglycoside-induced hearing loss.

5. The present study

As a result of the increasing MDR-TB epidemic in South Africa and the concomitant increase in aminoglycoside use more of our population will be at risk of developing aminoglycoside-induced hearing loss. It is therefore imperative that we investigate the frequency of mutations associated with aminoglycoside-induced hearing loss in our populations and determine whether certain sub-groups are at increased risk.

5.1. Multi-drug resistant tuberculosis (MDR-TB) patients

In the present study we investigated a group of patients at the Brooklyn Chest Hospital in Cape Town who are receiving high daily doses of aminoglycosides (streptomycin for retreatment TB cases, kanamycin for adults with MDR-TB and amikacin for children). These patients are admitted to the hospital for treatment of MDR-TB and stay at the hospital for a period of up to four months.

During this period they receive daily injections of either one of the above mentioned aminoglycosides as part of their treatment regime. These patients undergo routine monthly audiological examinations to monitor for any high frequency hearing loss as a result of aminoglycoside exposure.

5.2. South African family with streptomycin-induced hearing loss

In the present study we also investigated a family that had presented with streptomycin-induced hearing loss. This family was first reported in 1983 after eight members presented with severe perceptive deafness during treatment for TB (Viljoen, Sellars, and Beighton 1983). The affected family members had been admitted to the Groote Schuur Hospital in Cape Town for further investigation. A complete history of previous ear trauma or infections, renal dysfunction and specific diseases such as mumps was noted. The affected members were also asked about the specific time of onset of the deafness and whether or not they had experienced any tinnitus or vertigo in relation to the antituberculosis treatment. The affected members were subjected to a series of audiometric and speech tests to determine the extent of their hearing loss. At the time it was postulated that the family's susceptibility to streptomycin ototoxicity may be autosomal dominantly inherited. The affected members all claimed to have had normal hearing prior to the start of their TB treatment. The audiometric data confirmed perceptive deafness in all the patients, localised to the cochlea. The audiometric tests also demonstrated the characteristic initial effects of aminoglycoside toxicity, being the loss of hearing in the high frequency range with poor speech discrimination (Viljoen, Sellars, and Beighton 1983).

After the discovery of the A1555G mutation and its causal relationship with aminoglycoside-induced hearing loss in 1993 (Higashi 1989; Hutchin et al. 1993; Prezant et al. 1993) it was suspected that the family could harbour the A1555G mutation. The family was revisited in 1997 with an additional three members experiencing hearing loss as a result of TB treatment (Gardner et al. 1997). The hearing loss in the initial eight members had remained unchanged (**Figure 1.17**).
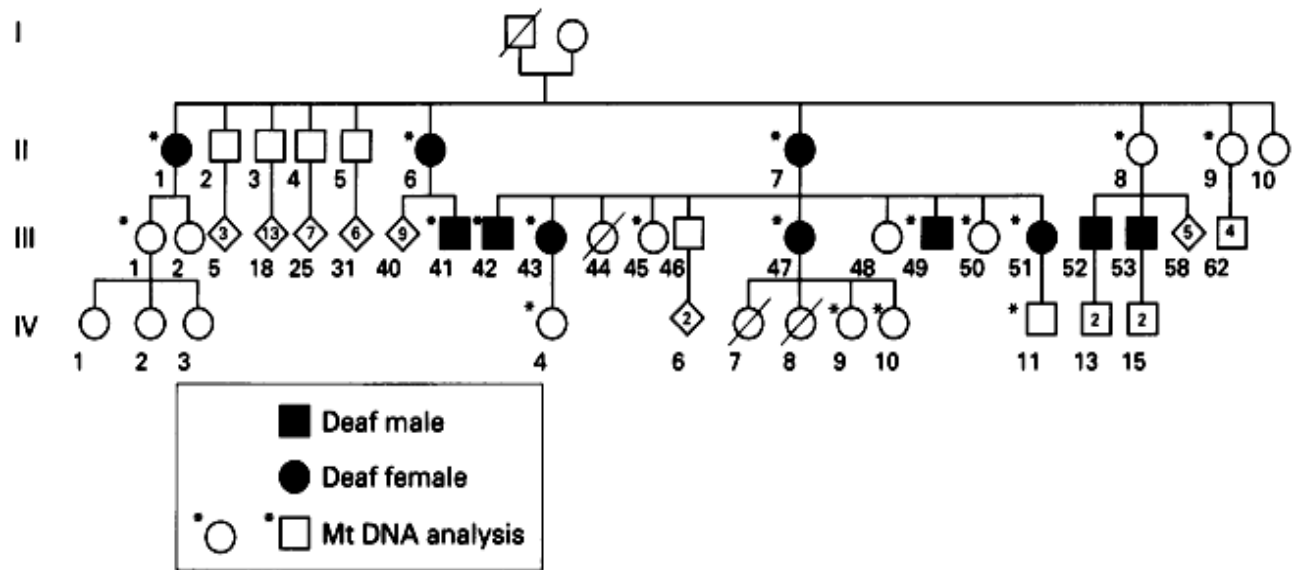
Figure 1.17: Pedigree of the South African family with streptomycin induced ototoxicity (Taken from: Gardner et al., 1997).

Molecular analysis was done on a total of 18 members and it was found that all 18 family harboured the A1555G mutation in the homoplasmic state (Gardner et al. 1997). Eleven family members were diagnosed with streptomycin-induced hearing loss due to their history of streptomycin use for the treatment of TB. An additional nine unaffected members were also identified to be mutation positive with no previous history of any aminoglycoside exposure. Therefore, in this family there are a number of individuals who are potential carriers of A1555G mutation and have an increased risk of developing hearing loss with any future exposure to aminoglycosides.

5.3. Aims and Objectives

The aim of the present study was to investigate the genetic aetiology of aminoglycoside-induced hearing loss in South African populations.

The objectives were the following:

1. To develop a rapid and inexpensive method for detection of the six mutations in *MT-RNR1* (A1555G, C1494T, T1095C, 961delT + insC$_{(n)}$, A827G and T1291C) associated with aminoglycoside-induced hearing loss.

2. To determine the frequencies of these known mutations, in a group of MDR-TB patients from the Brooklyn Chest Hospital in the Western Cape (who receive high doses of aminoglycosides daily as part of their treatment) as well as in control samples representative of the different South African sub-populations present in the Western Cape.

3. To identify novel mutations, in *MT-RNR1*, in those MDR-TB patients who have high frequency hearing loss but do not harbour any of the known mutations.

4. To develop a sensitive and cost effective method for the detection of heteroplasmic mutations.

5. In a South African family known to harbour the A1555G mutation we aimed to identify all mutation-positive individuals and to provide genetic reports informing them of their at-risk status.

6. To perform functional studies on the A1555G mutation.

Chapter 2

MATERIALS & METHODS

1. Summary of Methodology

The Single Nucleotide Polymorphism Multiplex system (Snapshot analysis) was used as a technique to develop a robust and cost effective method to genotype a cohort of MDR-TB patients and control populations for the six mutations associated with aminoglycoside-induced hearing loss. These MDR-TB patients are at risk of developing hearing loss due to their prolonged exposure to aminoglycoside antibiotics that forms part of their treatment for MDR-TB infections. The frequency of these mutations (A1555G, C1494T, T1095C, A827G, 961delT + insC$_{(n)}$ and T1291C) within South African populations is still unknown. Due to the current TB and MDR-TB epidemic that South Africa is facing determining the frequencies of these mutations is even more crucial than ever.

A search for novel variants in the mitochondrial *MT-RNR1* gene was conducted on a group of MDR-TB patients who have clear signs of ototoxicity but do not harbour any of the known mutations. Novel variant screening of *MT-RNR1* was done using High Resolution Melt (HRM) Analysis on the Rotor-Gene 6000. This technique has proven efficient in detecting sequence variants in a variety of different settings and is a rapid and cost effective method for doing mutation screening.

A subgroup of MDR-TB patients were chosen for sequencing of the mitochondrial genome to identify novel variants in other mitochondrial genes that may also be associated with aminoglycoside-induced hearing loss. The novel sequence variants identified were assessed for conservation at both the nucleotide and protein level to validate the importance of the variants.

Heteroplasmy is a rare condition in normal individuals, and is often only associated with mitochondrial DNA disease. Heteroplasmy occurs within and between cells and also between tissue and organs. Most of the pathogenic mitochondrial mutations are usually heteroplasmic and there is a certain threshold level at which the deleterious consequences of the mutation is no longer compensated for by the effect of the wild type DNA. Development of a sensitive and cost effective method for the detection of levels of heteroplasmy for the mutations associated with aminoglycoside-induced hearing loss is needed in order to determine the level of severity of the disease phenotype. To simulate levels of heteroplasmy present in the human cell, PCR fragments containing wild type or mutant alleles were cloned into *E.coli* the DNA isolated and combined in different ratios of wild type to mutant DNA to obtain various ratios of heteroplasmy. Levels of

heteroplasmy were determined by means of restriction fragment length polymorphism (RFLP) and electrophoresed on the ABI 3130xl Genetic Analyzer and agarose gels.

A South African family harbouring the A1555G mutation was genotyped for all six mutations associated with aminoglycoside-induced hearing loss using the ABI Prism® SNaPshot™ Multiplex system. Members from this family were also screened for certain modifying factors in *MT-TS1* and *TRMU* and the 35delG mutation in *GJB2*. In addition, mitochondrial haplogroup analysis was also performed on a member of this family in order to determine to which haplogroup the family belongs.

To date, functional studies have only been conducted on two (A1555G and C1494T) of the six mutations associated with aminoglycoside-induced hearing loss. To establish an assay to determine the effect of a mutation on mitochondrial function, we investigated the A1555G mutation using the MTT assay. Transformed lymphocytes from both wild type and A1555G-mutation positive individuals were subjected to 48 hours of incubation with different concentrations of streptomycin. The mitochondrial function of the cells was assessed using the MTT colorimetric assay. This assay is used to quantitatively measure cell survival and proliferation by means of a colour reaction. MTT assay measures the activity of various dehydrogenase enzymes that reduces the yellow MTT to formazan (purple) in living cells.

WPBTS Controls recruitment

N= 439

Of each population group
- Caucasian
- Afrikaner
- Mixed Ancestry
- Black

BCH Patients recruitment

N=115

MDR-TB patients on Aminoglycosides

South African Family

(A1555G Pos)

Family has a history of streptomycin- induced hearing loss

Family follow ups

SNaPshot analysis of 6 mutations in MT-RNR1.

A1555G C1494T
T1095C A827G
961delT T1291C

Develop a method to detect heteroplasmy by PCR-RFLP on ABI

35delG in Connexin 26

Search for modifiers by:
1) Haplogroup analysis
2) Nuclear genes
- TRMU
- GJB2
3) Mitochondrial gene
- MT-TS1

Assess mitochondrial function using the MTT assay

Diagnostic genetic reports

BCH patients with deafness & without any of the 6 known mutations

Whole mitochondrial genome sequencing

Identify novel variants

Investigate nucleotide and amino acid conservation across species

Patients were screened for novel mutations in *MT-RNR1*

Will use HRM to screen these patients

Samples with possible mutations will be sequenced to verify

Develop a rapid screening method to detect 35delG

HRM analysis

Samples with possible mutations will be sequenced

Allele frequencies of the 6 mutations in MT-RNR1 & 35delG will be determined

* Comparison of allele frequencies of the mutations between patients
and controls. Determine which mutations are most common in S.A. populations.
* Also determine what the frequencies are of the different mutations in the different ethnic groups
* Investigate a possible correlation between the mutations, deafness & a specific antibiotic

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2. Ethical approval

This study was approved by the University of Stellenbosch's Ethics Committee for Human Research (Study approval number N05/09/165). Written informed consent was obtained from all of the study participants (Appendix I).

3. Recruitment of Patients and Controls

A.   *Recruitment of patients currently receiving aminoglycoside treatment:*

Patients were recruited from the Brooklyn Chest Hospital in the Western Cape, South Africa. A total of 115 MDR-TB patients were included in the study. The group consisted of 57 Mixed Ancestry (50%), 52 Black (45%), 3 Cape Malay (2.6%), 2 Caucasians (1.7%) and 1 Somalian (0.8%) individual. Of the 115 patients 75 (63.5%) were female and 41 (34.5%) were male. The South African Mixed Ancestry population is an admixture of the indigenous African populations, Western European immigrants as well as slaves brought to South Africa from Madagascar, Malaysia and India (Nurse, Weiner, and Jenkins 1985).

At the time of the study, these patients were all receiving aminoglycoside antibiotics daily, for a period of 4 months, as standard treatment for multi-drug resistant (MDR-TB) (either streptomycin, kanamycin or amikacin). Regular hearing tests were administered on all patients to identify any high frequency hearing loss (Section 4, pg 63) as a result of aminoglycoside ototoxicity. Patients who had inner ear infections were excluded from the study.

B.   *Control subjects:*

Control subjects were recruited from the Western Province Blood Transfusion Service (WPBTS) in Cape Town. The subjects were representative of the 4 major ethnic groups found in the Western Cape and consisted of:

- 93 Afrikaner,

- 104 Caucasian,

- 112 Black and

- 130 Mixed Ancestry individuals.

C.   *Family with A1555G mutation*:

For the present study we recruited 97 individuals from this family which included children from as young as one month of age. Seven members already have hearing impairment following previous exposure to streptomycin for pulmonary tuberculosis (Gardner et al. 1997) with an additional four who are possibly affected but have not been clinically assessed by an audiologist.

4. Audiological Monitoring of MDR-TB patients

Audiological examinations were performed by trained audiologists at the Brooklyn Chest Hospital on all the study participants recruited for this study. Audiological examinations were performed on each patient at the start of and during their treatment with aminoglycosides. The first hearing test was performed within the first week of the patient's admittance to the hospital in order to establish a baseline hearing profile. Thereafter, the patient's hearing was monitored at regular intervals of approximately 4 weeks during their hospitalization. A final hearing test was performed when the patient was discharged from the hospital after about 4 months.

The severity of the hearing impairment was classified into five grades: mild: 20-39 decibels (dB), moderate: 40-54dB, moderate severe: 55-69dB, severe: 70-89dB and profound: 90db or greater.

All the tests were done in a sound-treated booth in the Audiology department at the Brooklyn Chest Hospital. A portable tympanometer was used for tympanometry and an Amplaid 308 audiometer and TDH-39 headphones were used for the conventional audiometry. All of the equipment was calibrated according to the manufacturers' specifications, prior to testing. Before commencing the entire test protocol, all tests to be performed was explained to the participant in the appropriate language. Clear instructions was given before each procedure, and repeated during the test if necessary. All patients were seated in a comfortable chair for the duration of the tests which lasted for approximately 30 minutes.

The following tests were conducted at each of the baseline and evaluation sessions: pure tone audiometer (subjective test), tympanometry and distortion product otoacoustic emissions (DPOAE) (objective tests).

Pure tone air conduction testing

This test involves presenting the patient with pure tones from 250 to 8000 Hertz (Hz), at octave intervals. The audiologist starts the testing at 0dB at 1000Hz using an ascending approach. The participant was asked to raise his/her hand every time a tone was detected. This response method was chosen because peripheral neuropathy is often present in TB-sufferers, which could interfere with their ability to press the button. The results were recorded in dB on an audiogram.

Tympanometry

With this test a probe is inserted into the external auditory meatus, whereby pressure is automatically applied onto the tympanic membrane. This test measures the compliance of the middle ear system, the ear canal volume and the middle ear pressure which is recorded by the automatic tympanometer. If any abnormalities were detected the patient was referred to the medical practitioner and the patient was excluded from the study.

DPOAE

Otoacoustic emissions are used to assess the functioning of the outer hair cells of the cochlea (Hall 2000). For this test, a probe is inserted into the external auditory meatus and two pure tones are presented to one ear at the same time. The frequency range for the input stimuli were from 1000 to 8000 Hz, with an $f_2{}^1/f_1{}^2$ ratio of 1.22 (f_2: higher frequency primary and f_1: lower frequency of the pair of eliciting stimuli). Three points per octave was recorded. $L_1{}^3$ (intensity of f_1) was equal to 65dB and $L_2{}^4$ (intensity of f_2) was 55dB. For the duration of this test the intensity of the presented signals was kept constant and the frequency varied systematically, progressing from the lowest to the highest frequency. The results were recorded in a DP-gram. The reliability and viability of the *DPOAEs* were enhanced by keeping the noise levels in the room where the test was conducted as low as possible (10dB).

5. DNA extraction and quantification

Peripheral blood samples were collected in EDTA-coated tubes from all adult study participants. DNA extraction was done by Mrs Ina le Roux and Miss Melissa du Plessis on whole blood using a modified phenol/chloroform method in the Molecules and Genes in Inherited Conditions (MAGIC) laboratory, of the Department of Biomedical Sciences (Appendix II) (Corfield et al. 1993).

Buccal swabs: For children under the age of 12 years, buccal swabs (Dacron) (Copan, Italy) were taken and genomic DNA extracted using the QIAamp® DNA Blood Mini Kit (Appendix Ⅲ) (Qiagen, Germany).

DNA quantification: After extraction, the concentration of each sample was determined on the Nanodrop® ND1000-UV-Vis spectrophotometer (NanoDrop Technologies, Inc. USA) following the manufacturer's instructions. The samples were then diluted down to 200ng/µl (for DNA extracted from blood) which was the working concentration for all later reactions. DNA extracted from buccal swabs was not diluted and were used at the concentration spectrophotometrically determined.

6. Primer Design and Polymerase Chain Reaction (PCR) amplification

All polymerase chain reaction (PCR) primers used in this study were designed using Primer 3 software (Rozen and Skalersky 2000) and sequences available from the Genbank database (http://www.ncbi.nlm.nih.gov/Entrez). Primers were synthesized by either the Synthetic DNA Laboratory (Department of Cellular and Molecular Biology, University of Cape Town, South Africa) or Integrated DNA Technologies. Melting Point temperatures (Tm) for each primer used in this study were manually calculated using the following formula:

$$Tm = 4(G+C) + 2(A+T)$$

6.1. ABI Prism® SNaPshot^{TM} Multiplex system (Snapshot analysis)

Primers were designed to PCR-amplify the entire mitochondrial *MT-RNR1* gene that contained all six mutations previously associated with aminoglycoside-induced deafness. A total of eight primers were designed for use in SNaPshot analysis. Primer set one was used for the amplification of the entire *MT-RNR1* containing all six of the mutations. A single SNaPshot primer was designed for each mutation in either the forward or reverse orientation, as shown in Table 2.1 and was used for single base extension during Snapshot analysis (Section 8, pg 79). Each SNaPshot primer had a different length in order to avoid any overlap between the final SNaPshot primer extension products when all the primers were combined in a single SNaPshot reaction.

Table 2.1: Primers used for amplification of the region containing the six mutations in *MT-RNR1* and primers used for SNaPshot analysis.

PCR Primers	Primer name	Primer Sequence	%GC	Primer Tm	Ta (°C)	Product size
	SET 1					
Forward	All 5 Fwd	5'-caa cca aac ccc aaa gac ac-3' (20bp)	50%	58	55	1.124bp
Reverse	Set A Rev	5'-gct cag agc ggt caa gtt aag-3' (21bp)	52%	60		
Orientation	**SNaPshot Primers**					
Reverse	A827G (Rev)	5'-gct tag tta aac ttt cgt tta ttg cta aag g-3' (31bp)	32%	82		N/A
Reverse	961delT (Rev)	5'-tca ggt gag ttt tag ctt tat tgg gg-3' (26bp)	42%	74		N/A
Forward	T1095C (Fwd)	5'-ctg gga tta gat acc cca cta tgc t-3' (25bp)	48%	74		N/A
Forward	C1494T (Fwd)	5'-cgt aca cac cgc ccg tca c-3' (19bp)	68%	64		N/A
Forward	A1555G (Fwd)	5'-tac gca ttt ata tag agg ag-3' (20bp)	35%	54		N/A
Reverse	T1291C (Rev)	5'-taa cgt ctt atc gtg ggt act tgc gct tac ttt gt-3' (35bp)	40%	63		N/A

bp: Base pairs, %GC: percentage Guanine and Cytosine, Tm: Melting temperature, Ta: annealing temperature, N/A: not applicable.

Amplification with primer Set1 produced a fragment of 1.124kb and included all six mutations **(Figure 2.1)**.

All 5 Fwd
→

cca accaaacccc aaagacaccc cccacagttt atgtagctta cctcctcaaa gcaatacact gaaaatgttt agacgggctc

acatcacccc ataaacaaat aggtttggtc ctagcctttc tattagctct tagtaagatt acacatgcaa gcatccccgt tccagtgagt

tcaccctcta aatcaccacg atcaaaagga acaagcatca agcacgcagc aatgcagctc aaaacgctta gcctagccac

A827G
accccacgg gaaacagcag tgatta*Acct ttagcaataa acgaaagttt aactaag*cta tactaacccc agggttggtc

aatttcgtgc cagccaccgc ggtcacacga ttaacccaag tcaatagaag ccggcgtaaa gagtgtttta gatcaccccc

961delT + ins C$_n$
*Tccccaataa agctaaaact cacctga*gtt gtaaaaaact ccagttgaca caaaatagac tacgaaagtg gctttaacat

T1095C
atctgaacac acaatagcta agacccaaa*c tgggattaga taccccacta tgctT*agccc taaacctcaa cagttaaatc

aacaaaactg ctcgccagaa cactacgagc cacagcttaa aactcaaagg acctggcggt gcttcatatc cctctagagg

agcctgttct gtaatcgata aaccccgatc aacctcacca cctcttgctc agcctatata ccgccatctt cagcaaaccc

T1291C
tgatgaaggc *Tacaaagtaa gcgcaagtac ccacgtaaag acgtta*ggtc aaggtgtagc ccatgaggtg gcaagaaatg

ggctacattt tctaccccag aaaactacga tagcccttat gaaacttaag ggtcgaaggt ggatttagca gtaaactaag

C1494T
agtagagtgc ttagttgaac agggccctga agcg*cgtaca caccgcccgt cacC*ctcctc aagtatactt caaaggacat

A1555G
ttaactaaaa cccc*tacgca tttatataga ggagA*caagt cgtaacatgg taagtgtact ggaaagtgca cttggacgaa

ccagagtgta gcttaacaca aagcacccaa cttacactta ggagatttca a**cttaacttg accgctctga gc**

←
Set A Rev

Figure 2.1: Representation of the 1.124kb fragment generated by PCR-amplification for SNaPshot analysis. Each of the six mutations (in capital letters) in the fragment is indicated by a different color together with their corresponding SNaPshot primers used during SNaPshot analysis.

6.2. Detection of the 35delG mutation in Connexin 26

For detection of the 35delG mutation in the *GJB2* previously reported primers were used to amplify a region in exon 2, in which the mutation is located. The forward primer is situated approximately 51bp into the intronic region and was taken from Xing *et al.,* 2006. The reverse primer is situated within the coding sequence (van Eyken et al., 2007). Table 2.2 shows the forward and reverse primer sequences as well as the corresponding primer Tm and PCR product size.

Table 2.2: Primers used for amplification of segment of exon 2 containing the 35delG mutations in *GJB2*.

PCR Primers	Primer Name	Primer Sequence	%GC	Primer Tm	Ta (°C)	Product size
Forward	**35 delG Fwd**	5'-cct gtg ttg tgt gca ttc gt-3' (20bp)	50%	60		
Reverse	**35delG van Eyken Rev**	5'-ctt tcc aat gct ggt gga gtg-3' (21bp)	52%	64	56	117bp

bp: Base pairs, %GC: percentage Guanine and Cytosine, Tm: Melting temperature, Ta: annealing temperature.

The PCR fragment produced from these primers is shown in **Figure 2.2**.
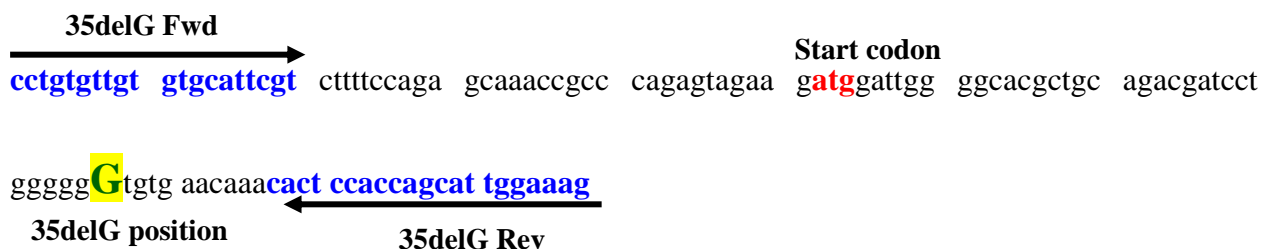
Figure 2.2: PCR fragment for the detection of the 35delG mutation in *GJB2*.

6.3. Detection of levels heteroplasmy of mutations associated with aminoglycoside induced deafness

For the detection of percentage heteroplasmy either the forward or reverse primers were labelled with a fluorescent dye (FAM) in order to detect the PCR product using the ABI Prism 3130xl® Genetic analyzer (Applied Biosystems, Foster City, CA, USA) . The primer sequences used for the detection of percentage of heteroplasmy for A1555G and $961delT + insC_{(n)}$ is shown in Table 2.3

Table 2.3: **Primers for the detection of heteroplasmy for the six mutations associated with aminoglycoside induced deafness.**

Mutation	Primer name	Primer Sequence	%GC	Primer Tm	Ta (°C)	Product size
961delT + insC(n)						
Forward	961delT Fwd	5' ggc tca cat cac ccc ata aa 3'(20bp)	50%	60	57	475bp
Reverse	961delT Rev (FAM labeled)	5' ggg cta agc ata gtg ggg ta 3'(20bp)	55%	59		
A1555G						
Forward	Set A Fwd (FAM labeled)	5' ccc cag aaa act acg ata gcc (21bp)	52%	60	60	298bp
Reverse	Set A Rev	5' gct cag agc ggt caa gtt aag 3'(21bp)	52%	60		

bp: Base pairs, %GC: percentage Guanine and Cytosine, Tm: Melting temperature, Ta: annealing temperature.

6.4. Detection of variants in tRNA$^{(Ser)(UCN)}$

For the detection of variants in the mitochondrial *MT-TS1* (tRNA$^{(Ser)(UCN)}$) gene, primers were designed to flank the target sequence. Due to the small size of all tRNA genes in the mitochondrial genome and the absence of introns, the targeted fragment included another tRNA gene, *MT-TD* (tRNA$^{(Asp)}$) which lies directly adjacent to tRNA$^{(Ser)(UCN)}$. Both the tRNA$^{(Asp)}$ and tRNA$^{(Ser)(UCN)}$ genes are situated between the two Cytochrome Oxidase 1 and 2 (*CO1* and *CO2*)

genes. Table 2.4 shows the primer sequences used for the detection of novel variants in the *tRNA*$^{(Ser)(UCN)}$ gene.

Table 2.4: Primers for the detection of novel variants in the mitochondrial *MT-TS1* gene.

PCR Primers	Primer name	Primer Sequence	%GC	Primer Tm	Ta (°C)	Product size
Forward	**tRNA Fwd**	5' agt gac tat atg gat gcc cc 3' (20bp)	50%	56	56ºC	286bp
Reverse	**tRNA Rev**	5' ggc gtg atc atg aaa ggt g 3' (19bp)	53%	60		

bp: Base pairs, %GC: percentage Guanine and Cytosine, Tm: Melting temperature, Ta: annealing temperature.

tRNA Fwd

agtgactata tggatgcccc ccaccctacc acacattcga agaacccgta tacataaaat ctaga**caaaa aaggaaggaa**

tcgaacccccc caaagctggt ttcaagccaa ccccatggcc tccatgactt tttcaaaaag gtattagaaa aaccatttca

taactttgtc aaagttaaat tataggctaa atcctatata tctta atggc acatgcagcg caagtaggtc tacaagacgc tacttcccct

atcatagaag agcttatcac ctttcatgat cacgcc

tRNA Rev
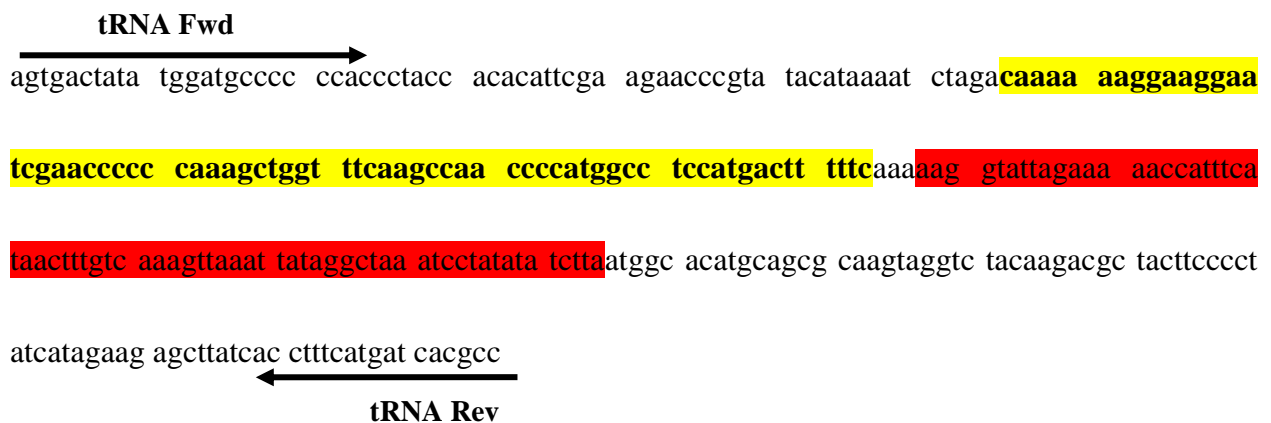
Figure 2.3: PCR fragment for the detection of novel variants in the mitochondrial *MT-TS1* gene. Amplification with these primers produced a fragment of 286bp. *MT-TS1* is highlighted in yellow and the *MT-TD* gene is highlighted in red.

6.5. Detection of the A10S variant in *TRMU*

Primers designed for the detection of the A10S variant in *TRMU* generated a fragment of 165bp. The A10S (G28T) variant is situated within the first exon of this gene. Table 2.5 shows the primer sequences used for the detection of this variant.

Table 2.5: Primers for detection of the A10S variant in the *TRMU*

PCR Primers	Primer Set	Primer Sequence	%GC	Primer Tm	Ta (°C)	Product size
Forward	**TRMU Fwd**	5' ctg cag ctg gcg aag ttg 3' (18bp)	61%	61	52ºC	165bp
Reverse	**TRMU Rev**	5' ggt ttc cgg gga cac att 3' (18bp)	55%	60		

bp: Base pairs, %GC: percentage Guanine and Cytosine, Tm: Melting temperature, Ta: annealing temperature.

TRMU Fwd

A10S

ctgcagctggcg aagttgggcg actggcggat gcaggccttg cggcacgtcg tgtgc**G**ccct gtccggcggc gtggacagcg

ccgtggccgc gctgctgctg aggcggagag gtgaggcgtc cgaggctccc gccccccgcc gagcgaatgt gtccccggaa acc

TRMU Rev
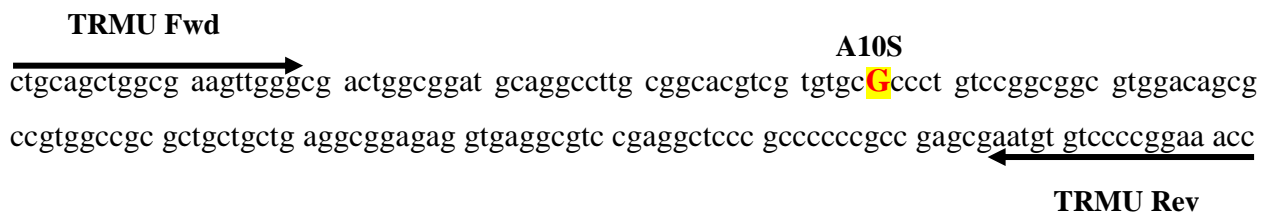
Figure 2.4: PCR fragment for the detection of the A10S variant in *TRMU*. Amplification with these primers produced a fragment of 165bp. The A10S variant is highlighted in yellow.

6.6. Detection of novel variants in *MT-RNR1*.

For detection of novel variants in *MT-RNR1*, the entire region of this gene was divided into overlapping fragments of between 180-280bp. Table 2.5 shows the primers sequences of each of the six sets (A to F) of overlapping fragments. Figure 2.4 shows the entire *MT-RNR1* with all six of the overlapping fragments.

Table 2.6: List of primers for the detection of novel variants in the mitochondrial *MT-RNR1* gene

Fragment	Primer Set	Primer Sequence	%GC	Primer Tm	Ta (°C)	Product size
A	All 5 Fwd	5'-gct tac ctc ctc aaa gca at-3' (20bp)	50%	60	58	272 bp
	All 5 Rev	5'-agg tta atc act gct gtt tc-3' (20bp)	40%	51		
B	Set 2 Fwd	5'-cac ggg aaa cag cag tga tt-3'(20bp)	50%	61	60	183 bp
	Set 2 Rev	5'-ctc agg tga gtt tta gct tt-3' (20bp)	40%	51		
C	Set 3 Fwd	5'-gat cac ccc ctc ccc aat aa-3' (20bp)	55%	63	62	190 bp
	Set 3 Rev	5'-ttc tgg cga gca gtt ttg tt-3' (20bp)	45%	60		
D	Set 4 Fwd	5'-gct tag ccc taa acc tca ac-3' (20bp)	50%	55	60	199 bp
	Set 4 Rev	5'-gcc ttc atc agg gtt tgc tg-3' (20bp)	55%	63		
E	Set 5 Fwd	5'-acc acc tct tgc tca gcc ta-3' (20bp)	55%	60	62	194 bp
	Set 5 Rev	5'-tgc taa atc cac ctt cga ccc tta3'(24bp)	45%	64		
F	Set 6 Fwd	5'-gaa act taa ggg tcg aag gt -3'(20bp)	52%	59°C	58	272 bp
	Set A Rev	5'-gct cag agc ggt caa gtt aag-3' (21bp)	52%	60		

Fwd: Forward, Rev: Reverse, bp: Base pairs, %GC: percentage Guanine and Cytosine, Tm: Melting temperature, Ta: annealing temperature

A Fwd

```
cca    accaaacccc  aaagacaccc  cccacagttt  atgtagctta  cctcctcaaa  gcaatacact
```

```
gaaaatgttt  agacgggctc  acatcacccc  ataaacaaat  aggtttggtc  ctagcctttc  tattagctct
```

```
tagtaagatt  acacatgcaa  gcatccccgt  tccagtgagt  tcaccctcta  aatcaccacg  atcaaaagga
```

B Fwd

```
acaagcatca  agcacgcagc  aatgcagctc  aaaacgctta  gcctagccac  accccacgg  gaaacagcag
```

A Rev

```
tgattaacct  ttagcaataa  acgaaagttt  aactaagcta  tactaacccc  agggttggtc  aatttcgtgc
```

A Rev

C Fwd

```
cagccaccgc  ggtcacacga  ttaacccaag  tcaatagaag  ccggcgtaaa  gagtgtttta  gatcaccccc
```

```
tccccaataa  agctaaaact  cacctgagtt  gtaaaaaact  ccagttgaca  caaaatagac  tacgaaagtg
```

B Rev

D Fwd

```
gctttaacat  atctgaacac  acaatagcta  agacccaaac  tgggattaga  taccccacta  tgcttagccc
```

C Rev

```
taaacctcaa  cagttaaatc  aacaaaactg  ctcgccagaa  cactacgagc  cacagcttaa  aactcaaagg
```

```
acctggcggt  gcttcatatc  cctctagagg  agcctgttct  gtaatcgata  aaccccgatc  aacctcacca
```

E Fwd

```
cctcttgctc  agcctatata  ccgccatctt  cagcaaaccc  tgatgaaggc  tacaaagtaa  gcgcaagtac
```

D Rev

```
ccacgtaaag  acgttaggtc  aaggtgtagc  ccatgaggtg  gcaagaaatg  ggctacattt  tctaccccag
```

F Fwd

```
aaaactacga  tagcccttat  gaaacttaag  ggtcgaaggt  ggatttagca  gtaaactaag  agtagagtgc
```

E Rev

```
ttagttgaac  agggccctga  agcgcgtaca  caccgcccgt  caccctcctc  aagtatactt  caaaggacat
```

```
ttaactaaaa  cccctacgca  tttatataga  ggagacaagt  cgtaacatgg  taagtgtact  ggaaagtgca
```

```
cttggacgaa  ccagagtgta  gcttaacaca  aagcacccaa  cttacactta  ggagatttca  acttaacttg
```

```
accgctctga  gc
```

F Rev

Figure 2.5: Sequence of *MT-RNR1*. The six overlapping fragments and their corresponding primers all indicated in various colours. Fragment A: Red; B: Blue; C: Green; D: Purple; E: Orange; F: Pink.

6.7. Polymerase Chain Reaction (PCR)

6.7.1. PCR Amplificationon the GeneAmp® PCR system 2720 Thermal Cycler

200ng genomic DNA was used as template for all the PCR amplifications. PCR reactions was performed in 50µl reactions using the GeneAmp® PCR system 2720 Thermal Cycler (Applied Biosystems). Each PCR reaction consisted of 10pmol of each primer (section 5), 75µM dinucleotide triphosphates (dATP, dTTP, dCTP, dGTP) (Bioline Ltd, London, UK), 5% dimethyl sulfoxide (DMSO) (only used for *TRMU* primers discussed in section 6.5, pg 72), 0.5 Units BIOTAQ^{TM} DNA Polymerase (Bioline Ltd, London, UK), 1×NH$_4$ Buffer (Bioline Ltd, London, UK), 1.5mM MgCl$_2$ (Bioline Ltd, London, UK) and ddH$_2$O (Fresenius Kabi) to a final volume of 50µl.

The following PCR cycling conditions were followed: an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, varying annealing temperatures (depending on the primer set used) for 30sec, and extension at 72°C for 45sec. A final extension step of 72°C for 5min was added to the conditions. All PCR products were resolved by electrophoresis on 1% or 2% Agarose (Whitehead Scientific, South Africa) gels (Section 9, pg 81). After amplification all PCR products were stored at 4°C until further use.

6.7.2. Amplification on the Rotor-Gene 6000

For PCR amplification on the Rotor-Gene 6000 (Corbett Life Science, Australia) all DNA sample concentrations were diluted to 10ng/µl. The PCR reaction setup required the addition of the intercalating dye SYTO9 (1µM) (Invitrogen, USA). PCR cycling conditions were as follows: 94°C for 5min, 94°C for 15sec, varying annealing temperatures (depending on the primer set used) for 15sec, and extension at 72°C for 20sec. A final extension step of 72°C for 20sec was added to the conditions. After amplification all PCR products were stored at 4°C in the dark until further use.

7. Gel electrophoresis

7.1. Agarose gel electrophoresis

For the verification of the success of PCR amplification all PCR products were subjected to Agarose gel electrophoresis. All gels were made up to a specific percentage (w/v) depending on the size of the fragment. The appropriate amount of Agarose (Whitehead Scientific, South

Africa) was weighed out and dissolved in 1×Sodium Borate (SB) (Appendix III) buffer at a high temperature. A total of 2µl of 1µg/µl Ethidium Bromide was added to the gel solution for visualisation of DNA fragments under an Ultra Violet (UV) light. Eight µl of each sample was mixed with approximately 2µl of Bromophenol Blue (Appendix III) loading dye. Each sample was then loaded into a separate well of the gel. Two µl of 100bp or 200bp DNA ladder depending on the size of the fragment (Promega, USA) was also loaded in a well to serve as a size standard against which the size of the PCR fragment can be verified. Samples were resolved by electrophoresis at 240 volts (V) for 15 – 20 min in 1×SB running buffer. After electrophoresis samples were viewed under a UV light (Transilluminator model LMS-26E, USA) and photographed.

7.2. Non-denaturing polyacrylamide gel electrophoresis (PAGE)

For the detection of products from PCR-RFLP analysis, non-denaturing polyacrylamide gels were used due to better resolving power compared to agarose gels. The method involves the size separation of DNA fragments using a 12% polyacrylamide solution (Promega, USA) (Appendix III).

7.2.1. PAGE Gel Casting

Two 72mm × 100mm or 180mm × 210mm glass plates were used for casting of the gel. Plates were washed beforehand with Cal-liquid hand soap (Cal-Chem, South Africa), rinsed and dried with paper towels. The plates were treated with 70% ethanol sprayed onto the surface of the plates and wiped clean with a paper towel. One millimetre spacers were sprayed with 70% ethanol and placed onto the back plate, thereafter the front plate was placed on top of the spacers to create a space for the later insertion of the comb. The two plates were clamped firmly together and sealed at the bottom and sides with gel-sealing tape (Sigma, Germany). Finally, the gel solution was poured between the two plates and the well-forming comb inserted in between the plates. The gel was allowed to polymerise for approximately 45 minutes before use.

7.2.2. Electrophoresis

After polymerisation of the gel, all the gel sealing tape was removed from all sides of the plates and the well-forming comb was also removed. The gel was vertically mounted and clamped onto the gel electrophoresis apparatus (Scigen vertical apparatus, Whitehead Scientific, South Africa). The buffer chambers were filled with 1×TBE electrophoresis buffer (Appendix III) and the wells in the gel were cleaned with the buffer to remove any acrylamide residue. Twelve µl of digested product was mixed with 10µl bromophenol blue loading dye to give the sample density and aid in the visualisation. Afterwards samples, including an undigested sample and digested (*Pst1)* lambda DNA (Promega Corp, Madison Wisconsin USA) (as DNA ladder), were loaded into separate wells and electrophoretically separated for 1 and a half hours at 240 volts at room temperature.

7.2.3. Silver staining

After completion of electrophoresis of samples, the gel was dismantled and the gel gently removed from the glass plates. The gel was placed in a plastic tray and rinsed with ddH$_2$O. After rinsing the gel was covered with Staining solution, 0.1% Silver Nitrate (AgNO$_3$) (Solution 1) (Appendix III) and placed on a shaker (Labdesign, Maraisburg) for 10 minutes. After 10 minutes, the solution 1 was decanted and the gel rinsed with ddH$_2$O. Subsequently, the gel was covered with the developing solution (Solution 2) (Appendix III) and placed on the shaker for approximately 30 minutes or until the stained bands were visible. After staining the gel was sealed in a plastic sleeve for future reference.

7.3. Electrophoresis on ABI 3130xl

Electrophoresis of fragments were done by the Core Sequencing Facility of the Department of Genetics, University of Stellenbosch using the BigDye terminator V3.1 Ready reaction kit (Applied Biosystems, Foster City, CA, USA) on the ABI 3130xl ® Genetic analyzer (Applied Biosystems Inc, Foster City, CA, USA). Two µl of the sample was mixed with 0.5 µl Liz 120 size standard (Applied Biosystems, Foster City, CA, USA) and 9 µl of HiDi formamide prior to denaturing of the products for five minutes at 95°C. Directly after heating of the samples they were placed on ice for approximately five minutes before electrophoresis. Samples were then loaded on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and electrophoresed using a 50 centimetre (cm) capillary (Applied Biosystems, Foster City, CA,

and POP 7 polymer. All samples were genotyped using the GeneMapper software V3.7 (Applied Biosystems, Foster City, CA, USA).

8. ABI Prism® SNaPshot™ Multiplex system (Snapshot analysis)

For the detection of the six mutations associated with aminoglycoside-induced deafness, the ABI Prism® SNaPshot™ multiplex system was used as a multiplex mutation detection technique. SNaPshot allows for the detection of several single nucleotide polymorphisms (SNPs) within a single reaction. SNaPshot analysis makes use of a single base primer extension technique in the presence of fluorescently labelled dNTPs **(Figure 2.6)** (Makridakis and Reichardt 2001).
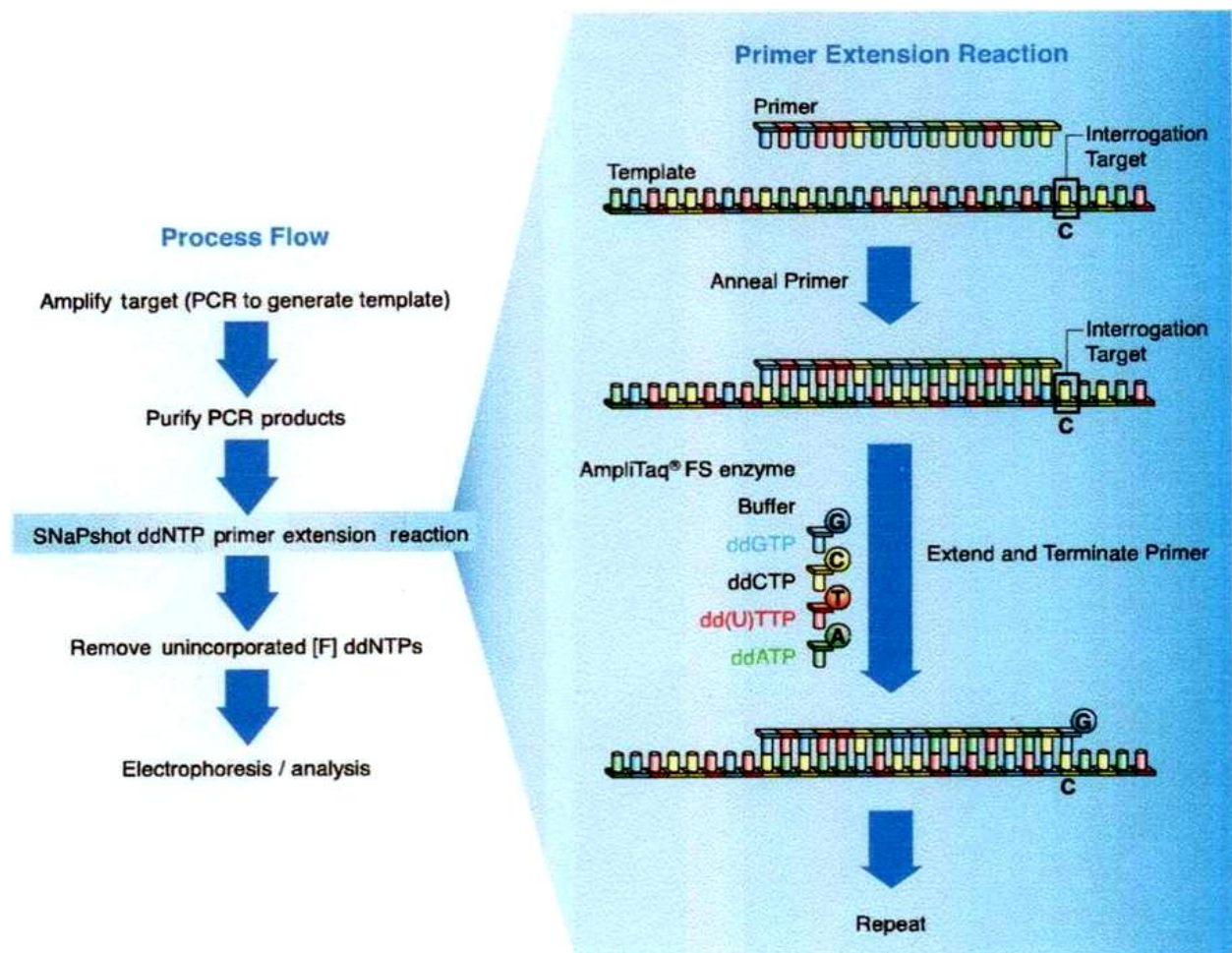
Figure 2.6: Schematic overview of the SNaPshot primer extension technique

(Taken from http://www.appliedbiosystems.com)

The SNaPshot technique is a stepwise procedure that involves the PCR amplification of a fragment containing all the SNPs of interest. PCR amplification of the target fragment was performed as discussed in section 6.7, pg 74. After amplification PCR products were purified in order to remove all unincorporated dNTPs and excess primers. Purification was done by adding 1U of shrimp alkaline phosphatase (SAP) (Promega, USA) and 0.2U *Exonuclease I* (Exo I) (AEC Amersham) to 3µl of PCR product. Samples were then incubated at 37°C for 60min followed by 75°C for 30min allowing inactivation of the enzymes. Each SNaPshot reaction was carried out in a final volume of 10µl that consisted of 3µl of purified PCR product, 3µl SNaPshot Ready Reaction Mix (Applied Biosystems Inc, Foster City, CA, USA) and 1.67µl of a mixture of each of the six extension primers (Section 6.1). Each of the six primers was at a concentration of 1.8µM except for the A1555G primer that was at a concentration of 2.9µM. Each sample was then subjected to thermal cycling according to the manufacturer's instructions in a GeneAmp 9700 (Applied Biosystems Inc, Foster City, CA, USA). Cycling conditions for the single base extension consisted of 27 cycles of 96°C for 10sec, 50°C for 5sec and 60°C for 30sec. Following the primer extension, all unincorporated fluorescently labeled ddNTPs were removed by adding 1U of SAP followed by incubation for 60min at 37°C then at 75°C for 30min to inactivate the SAP enzyme. Samples were denatured in a loading mix containing 1µl of product, 0.5µl of GeneScan™-120LIZ™ size standard (Applied Biosystems, Foster City, CA, USA) and 9µl HiDi formamide (Applied Biosystems, Foster City, CA, USA) . The LIZ size standard was used in order to determine the size of each fragment. The samples were loaded on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and electrophoresed in a filterset E5; on a 36cm capillary with POP7 polymer. 1X TBE (Appendix III) was used as running buffer according to custom-made run module conditions. The allele at each SNP or mutation site is generated into a peak (**Figure 2.7**). Samples were genotyped and data analyzed using GeneMapper software version 3.7 (Applied Biosystems, Foster City, CA, USA).
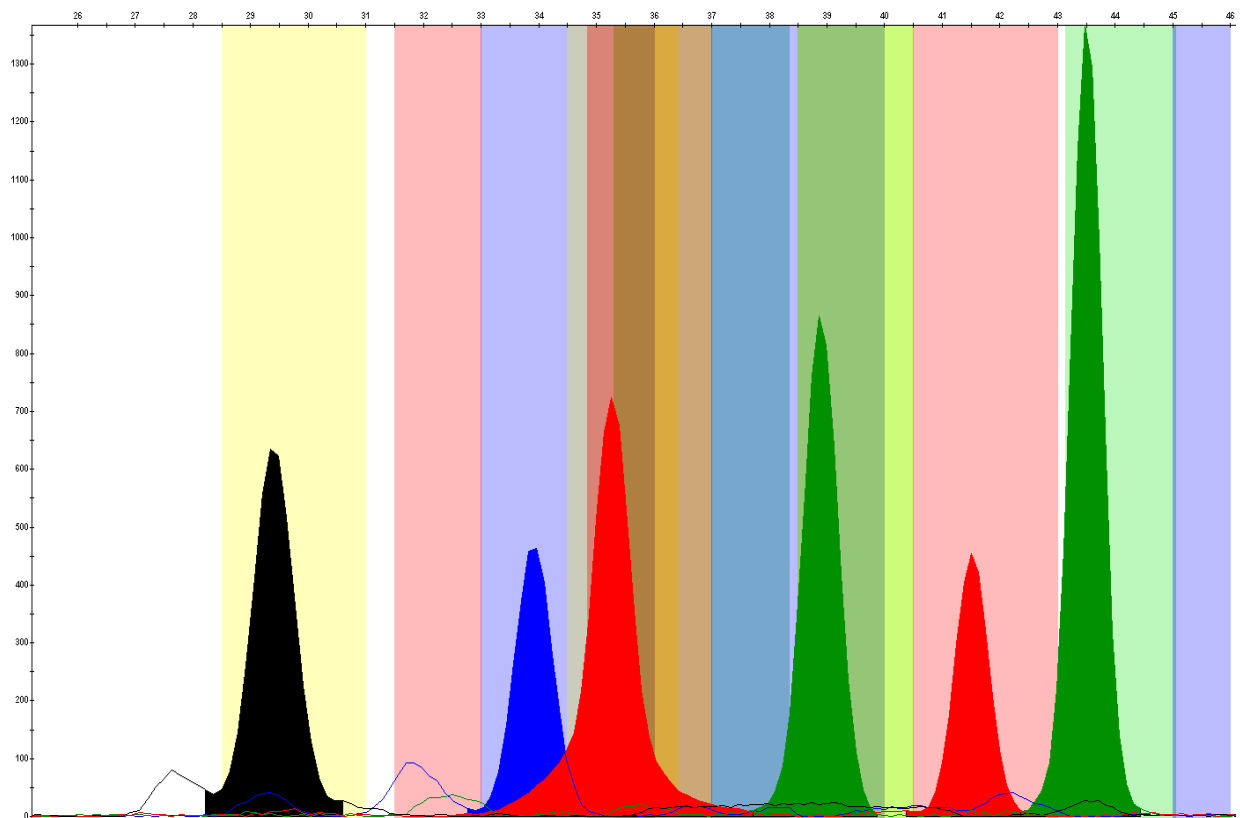
Figure 2.7: **Representation of an electropherogram demonstrating allele calling for SNPs. Blue: G allele, Green: A allele, Red: T allele, Black: C allele** (Bardien et al. 2009).

9. Mutation detection using High Resolution Melt (HRM) Analysis

After amplification, the PCR products underwent a high resolution melt (HRM) on the Rotor-Gene 6000 analyzer (Corbett Life Science, Australia). This technique is used to characterize a DNA sample according to a shift in fluorescence as the PCR product dissociates from double stranded DNA to single stranded DNA with an increase in temperature (**Figure 2.8**).
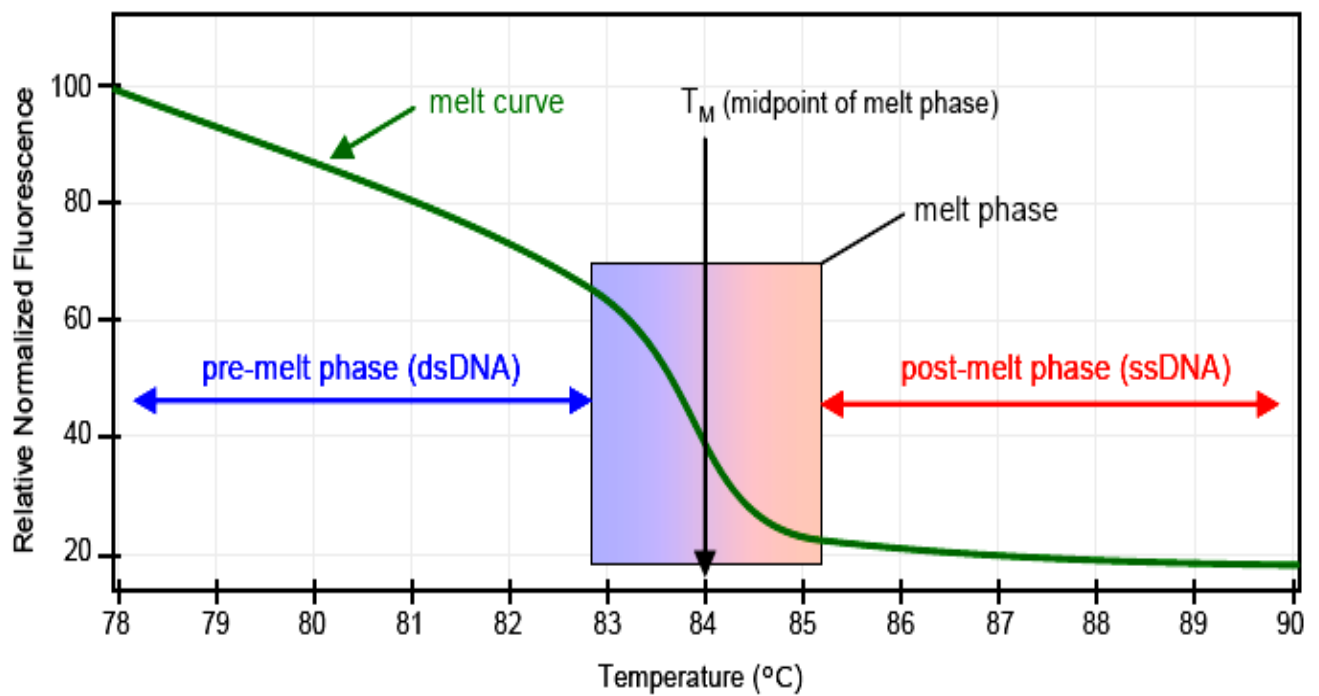
Figure 2.8: Representation of a typical HRM melt curve. The melt curve demonstrates the transition from high fluorescence of the pre-melt phase to a sharp decrease in fluorescence as the intercalating dye is released. The intercalating dye is released as the double stranded DNA dissociates (melts) into single stranded DNA. The midpoint of the melt phase defines the temperature of melting (T_m) of the particular DNA fragment (Taken from: http://www.corbettlifescience.net/public/Rotor-Gene%206000/hrm_corprotocol.pdf).

SYTO 9 (Invitrogen, USA) is one of the fluorescent dyes used in HRM analysis and it intercalates with double stranded DNA (**Figure 2.9**). At the beginning of a melt when only double stranded products are present, the fluorescence is high. As the products dissociate from double stranded to single stranded DNA, the dye is released resulting in a decrease in fluorescence and a melt curve is observed.
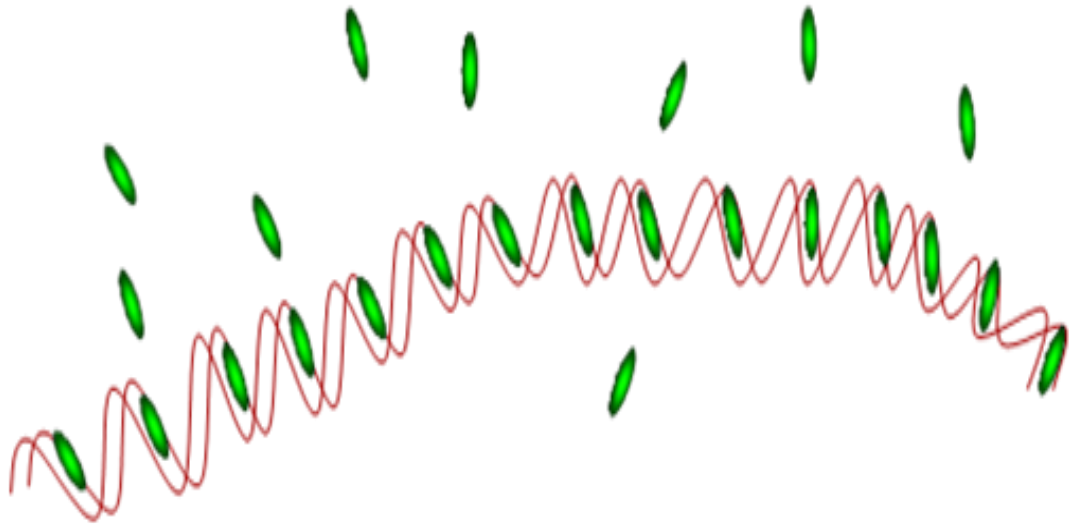
Figure 2.9: Schematic illustration of the binding of the intercalating dye Syto 9 to double stranded DNA (Taken from: http://www.corbettlifescience.net/public/Rotor-Gene%206000/hrm_corprotocol.pdf).

Each DNA sample has its own unique melt curve, so that when an unknown mutation or SNP is present, in a particular sample, the melt curve will differ from that of a wild type sample thereby indicating that a sequence variation is present.

Samples with different melting profiles to that of known wild types were sequenced to verify whether a variant was present.

10. Automated DNA sequencing analysis
10.1. Purification of PCR products

In some cases, purification of PCR products was needed for subsequent sequencing of products. In each case, DNA was purified using *Exonuclease I* (USB Corporation, USA) and Shrimp Alkaline Phosphatase (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. Eight µl of PCR product was carried over to a new 0.2 µl PCR tube (Whitehead Scientific, South Africa), 5U of *Exonuclease I* (Exo1) and 5U of Shrimp Alkaline Phosphatase (SAP) was added to each sample. The samples were then incubated at 37°C for 15min followed by 80°C for 15min to allow inactivation of the enzymes.

After purification, the concentration of each sample was determined on the Nanodrop® ND1000-UV-Vis spectrophotometer (NanoDrop Technologies, Inc. USA) following the manufacturer's instructions. The samples were then diluted down to 30ng/µl

10.2. Automated Sequencing Analysis

For all sequencing reactions the same primers were used as during the PCR amplification of the products. For sequencing of the cloned inserts vector specific primers (T7) were used for the reaction. Primers used for sequencing reactions were diluted to 1.1µM. All automated sequencing reactions of PCR amplified products and cloned inserts was performed at the Core Sequencing Facility of the Department of Genetics, University of Stellenbosch using the BigDye terminator V3.1 Ready reaction kit on the ABI 3130xl ® Genetic analyzer (Applied Biosystems Inc, Foster City, CA, USA).

10.3 Analysis of DNA Sequences

Analysis of sequencing data was done using the BioEdit version 7.0.5 software (Hall 1999). DNAman™ version 4 software (Lynnion Biosoft Corp©) were used to analyse all sequence alignments for cloned inserts and PCR fragments. Sequence alignments were done in order to verify that the insert of interest was present in the vector.

11. Whole mitochondrial DNA sequencing

Sequencing of the mitochondrial genome was performed on a group of eight multi-drug resistant tuberculosis (MDR-TB) samples. None of these samples had any of the known aminoglycoside-induced deafness mutations but was suffering from ototoxicity. DNA from each of the eight patients was sent to the Department of Clinical Biochemistry and Immunology, Statens Serum Institute, Copenhagen, Denmark where the whole mitochondrial sequencing was performed.

12. PCR-RFLP analysis

After PCR amplification, samples were digested with the appropriate restriction enzymes (Table 2.7), 10μl of the PCR product was added to a 0.5μl microfuge tube. Subsequently, 2μl 10×buffer, 2-5U restriction enzyme (Promega Corp, Madison Wisconsin USA) or (Fermentas life Sciences, UK) and water was added to make up a final volume of 20μl. Samples were incubated overnight at 37°C to allow digestion of the PCR fragment. After digestion, samples were electrophoresed on either ABI 3130xl Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA) or PAGE gels.

Table 2.7: Restriction enzymes used for the detection of each mutation.

Mutation	Restriction Enzyme	Recognition Site	Company
A1555G	Alw26I	gtctc	Fermentas
961delT + insC(n)	MnlI	cctc	Fermentas
G719A	EcoRI	g/aattc	Promega

13. Cloning

The phenomenon of heteroplasmy, where both mutant and wild type copies of mitochondrial DNA is present in the cell is common in mitochondrial DNA disorders. The proportion of mutant to wild type copies in the cells influence the phenotypic manifestation of the disease. In the case of non-syndromic hearing loss the proportion of the associated mutation influence the severity of the hearing loss ranging from moderate to profound (Guan, Fischel-Ghodsian, and Attardi 1996; Guan, Fischel-Ghodsian, and Attardi 2000; Guan, Fischel-Ghodsian, and Attardi 2001).

For the detection of different levels of heteroplasmy for the A1555G and 961delT + insC$_{(n)}$ mutations associated with aminoglycoside induced deafness a PCR fragment that contains a specific mutation of interest was cloned into a vector. In this study PCR fragments were cloned into the pGEM®-T Easy vector (Promega Corp, Madison Wisconsin USA) using the pGEM®-T Easy vector system. pGEM®-T Easy vectors are used in a variety of different applications from

cloning PCR products to blue/white screening for recombinants. The pGEM®-T Easy vector was prepared by cutting the vector with *Eco*R V and adding an additional 3' terminal thymidine (3'-T) to both ends of the insertion site. Therefore improving ligation efficiency of the PCR product into the plasmid by avoiding the recircularisation of the vector and providing compatible overhangs for the PCR products generated by certain polymerases. The multiple cloning region of the vector is located within the coding region of the enzyme ß-galactosidase. Upon successful cloning of an insert into the pGEM®-T Easy vector the normal coding sequence of the ß-galactosidase gene is disrupted. As a result, clones that contain the PCR insert will produce white colonies with clones containing no insert producing blue colonies in the presence of the substrate XGAL when grown on agar plates containing ampicillin.

13.1. Preparation of *E.coli* DH5α competent cells for bacterial transformation

In order to facilitate the selection and purification of constructs containing the PCR fragment of interest, ligation reactions (Section 8.2) were transformed into competent *E.coli* DH5α cells. For the preparation of competent *E.coli* DH5α cells a scrape of frozen (-70°C) *E.coli* DH5α cells were inoculated into 10ml LB media (Appendix Ш). The culture was incubated overnight at 37°C in a YIH DER model LM-530 shaking incubator (SCILAB instrument CO, Ltd, Taipei, Taiwan) at 200rpm. After inoculation, 200µl of the overnight culture was inoculated into a 2L Erlenmeyer flask (sealed with a cotton wool bung and tin foil lid) containing 200ml LB media. The bacterial cells were allowed to grow at room temperature, shaking at 85rpm, overnight or till the cells had grown to mid log phase (Optical density: 0.4-0.6nm). 200ml of the broth were then aliquoted into 4×50ml sterile Falcon polypropylene tubes and centrifuged at 3000rpm for 15min at 4°C. All steps to follow were done on ice.

After centrifugation the supernatant was discarded and the pellet was gently resuspended in one-third (16ml) of ice-cold CAP buffer (Appendix Ш). Cells were re-pelleted by centrifugation at 3000rpm for 15min at 4°C and the supernatant discarded. The cell pellet was gently resuspended in one-twelfth volume (4ml) CAP buffer. 200µl aliquots were prepared in 2ml microfuge tubes. Tubes were left overnight at 4°C and subsequently stored at -70°C until further use.

13.2. Ligation of wild type and mutant samples into vector

A DNA fragment that contains the mutation of interest was PCR amplified as discussed in section 6.7. After amplification, PCR products were diluted to 50ng/µl. Products were then ligated into the pGEM®-T Easy vector according to manufacturer's instructions (Appendix Ш). Ligations were allowed to take place overnight at room temperature.

13.3. Transformation of plasmid into *E.coli*

After ligation, 4µl of the ligation reaction was used to transform 200µl of competent cells that was allowed to thaw undisturbed on ice for 15-30min. After addition of the ligation reactions the mixture was mixed gently and left on ice for 20-30min. The 200µl of competent cells were transformed via heat shock by incubating the mixes for 45sec at 41°C. Thereafter the mixes were left at room temperature for 2 min. 1ml of LB media was added to the cells and the cells were incubated on a shaking incubator for 1 hour at 37°C. Afterwards the cells were plated on LB agar plates (Appendix Ш) to which 15mM of Isopropyl-beta-D-thiogalactopyranoside (IPTG), 5mg/ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (XGAL) and 100µg/ml ampicillin was added. Plates were then incubated at 37°C overnight.

Following incubation overnight, plates were checked for colony growth. If colony growth was observed a number of white colonies (believed to contain the PCR fragment of interest as discussed in section 8) were picked from the plates and subjected to PCR amplification. After amplification, products were electrophoresed as discussed in section 7.1 to check which colonies contained the correct sized fragment. Colonies with the correct fragment size were inoculated overnight with 10ml LB media together with 20µl ampicillin in a 50ml Falcon polypropylene tube at 37°C overnight in a shaking incubator.

13.4. Bacterial plasmid purification

Before plasmid DNA was isolated, freezer colonies were prepared by taking 500µl of the overnight cultures and adding 500µl of 50% glycerol into a 2ml microfuge tube. Tubes were frozen away at -70°C for later use. The remaining volume of the cultures was centrifuged for 10min at 3000rpm. After centrifugation the supernatant was discarded and plasmid DNA was

isolated using the GeneJET™ Plasmid Miniprep Kit (Fermentas life Sciences, UK) according the manufacturer's instructions.

14. Heteroplasmic Ratios

In order to generate templates of varying mutant to wild type DNA ratios, PCR fragments of wild type and mutant samples were cloned into *E.coli* (Section13.3), the DNA was isolated and purified (Section 13.4) and the appropriate mutant to wild type ratios of 100%, 50%, 25%, 12.5% and 6.25% were made. The different ratios were set up in order to simulate heteroplasmic ratios observed within the human cell.

15. Mitochondrial Haplogroup assignment

Mitochondrial haplogroup analysis reconstructs the evolutionary history of all the mitochondrial DNA lineages, found in all living people, to a common ancestor. This common ancestor is sometimes referred to as the "Mitochondrial Eve". This ancestor lived in Africa, about 150 000 years ago and lies at the root of all the maternal ancestries of all 6 billion people on earth. Since mitochondrial DNA is maternally inherited every person is a direct maternal descendant of this "Mitochondrial Eve". The hyper variable region in the mitochondrial genome is the most polymorphic and it is thus this region that is focused on when constructing an individual's mitochondrial haplogroup.

For this study, mitochondrial haplogroup analysis was done on an individual from the South African family with streptomycin induced deafness. The analysis was conducted at the Human Genomic Diversity and Disease Research Unit (University of the Witwatersrand, Johannesburg, South Africa).

16. Lymphocyte separation and transformation

Blood was collected from two family members (one member with the A1555G mutation and another wild type for the mutation) of the South African family with streptomycin-induced hearing loss. All lymphocyte separation and transformations was performed by Mrs Ina le Roux and is discussed in detail in Appendix IV.

17. MTT assay

MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assays are used to quantitatively measure cell survival and proliferation by means of a colour reaction. MTT measures the activity of various dehydrogenase enzymes (Slater et al., 1963) that reduces the yellow MTT to formazan (purple). The tetrazolium ring is cleaved in active mitochondria therefore the colour reaction can only take place in living cells (Mosmann 1983). This assay has also been used to determine the cytotoxicity of certain toxic medicinal agents (Mosmann 1983; Skehan et al. 1990; van de Loosdrecht et al. 1994). The reduction of MTT to formazan can only take place in cells that are metabolically active (i.e. living cells). In cells that are dead, either due to necrosis or apoptosis, no colour reaction will take place due to the fact that there are no active mitochondria or metabolically active enzymes to reduce the MTT to purple formazan.

The effect of streptomycin on the cell survival for both mutation positive (MT) and mutation negative (WT) cell lines for the A1555G mutation was tested using the MTT colorimetric assay. Both the wild type and the mutant transformed lymphocytes were recultured from the frozen stocks in a small cell culture flask ($25cm^3$) (Sigma, St Louis, MO, USA) that contained 1.5 millilitre (ml) RPMI medium. The cells were then incubated in a Ferma termosteri-cycle 5% carbon dioxide (CO_2) incubator (Farma International, Miami, Florida, USA) for 1 week in an upright position at 37ºC, 5% CO_2 and 90% humidity. Every second day the cells were examined under a Nikon TMS light microscope (Nikon, Tokyo, Japan). If sufficient growth of cells were observed, 1ml RPMI 1640 growth media was added and the cell culture flask was incubated again in a horizontal position and fed two to three times weekly with 1-2ml RPMI 1640 growth media until the total volume in the flask reached 5ml and then passaged into a big culture flask ($75cm^3$) (Sigma, St Louis, MO, USA).

When the appropriate volume of cells was reached, the cells were counted on a Neubauer haemocytometer (**Figure 2.10**). A glass coverslip was placed over the counting chamber and a total of 10μl of cells from each cell line was loaded into the V-shaped wells and allowed to fill the area under the coverslip through capillary action. The counting chamber was placed under the Nikon TMS light microscope (Nikon, Tokyo, Japan) stage and the counting area was brought into focus under low magnification to do the cell count. The organisation of the counting area is shown in F**igure 2.10.** The number of cells per ml was determined as follows: The number of cells was counted in the two of the four squares shaded in yellow in **Figure 2.10**. The average

number of cells for the two blocks were calculated and multiplied by 10^4 (this is a constant that is used because the depth of the haemocytometer is 0.1mm).

Number of cells/ml=number of cells $\times$ dilution factor $\times 10^4$
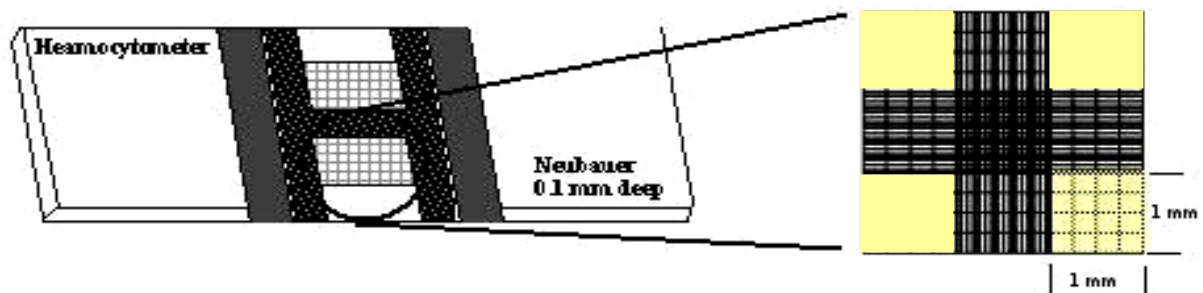
Figure 2.10: **Haemocytometer used for cell counting.** Two blocks indicated in yellow were counted for each cell line and the average of the two blocks was calculated to determine the total cells per ml.

A total of 10^5 cells/ml for each cell line was needed for the MTT assay. The entire MTT assay was performed in a 96well tissue culture plate (Greiner Bio-one, Frickenhausen, Germany) with a clear bottom. After the appropriate dilutions of each of the cell lines was made, a 100µl of the WT cell suspension was added to rows A, B and G and 100µl of the MT cell suspension was added to rows D and E (**Figure 2.11**). A further 100µl of RPMI growth medium was added to each of the above-mentioned rows and 200 µl of the RPMI growth medium was added to row H (Blank). The cells were incubated for 24 hours in a Ferma termosteri-cycle 5% carbon dioxide (CO_2) incubator (Farma International, Miami, Florida, USA). After 24 hours the cells were centrifuged at 800 r.p.m using a Sorval® GLC-4 general laboratory centrifuge (Separations Scientific, Johannesburg, South Africa) for one minute to pellet the cells at the bottom of the wells. Following centrifugation, the medium from all the wells were carefully removed (taking care not to dislodge the cell pellet) and 100 µl fresh RPMI growth medium was added to all the wells. A serial dilution of Streptomycin sulphate was prepared in RPMI medium at the following concentrations: 200µg/ml, 20 µg/ml, 2µg/ml, 0.2µg/ml, 0.02µg/ml, 0.002µg/ml and 0.0002µg/ml. A total of 100µl of each of the different drug concentrations were added to the

two different cell lines (WT and MT) in triplicate but not in row G (positive control) **(Figure 2.11)**. Thereafter the cells were incubated for another 48 hours in a Ferma termosteri-cycle 5% carbon dioxide (CO_2) incubator (Farma International, Miami, Florida, USA). Following incubation, 25µl sterile MTT was added to each well and incubated for another four hours. Thereafter, the cells were centrifuged for 10 minutes at 200 r.p.m using a Sorval® GLC-4 general laboratory centrifuge (Seperations scientific, Johannesburg, South Africa). The medium was carefully aspirated off and 100µl DMSO was added to all the wells to dissolve the formazan crystals. The plate was shaken on a rotational shaker for approximately two minutes in order to dissolve the purple crystals. When all the crystals were dissolved, the absorbance in each well was read on at 540 nanometres (nm) using a Synergy HT Luminometer (Bio-Tek Instruments Inc, Vermont, USA).
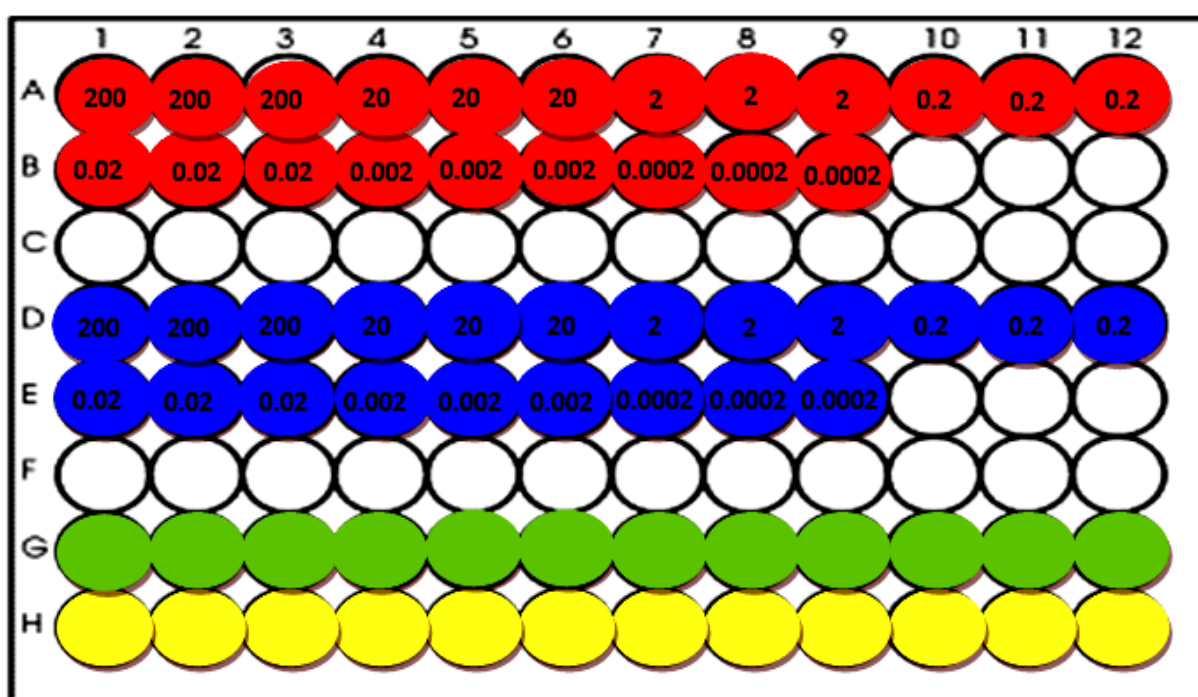
Figure 2.11: Sample layout for the MTT assay to measure the effect of different concentrations of Streptomycin on both mutation positive and mutation negative cell lines for the A1555G mutation. Row H (yellow) is the blank that only contains the RPMI medium. Row G is the positive control that contains only cells and medium but no streptomycin. The WT cell line (A1555) was added to rows A and B (Red) and the MT cell line (G1555) was added to rows D and E (Blue). The streptomycin drug was diluted to different concentrations ranging from 200µg/ml to 0.0002µg/ml. The drug at each of the different concentrations was added to the two different cell lines in triplicate as indicated on the figure.

Chapter 3

RESULTS

1. Detection of the six known mutations associated with aminoglycoside-induced hearing loss

Six mutations (A1555G, C1494T, T1095C, A827G, 961delT + insC$_{(n)}$ and T1291C) in the mitochondrial *MT-RNR1* gene are associated with aminoglycoside-induced hearing loss. We developed a rapid and cost effective method to simultaneously detect all of these mutations within a single reaction. We aimed to determine the frequency of these mutations in a cohort of 115 MDR-TB patients who are at high risk of developing aminoglycoside-induced hearing loss if they are carriers of one of these mutations. The frequencies of these mutations were also determined in unrelated control samples representative of four different ethnic groups in the Western Cape region of South Africa.

1.1. Polymerase chain reaction (PCR) amplification

The entire *MT-RNR1* gene was PCR amplified in a fragment of 1,124bp which included all six of the mutations previously associated with aminoglycoside induced hearing loss. After amplification, the products were resolved on 1% agarose gels to confirm the successful amplification of the fragment. A representative example of an agarose gel with the amplified products is shown in **Figure 3.1**. The correct size of the amplified product was determined by loading a 200bp molecular marker in the first lane of the gel. A negative control sample (containing no DNA) was always included in all PCR reactions to exclude any possible PCR contamination.
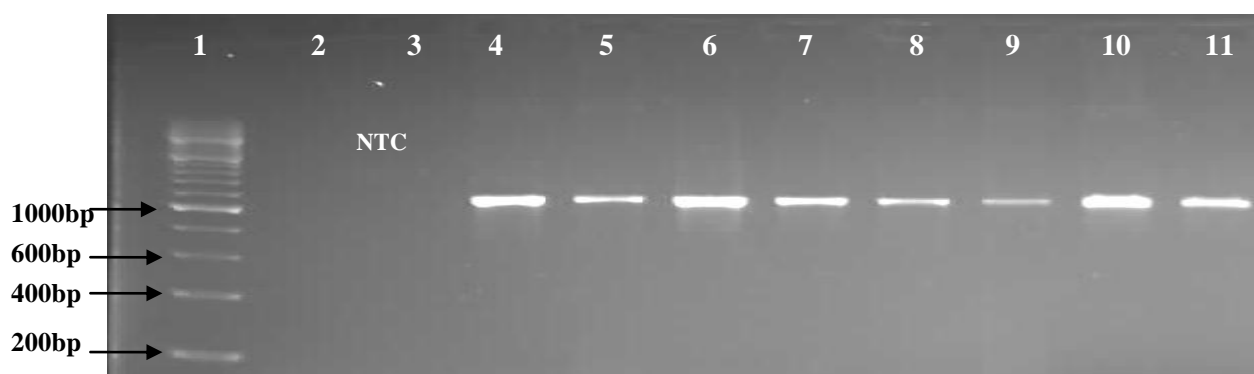
Figure 3.1: **1,124bp PCR fragment of *MT-RNR1***. The gel was stained with 1µg/µl Ethidium Bromide. Lane 1: size marker; Lanes 2 and 3: non-template control (NTC); Lanes 4 – 11: PCR Products. Samples were resolved by electrophoresis at 240 volts (V) for 15 – 20 min in 1×SB running buffer. After electrophoresis the gel was viewed under a UV light and photographed.

1.2. ABI Prism® SNaPshot™ Multiplex system (Snapshot analysis)

After PCR amplification, all samples, including a negative control, was analyzed using SNaPshot analysis (Chapter 2 Section 12) preformed at the Core Sequencing Facility of the Department of Genetics, University of Stellenbosch using the ABI 3130xl ® Genetic analyzer. SNaPshot analysis was chosen as the mutation detection method of choice since more than one single nucleotide polymorphism (SNPs) or mutation can be genotyped within one reaction, making this method more cost effective and less labour intensive to use. **Figure 3.2** represents an electropherogram demonstrating the allele calling for the six mutations in *MT-RNR1*. ABI GeneMapper software was used to assign bins to each of the five loci in order to facilitate correct allele scoring and quality checking. Initially, we only included the five most reported mutations in our SNaPshot analysis (Bardien et al. 2009). It was later decided to include another mutation, T1291C which had been reported in Spanish individuals with non-syndromic deafness (Ballana et al. 2006b).

The SNaPshot method underwent a series of lengthy optimisation steps which involved editing of the sequence of the six different SNaPshot primers in order to minimize any secondary structure formation or, primer dimer formation. Furthermore, the length of the SNaPshot extension primers were lengthened or shortened until adequate separation of the different alleles for each of the six mutations were obtained. To determine the positions of the peaks produced for each of the alleles, both wild type and mutant alleles for the A1555G, C1494T and T1095C mutations were used. Since this is mitochondrial DNA and the mutations are all homoplasmic, for each mutation, only one allele will be present.

Locus:		1494		1095	1555	961	827	1291
Allele present:		C		T	A	*T	*A	*T
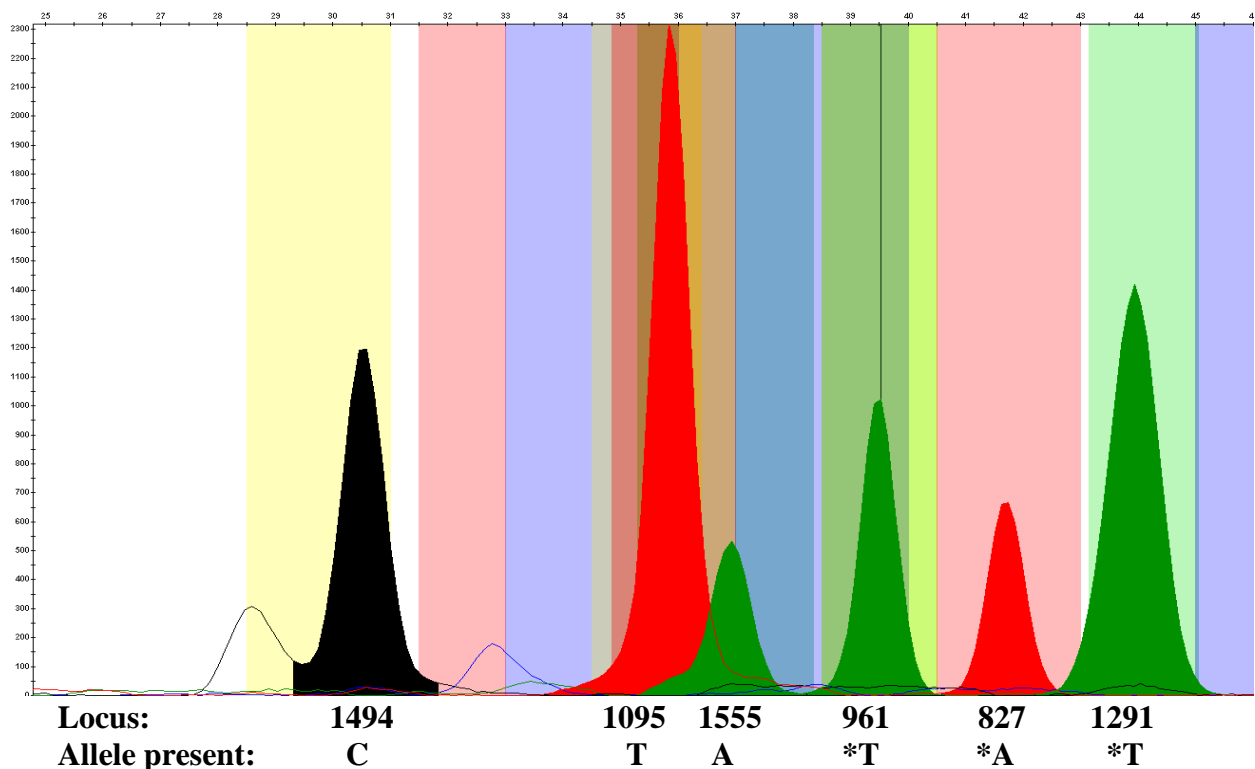
Figure 3.2: SNaPshot analysis of the six mutations associated with aminoglycoside-induced deafness (C1494T, T1095C, A1555G, 961delT +insC$_{(n)}$, A827G and T1291C). The different peaks shown in this electropherogram represent wild type alleles, for one individual, at all six loci.

* Is representative of the SNaPshot extension primers for 961delT +insC$_{(n)}$, A827G and T1291C that is in the reverse orientation.

In the above figure the genotypes are represented as follows: For C1494T, either a black peak (C allele) or a red peak (T allele) will be present. For T1095C, a red peak (T allele) or a black peak (C allele). For A1555G, a green peak (A allele) or a blue peak (G allele). For 961delT +insC$_{(n)}$, a green peak (A allele; T on forward strand) or a blue peak (G allele; C on forward strand) as the extension primer is in the reverse orientation. For A827G, with the extension primer also in the reverse orientation, a red peak (T allele; A on forward strand) or a black peak (C allele; G on forward strand). For T1291C, the extension primer is in the reverse orientation, a green peak (A allele; T on forward strand) or a blue peak (G allele; C on forward strand).

The SNaPshot method was able to successfully identify mutation-positive samples. Initially the method was designed to detect the 961delT +insC$_{(n)}$ mutation. Although other types of mutations (Li et al. 2004b; Li et al. 2004a) have been reported at this position we decided to only focus on the 961delT +insC$_{(n)}$ mutation since it has been reported in a variety of different populations. During the screening of the samples we detected a black peak (C allele) at the position where we normally expected to see either a blue (representing the C allele) or green (representing the T

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allele) peak (SNaPshot primer for 961delT +insC$_{(n)}$ mutation is in the reverse orientation). Upon further investigation we realised that the black peak was representative of the G allele for the T961G mutation. This mutation has previously only been detected in one other population group of Caucasian ethnicity (Li et al. 2004a). **Figure 3.3** is a representative electropherogram of the SNaPshot result in which we detected the G allele of T961G.

Locus:	1494	1095	1555	961	827	1291
Allele present:	C	T	A	G	A	T
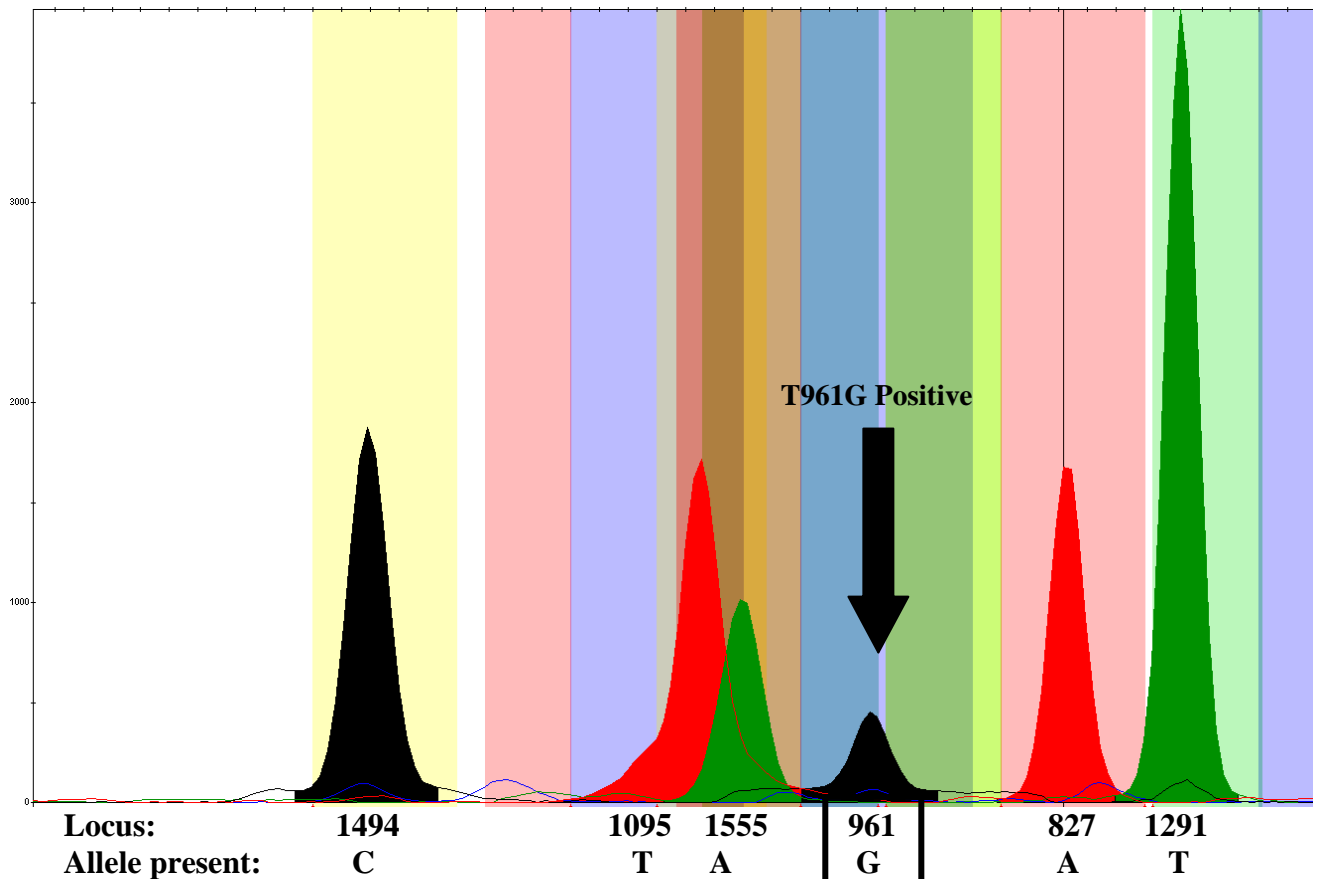
Figure 3.3: Electropherogram of the SNaPshot result for a sample with the T961G mutation. The different peaks shown in this electropherogram represent wild type alleles, for one individual, at five loci (1494, 1095, 1555, 827, 1291) and a mutant G allele at the 961 locus, black peak (C allele; G on the forward strand) as the extension primer is in the reverse orientation.

Figure 3.4 and **Figure 3.5** are representative electropherograms of the SNaPshot results in which we detected the G allele of A1555G and the G allele of the A827G mutations, respectively.
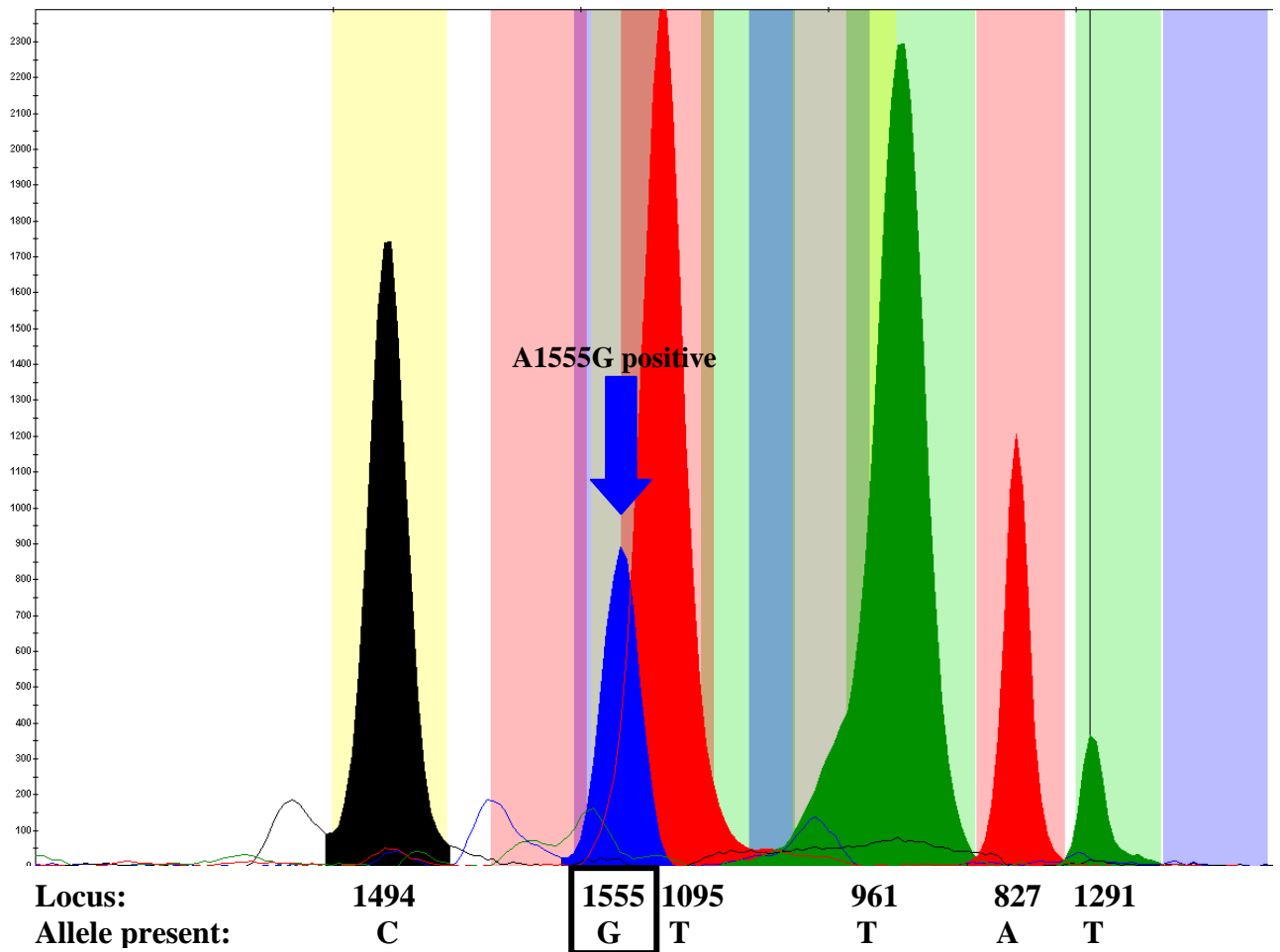
Figure 3.4: Electropherogram for a sample with the A1555G mutation. The different peaks shown in this electropherogram represent wild type alleles, for one individual, at five loci (1494, 1095, 961, 827, 1291) and a mutant G allele at the 1555 locus, blue peak (G allele).

Locus:	1494	1095	1555	827	961	1291
Allele present:	C	T	A	G	T	T
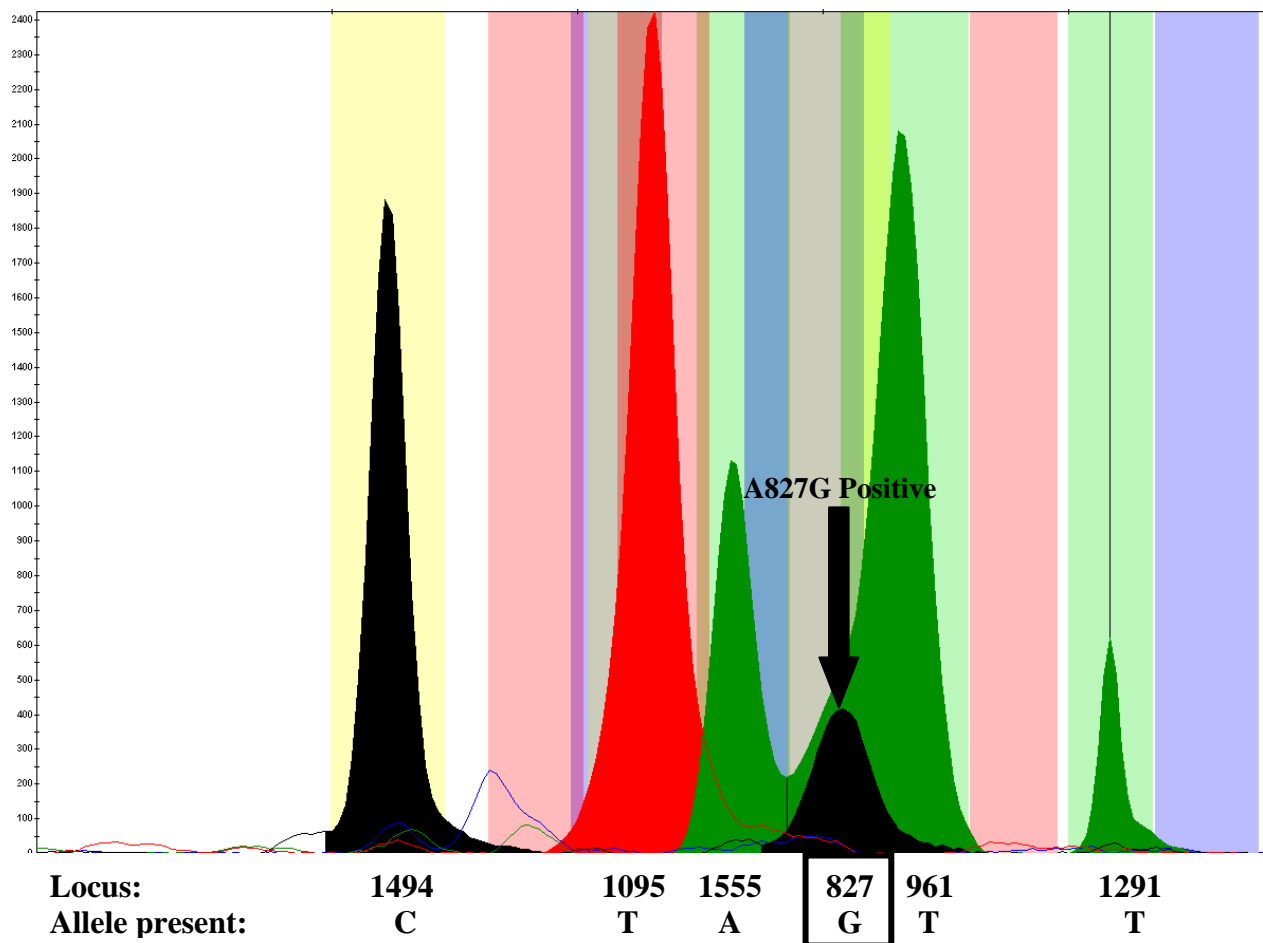
Figure 3.5: Electropherogram for a sample with the A827G mutation. The different peaks shown in this electropherogram represent wild type alleles, for one individual, at five loci (1494, 1095, 1555, 961, 1291) and a mutant G allele at the 827 locus, black peak (C allele; G on the forward strand) as the extension primer is in the reverse orientation.

1.3.   DNA sequencing

All samples that were detected as being mutation positive for any one of the six mutations were subjected to direct sequencing in order to confirm the presence of the mutant allele. The resultant chromatograms for individuals mutation positive for A1555G (**Figure 3.6a**) and A827G (**Figure 3.6b**). The two mutations at 961, are shown in **Figure 3.7 a-c**. The sequencing results confirmed that the mutations were in homoplasmic form.

(a) A1555G

(b) A827G

Figure 3.6: Chromatograms representative of mutation positive samples. (a) A1555G (b) A827G

(a) Wild type at position 961

(b) T961G

(c) 961delT + insC$_{(n)}$

Figure 3.7: Chromatograms representative of wild type and mutant-positive samples at position 961. (a) Wild type at position 961 (b) T961G (c) 961delT +insC$_{(n)}$

There was 100% concordance between the SNaPshot results and the results of the automated sequencing regarding the detection of these mutations. This indicates the efficacy and accuracy of the SNaPshot method for the detection of these mutations.

1.4. Statistical analysis

A summary of the mutations detected in the patients and controls using SNaPshot analysis is provided in Table 3.1. The C1494T, T1095C and T1291C mutations were not detected in any of the MDR-TB or control groups. The A1555G mutation was not detected in the MDR-TB patients but was detected in 0.9% (one of the 112) of the Black control samples. It was detected in a heteroplasmic state in 1.1% (one of the 93) of the Afrikaner controls. The A827G mutation was detected in 0.9% (one of the 115) of the MDR-TB patients and in 1.1% (one of the 93) of the Afrikaner control group. However, the MDR-TB patient (Somalian) in which the A827G mutation was detected had no signs of ototoxicity. The patient was later lost to follow-up. The 961delT + insC$_{(n)}$ mutation was found in relatively high frequencies in a number of the MDR-TB patients as well as the control groups. This mutation was detected in 3.5% (four of the 115) of the MDR-TB patients, who had no signs of ototoxicity. It was also detected at a frequency of 1.5% (two of the 130) in the Mixed Ancestry controls, 7.1% (eight of the 112) of the Black controls and in 1.1% (one of the 93) of the Afrikaner controls. The T961G mutation was only detected in the Caucasian and Afrikaner control groups at a frequency of 2.9% (three out of 104) and 3.2% (three of the 93), respectively **(Table 3.1)**. The high frequencies observed for the 961delT + insC$_{(n)}$ and T961G variants indicates that they are probably non-pathogenic polymorphisms.

Table 3.1: Allele frequencies for the six mutations in the mitochondrial *MT-RNR1* gene associated with aminoglycoside-induced hearing loss.

Mutation							
Samples	**A1555G**	**C1494T**	**T1095C**	**A827G**	**961delT+insC(n)**	**T961G**	**T1291C**
MDR-TB patients N=115	0	0	0	1/115 (0.9)	4/115 (3.5)	0	0
Mixed Ancestry Controls N=130	0	0	0	0	2/130 (1.5)	0	0
Black Controls N=112	1/112 (0.9)	0	0	0	8/112 (7.1)	0	0
Caucasian Controls N=104	0	0	0	0	0	3/104 (2.9)	0
Afrikaner Controls N=93	1/93 (1.1) Heteroplasmic	0	0	1/93 (1.1)	1/93 (1.1)	3/93 (3.2)	0

Percentage frequencies are indicated in brackets

These results indicate that the 961delT + insC$_{(n)}$ and the T961G mutations are very common in many population groups thus indicating that these mutations might be common polymorphisms and might not play a role in the pathogenesis of aminoglycoside-induced hearing loss. The T961G variant was found solely in Caucasian samples and it has been detected previously in American Caucasian individuals (Li et al. 2004a). This indicates that this variant might be a common polymorphism in the Caucasian population.

The high frequency of the A1555G mutation in the black controls is alarming since the incidence of TB and MDR-TB is high in this population group putting them at risk of developing aminoglycoside-induced hearing loss. The A827G mutation was found in a Somalian individual, however it was also detected at a frequency of 1% in the Afrikaner control population. Thus further work is needed to determine if the A827G mutation is pathogenic.

2.   Screening of the 35delG mutation in the *GJB2*

Mutation screening for the 35delG mutation in *GJB2* was done in order to eliminate any other possible genetic causes of deafness in the MDR-TB patients. The 35delG mutation accounts for up to 75-80% of all autosomal recessive deafness cases related to *GJB2* (Gasparini et al. 2000; Morell et al. 1998). The 35delG mutation is a deletion of a guanine residue in an area of six G's at position 35 of the cDNA and leads to a prematurely truncated protein (Carrasquillo et al. 1997; Morell et al. 1998). High Resolution Melt analysis (HRM) was attempted to develop a cost effective screening method to detect this mutation in our study participants.

2.1.   Polymerase chain reaction (PCR) amplification

A region of 117bp, within the only coding exon (exon 2) of *GJB2* was PCR amplified. A fluorescent intercalating dye Syto 9 was included in the PCR reaction which binds to all double stranded DNA molecules and is released from the DNA at high temperatures when DNA denatures. The presence of this dye is used to track the dissociation of the double stranded DNA molecules to single stranded molecules with the increase in temperature. The DNA of 101 MDR-TB patients were amplified by means of PCR and subjected to HRM analysis to detect the 35delG mutation.

2.2.   High resolution melt (HRM) analysis

High resolution Melt (HRM) analysis is a technique used to characterize a DNA sample according to a shift in fluorescence as the PCR product dissociates from double stranded DNA to single stranded DNA with an increase in temperature (Chapter 2 Section 13). The shift in melting curve is then used to detect either known or unknown single nucleotide polymorphisms (SNPs) or other mutations within a sample or a group of samples. All HRM analysis was performed on the Rotor-Gene 6000 analyzer (Corbett Life Science, Australia). **Figure 3.8** shows the melt peaks for a wild type sample, a heterozygote and a homozygote for the 35delG mutation. The melt peaks demonstrates the unique melting profile for each sample from which differences between samples can be detected.
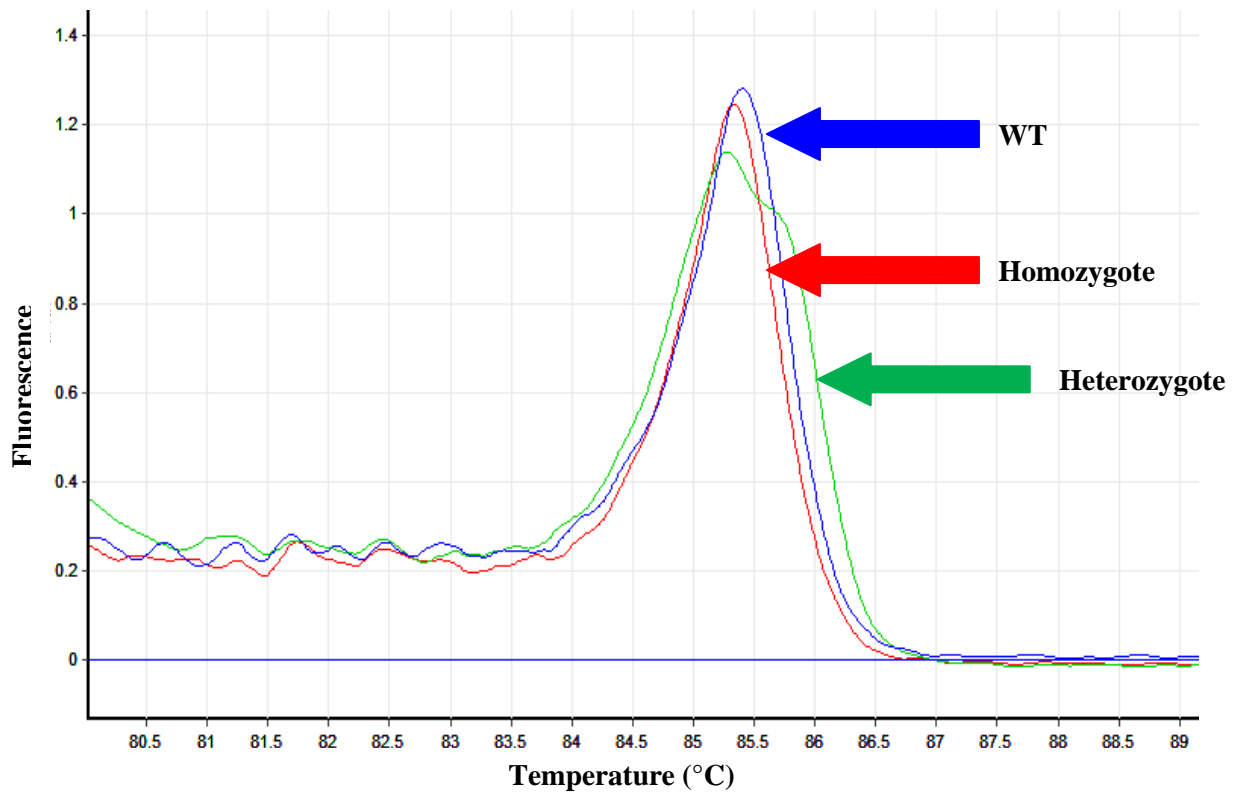
Figure 3.8: **Melt peaks for the 35delG mutation using HRM analysis.** Three samples with known 35delG genotypes were subjected to HRM. The wild type sample is indicated by the blue arrow with the homozygote deletion indicated in the red arrow and the heterozygote deletion indicated by the green arrow.

The differences in melt profiles between samples can also be viewed in another way by analysing either the normalised graph or the difference graph. The normalised graph (**Figure 3.9**) shows a basic representation of different genotypes based on either shifting of the curve to the left or right, or a change in the shape of the curve. A sample that has a homozygous change will have a shift in the curve either to a lower or higher melting temperature whereas a sample with a heterozygous change will have a different curve shape compared to the wild type sample.
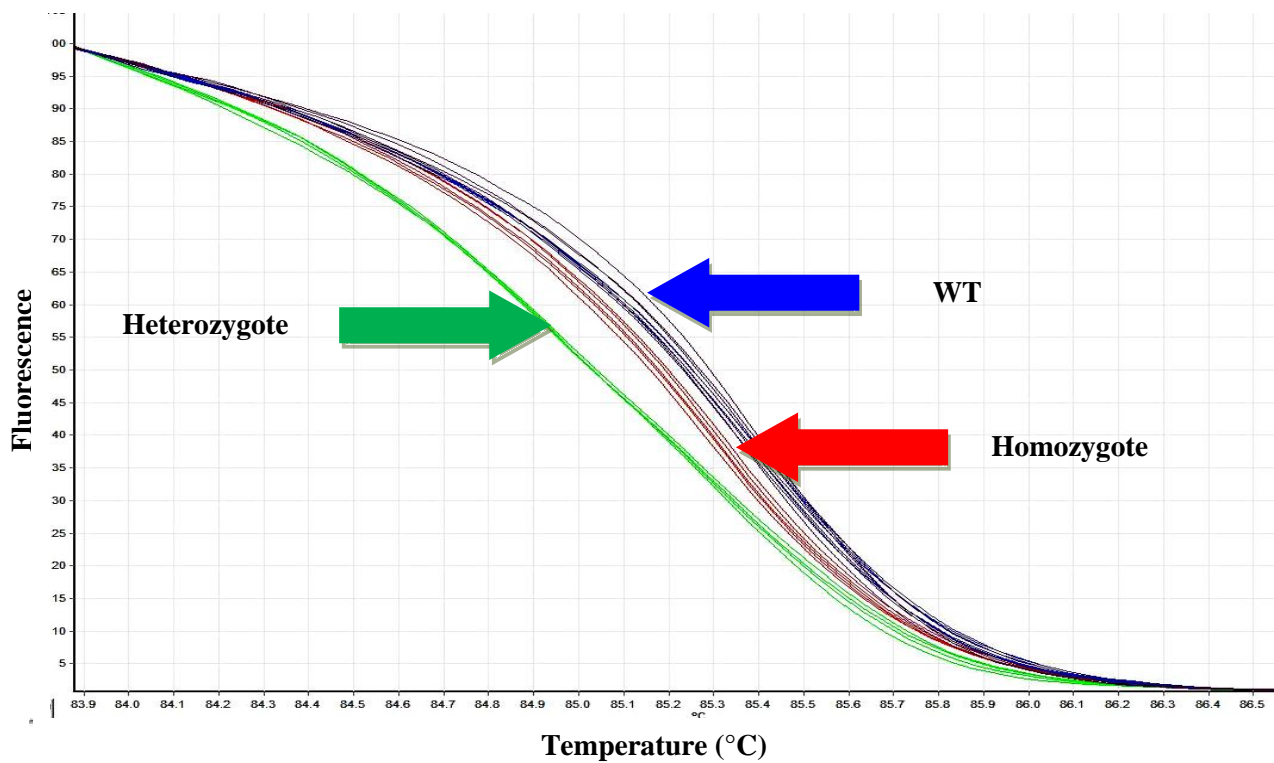
Figure 3.9: Normalised graph for 35delG mutation with HRM analysis. The wild type sample is indicated by the blue arrow, with the homozygote deletion indicated in the red arrow. The heterozygote deletion indicated by the green arrow has a clear change in shape of the curve compared to the wild type sample.

The difference graph (**Figure 3.10**) shows the difference in fluorescence of a sample compared to a selected control (reference sample) as the temperature increases. The difference graph provides an alternative view of the differences between samples.
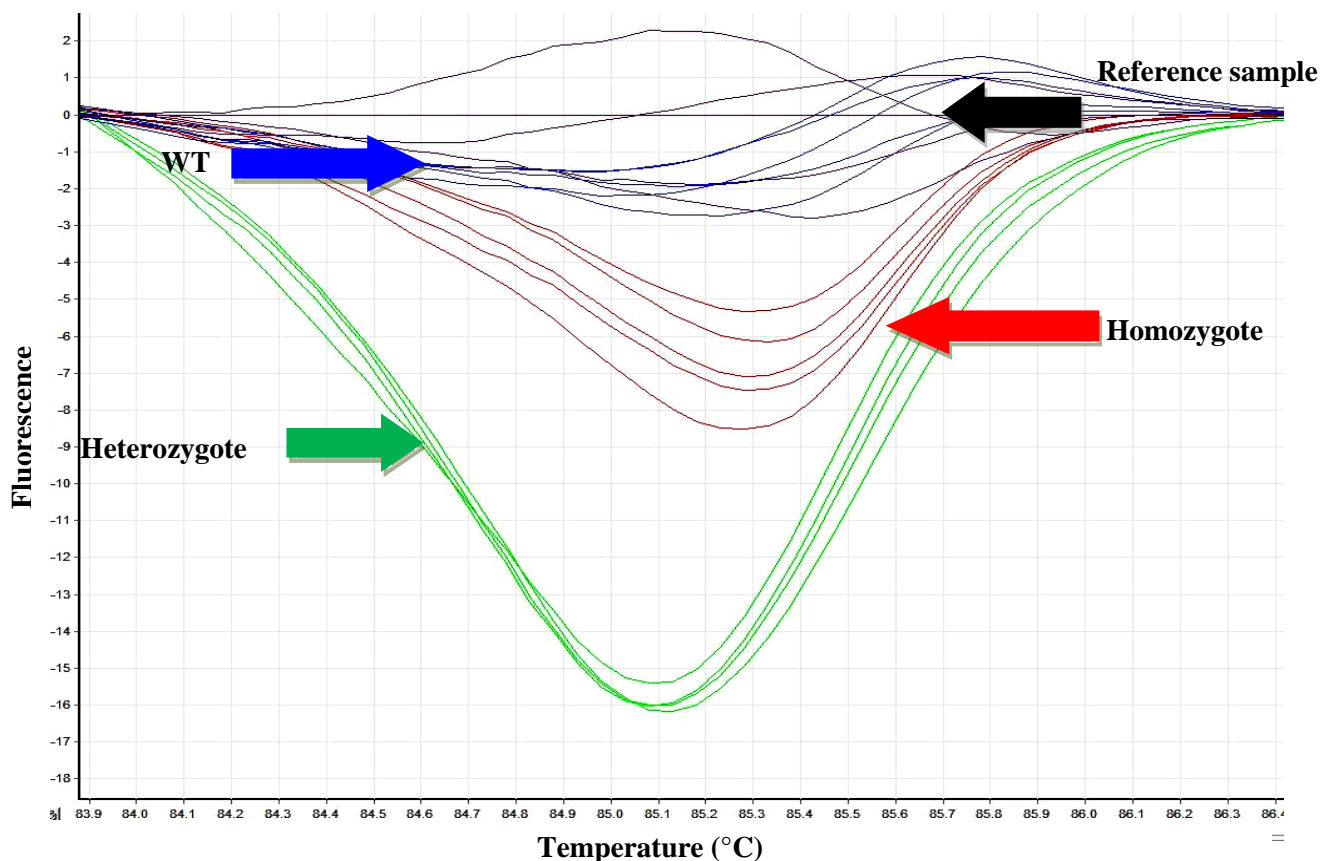
Figure 3.10: Difference graph of the 35delG mutation with HRM analysis. The wild type sample is indicated by the blue arrow with the homozygote deletion indicated in the red arrow and the heterozygote deletion indicated by the green arrow.

After numerous attempts and consultations with the Rotor-Gene 6000 developers, the homozygote mutant allele could not be distinguished from the homozygous wild type samples. On the difference graph, a difference could be detected but this difference was not consistently observed and could not unequivocally be distinguished from the wild type. The best results obtained are shown in **Figures 3.9 and 3.10**. However, when the MDR-TB samples were screened the results for many of these samples were equivocal. This may be due to the fact that the homozygote deletion does not significantly alter the melting profile compared to a wild type sample.

2.3. DNA Sequencing

It was therefore decided to sequence all of the 101 MDR-TB samples for screening of the 35delG mutation. DNA sequencing is far more expensive and labour-intensive than HRM analysis, however, using this method we were able to successfully screen for the presence of the 35delG mutation. Chromatograms for positive controls for wild type, heterozygous and

homozygous samples are shown in **Figure 3.11 a-c**. Direct sequencing of the 101 MDR-TB patients did not detect the 35delG mutation in any of the samples in either heterozygous or homozygous form thus excluding this mutation as a possible cause of deafness in these patients.

(a) Wild Type

(b) 35delG Homozygous Deletion

(c) 35delG Heterozygous Deletion
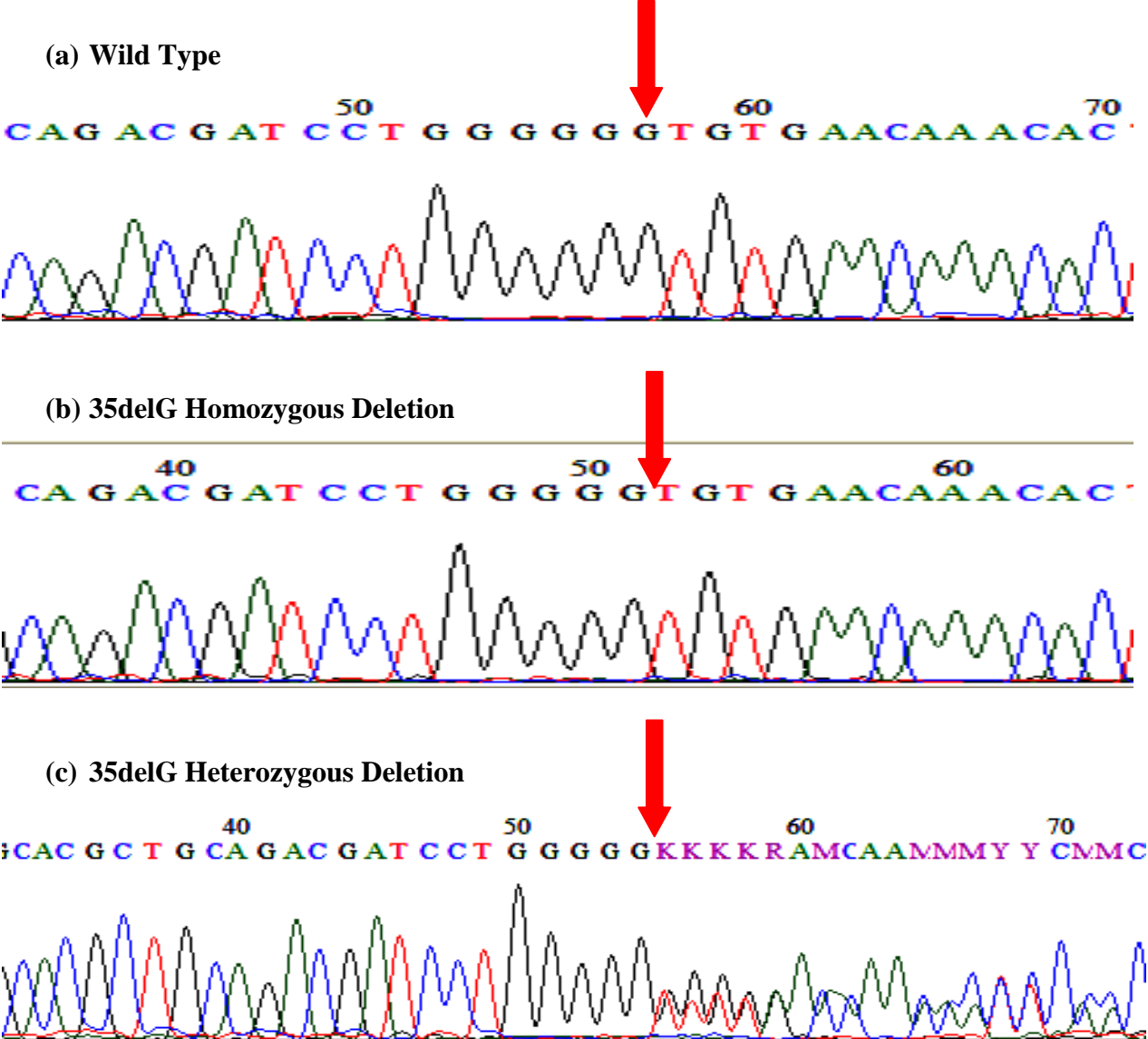
Figure 3.11: Chromatogram of the 35delG mutation in *GJB2*. (a) Representative chromatogram of a sample wild type for the 35delG mutation, indicated by the red arrow. (b) A sample with a homozygous deletion for the 35delG mutation, indicated by the red arrow. (c) A heterozygous deletion of the 35delG mutation, indicated by the red arrow.

3. Detection of novel variants in *MT-RNR1* using high resolution melt (HRM) analysis

The search for novel variants was done in a group of 34 MDR-TB patients who were experiencing ototoxicity but did not have any of the known mutations associated with aminoglycoside-induced hearing loss, as determined by SNaPshot analysis. Signs of ototoxicity, represented by hearing loss in the high frequency range, **(Figure 3.12)** for each patient were assessed by an audiologist at the Brooklyn Chest Hospital.
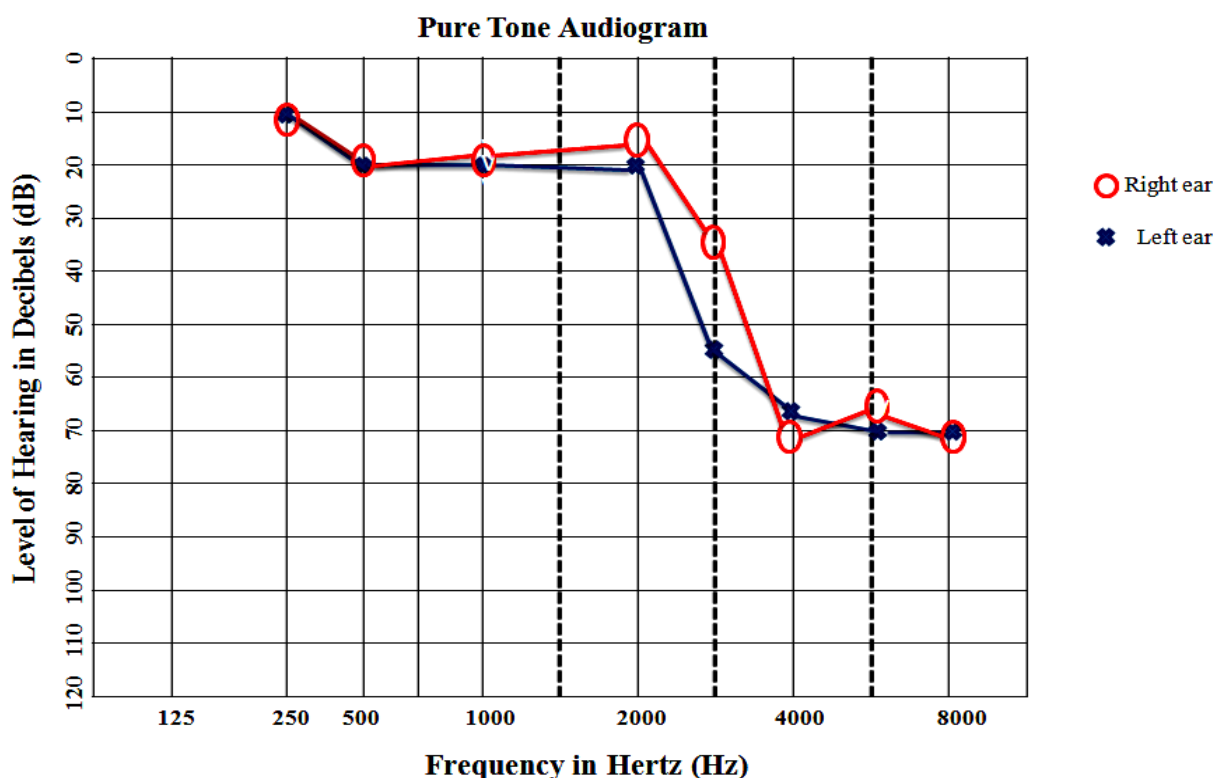
Figure 3.12: Audiogram of one of the MDR-TB patients with ototoxicity. The measurements for the left ear are indicated by the crosses with the circles indicating the measurements for the right ear.

HRM analysis was used to detect novel variants in the mitochondrial *MT-RNR1* gene. For HRM analysis, the amplified PCR fragment needs to be between 100bp and 300bp in size. This is due to the fact that a small change of only 1bp affects the melt profile of a 100bp fragment more than in a 500bp fragment. Therefore, the *MT-RNR1* gene (±1kb) was divided up into six overlapping PCR fragments of approximately 200bp each (**Figure 2.5, pg 73**), which would facilitate the detection of a variant in this gene.

3.1. Polymerase chain reaction (PCR) amplification

All six fragments were successfully amplified by PCR amplification. PCR amplification for four of the six fragments (C-F) was performed on the Rotor-Gene 6000 (Corbett Life Science, Australia) (Chapter 2 Section 6.7) and followed by HRM analysis. The remaining two sets (A-B) were amplified using the GeneAmp® PCR system 2720 Thermal Cycler, after repeated failure of amplification of the correct sized fragments on the Rotor-Gene 6000. Following the successful amplification, HRM analysis was performed on the Rotor-Gene 6000. Amplified fragments for all six set of primers were confirmed to be the correct size by electrophoresis on a 2% agarose gel. Representative examples of agarose gels with the amplified product of fragments A and B are shown in **Figures 3.13** and **Figure 3.14**. The correct size of the amplified product was determined by loading a 100bp or 200bp molecular marker in the first lane of the gel. A negative control sample (containing no DNA) was always included to exclude any possible PCR contamination.
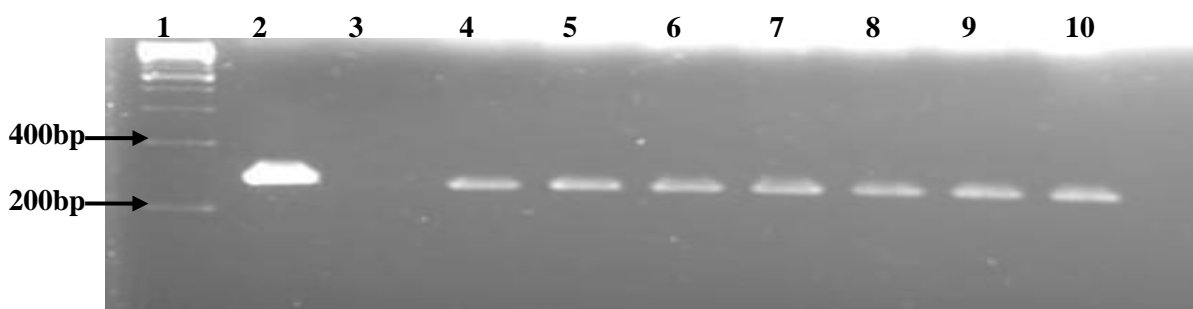
Figure 3.13: Amplified products of fragment A (*MT-RNR1* gene). Fragment was amplified with primer sets A Fwd and A Rev and produced a fragment of 272bp. Lane 1: Molecular marker; Lane 2: 272bp-sized fragment; Lane 3: Negative Control (NTC); Lanes 4-10: PCR products.
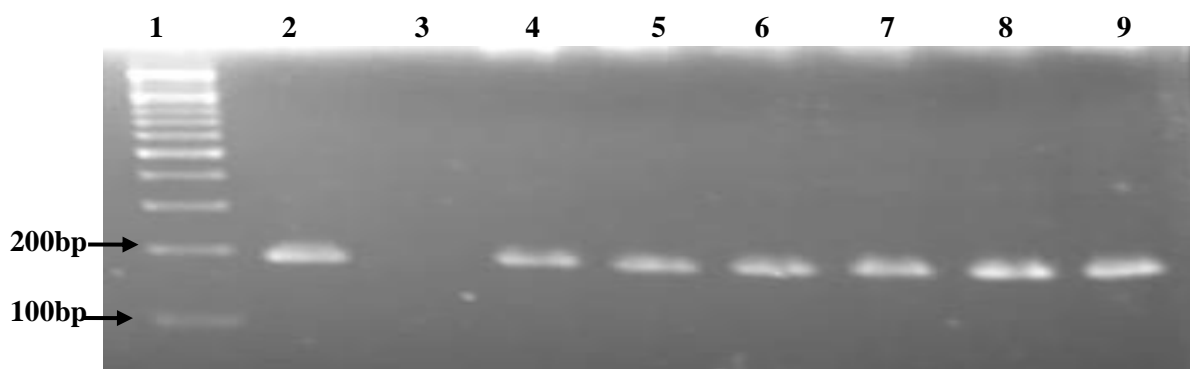
Figure 3.14: Amplified products of fragment B (*MT-RNR1* gene). Fragment was amplified with primer B Fwd and B Rev and produced a fragment of 183bp. Lane 1: Molecular marker; Lane 2: 183bp-sized fragment; Lane 3: Negative Control (NTC); Lanes 4-9: PCR products.

The PCR fragments for primer sets C, D, E and F were amplified on the Rotor-Gene 6000 (Corbett Life Science, Australia). Incorporating the SYTO 9 dye allows for the visual inspection of the exponential amplification of the products during amplification on the Rotor-Gene 6000. During the amplification a characteristic sigmoidal curve was generated **Figure 3.15**.
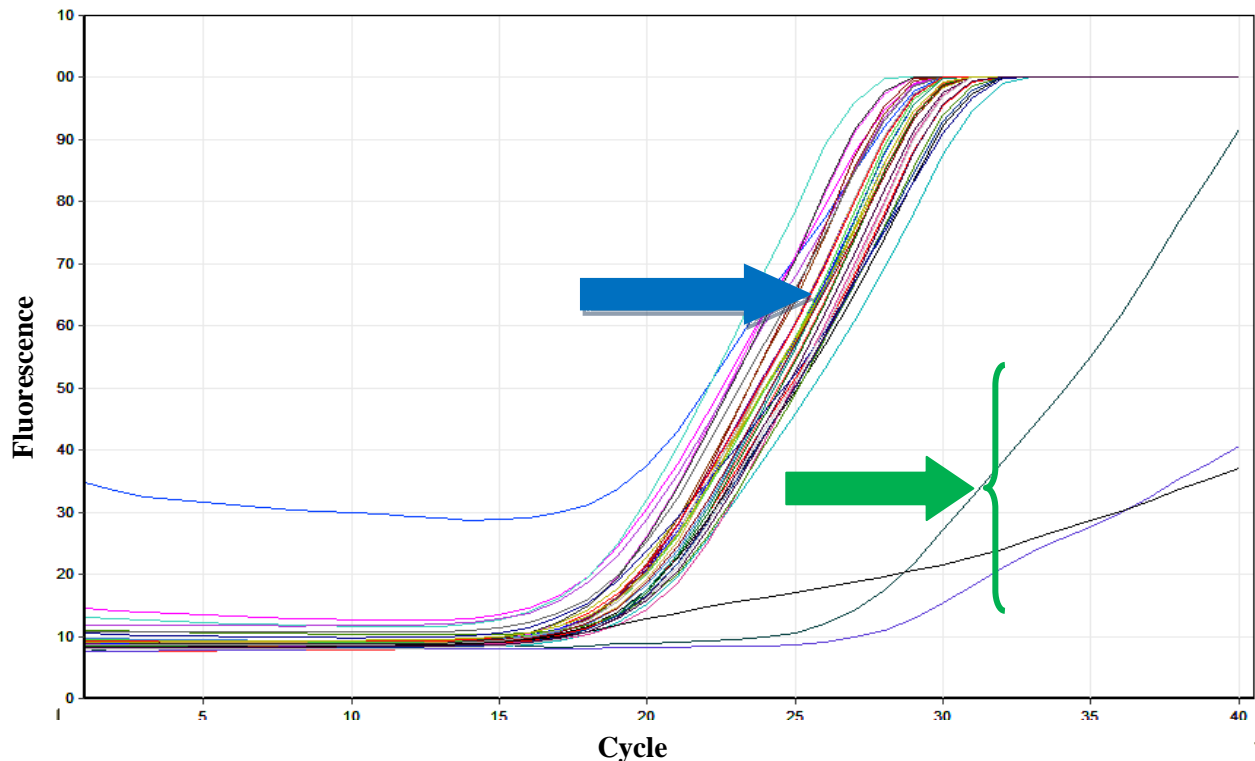
Figure 3.15: Sigmoidal amplification curve for fragment C on the Rotor-Gene 6000. Starting concentration of all samples was at 10ng/μl. A characteristic sigmoidal pattern can be seen where the concentration of the DNA remains low for up to 17 cycles and then increases exponentially until it plateaus after 33 cycles. The blue arrow indicates the group of samples amplified with the set C primers. The green arrow indicates three samples that failed to amplify successfully.

All samples that did not amplify successfully during the amplification process were not analysed during later HRM analysis. The samples that did not PCR amplify were sequenced in order to not miss any possible sequence variants. Similar amplification curves were obtained for fragments D, E and F.

3.2. High resolution melt (HRM) analysis

After amplification of the different products, samples were subjected to HRM analysis in order to detect possible variations using the melting profiles generated. In every HRM run a negative control (no DNA added to the PCR reaction) and where possible, a positive control (a sample

harbouring one of the mutations associated with aminoglycoside-induced hearing loss) (**Table 3.2**) were included.

Table 3.2: Mutation positive controls included as positive controls in HRM analysis.

Fragment	Mutation positive control
A	A827G
B	A827G 961delT + insC$_{(n)}$
C	T1095C
D	None
E	None
F	C1494T A1555G

Samples for each of the six sets of fragments were analyzed for any possible variation from the wild type sample using the melt curves, normalised curves and difference curves as discussed in section 2.2. Results for only the sets (sets A, C, D and E) in which sequence variants were successfully detected are shown.

Analysis of the different HRM graphs for fragment A indicated that some samples might have some possible variants in that part of *MT-RNR1*. **Figures 3.16** to **Figure 3.18** are representative of the graphs (Figure 3.16: melt curve, Figure 3.17: normalised graph and Figure 3.18: difference graph) obtained.
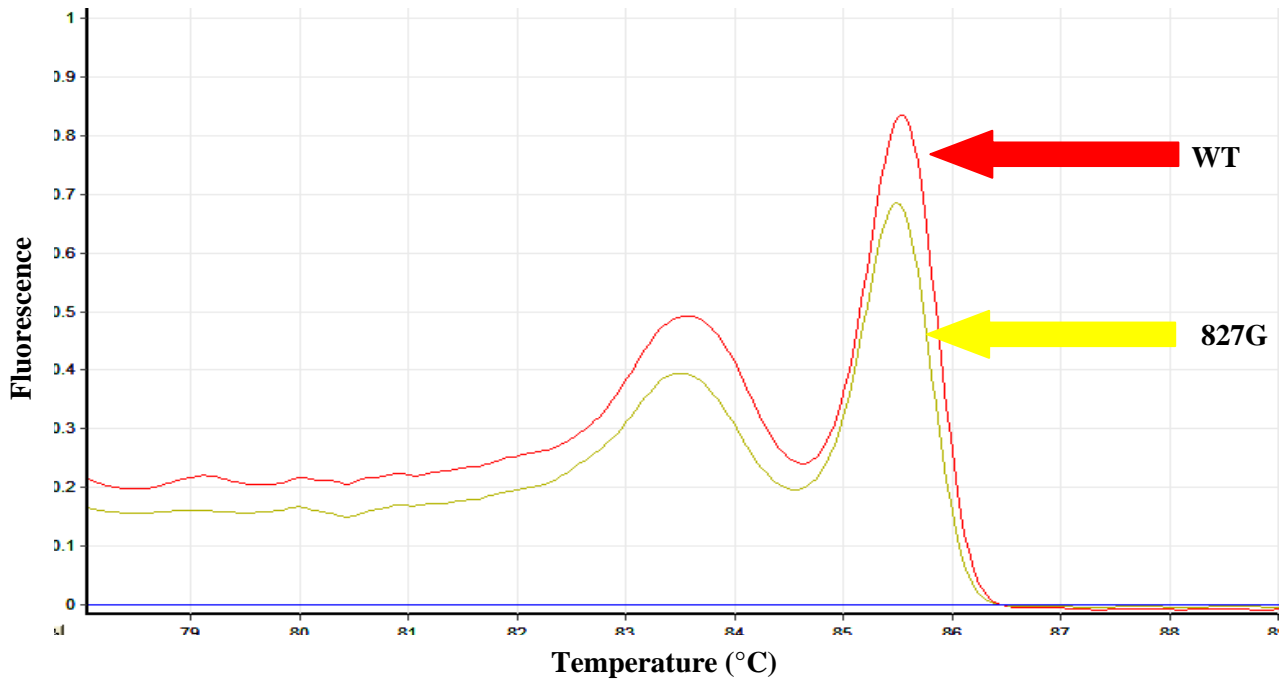
Figure 3.16: Melt curve analysis of fragment A. Red arrow: wild type; Yellow arrow: positive control for G827.
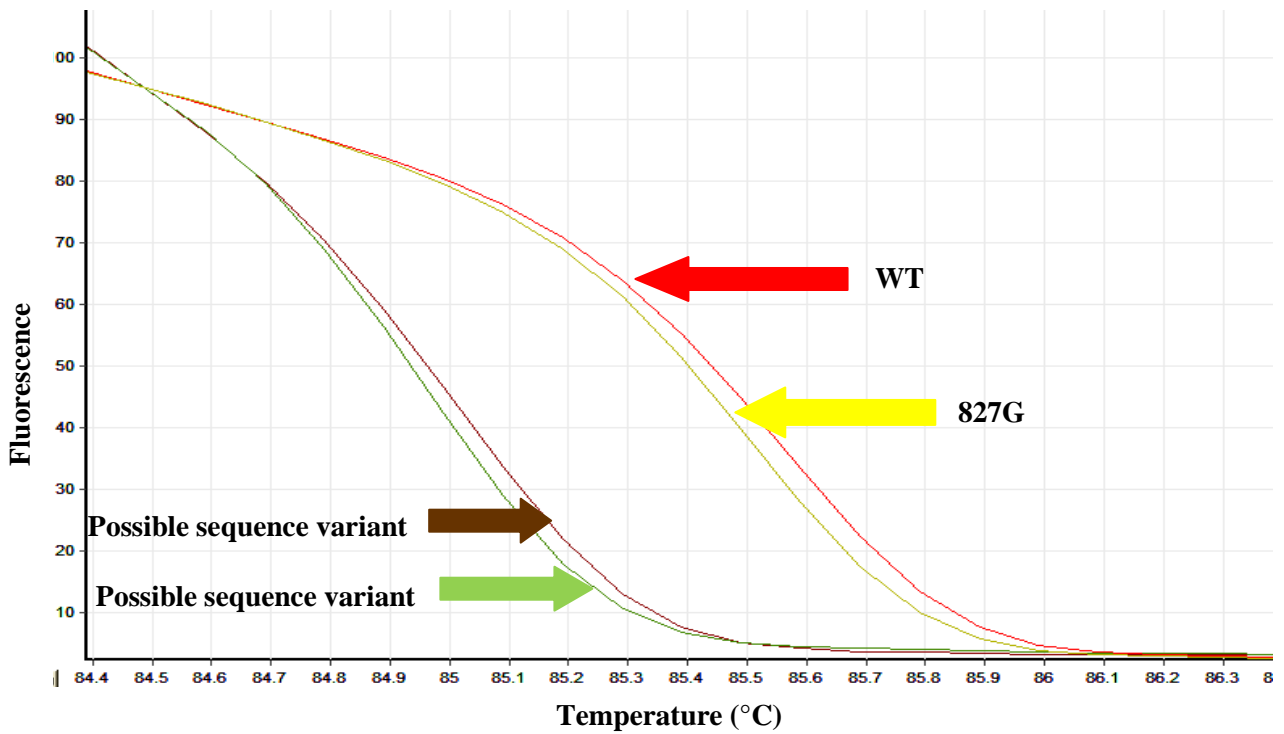
Figure 3.17: Normalised graph for fragment A. Red arrow: wild type; Yellow arrow: positive control for G827. Brown arrow: Possible sequence variant; Green arrow: Possible sequence variant.
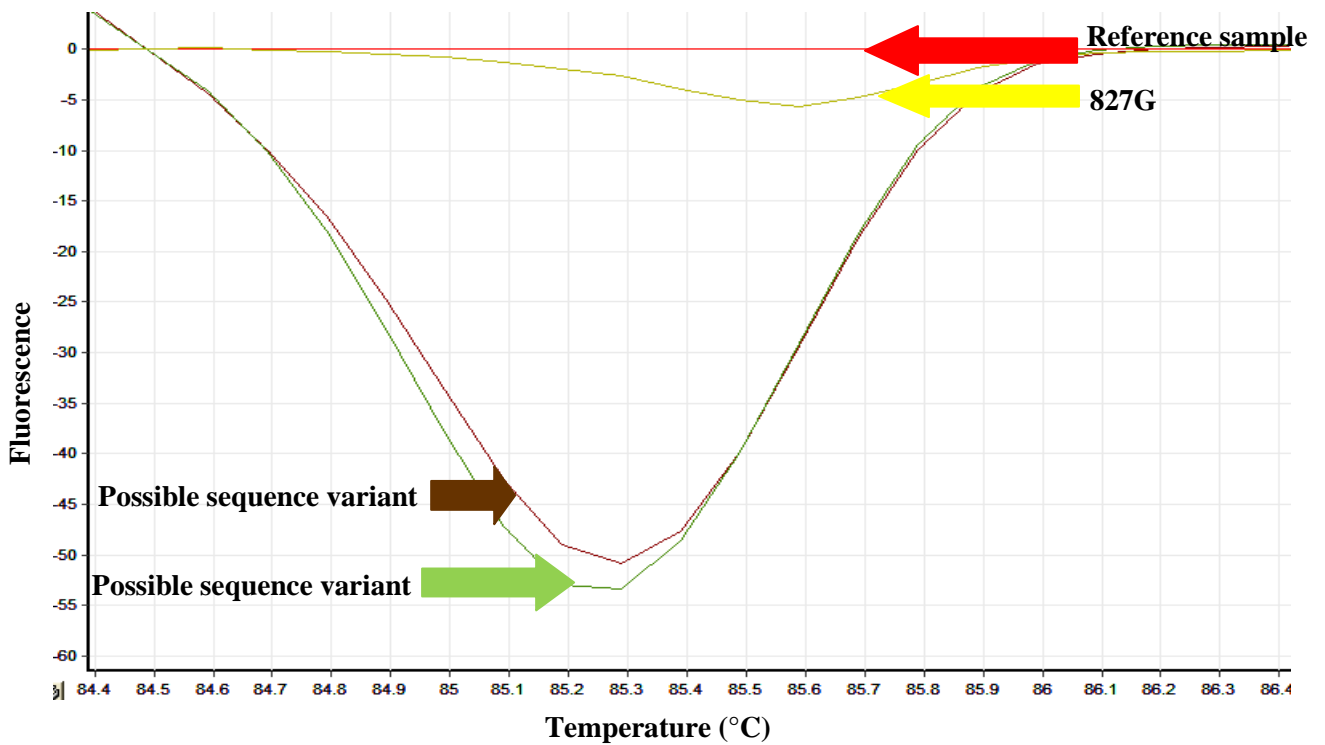
Figure 3.18: Difference graph of fragment A. The difference graph illustrates the difference in fluorescence between a sample compared to a reference sample. Red arrow: wild type; Yellow arrow: positive control for G827. Brown arrow: Possible sequence variant; Green arrow: Possible sequence variant.

Similarly, analysis of the different HRM graphs for fragments C, D and E indicated that some samples might have possible variants present in *MT-RNR1*. **Figures 3.19** to **Figure 3.27** are representative of the different graphs obtained for fragments C, D and E (melt curves, normalised graphs and difference graphs) analysed for possible novel variants in *MT-RNR1*.
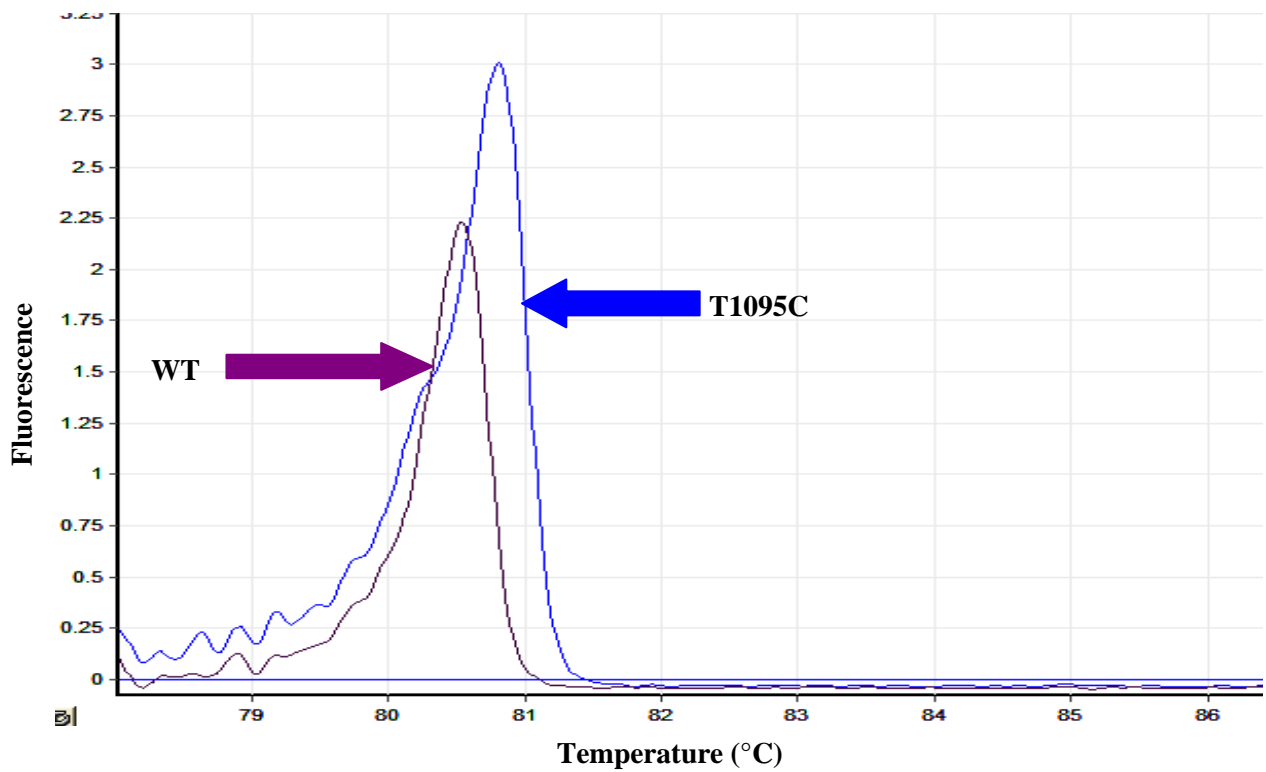
Figure 3.19: Melt curve of fragment C. Purple arrow: wild type; Blue arrow: positive control for T1095C.
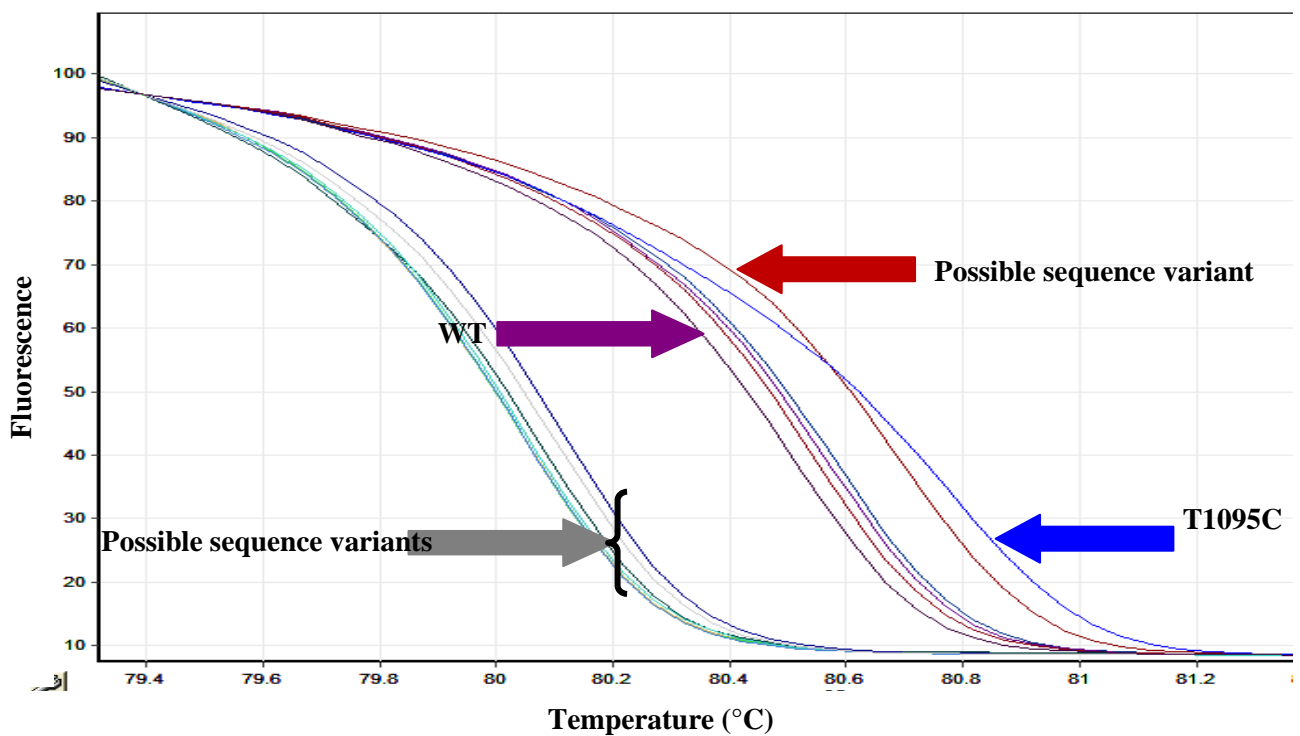
Figure 3.20: Normalised graph for fragment C. Purple arrow: wild type; Blue arrow: positive control for T1095C. The gray arrow indicates a group of samples with a possible variant. The brown arrow indicates another sample with a possible variant with a shift in the curve to right which indicates that the sample melted at a higher temperature compared to the wild type sample.
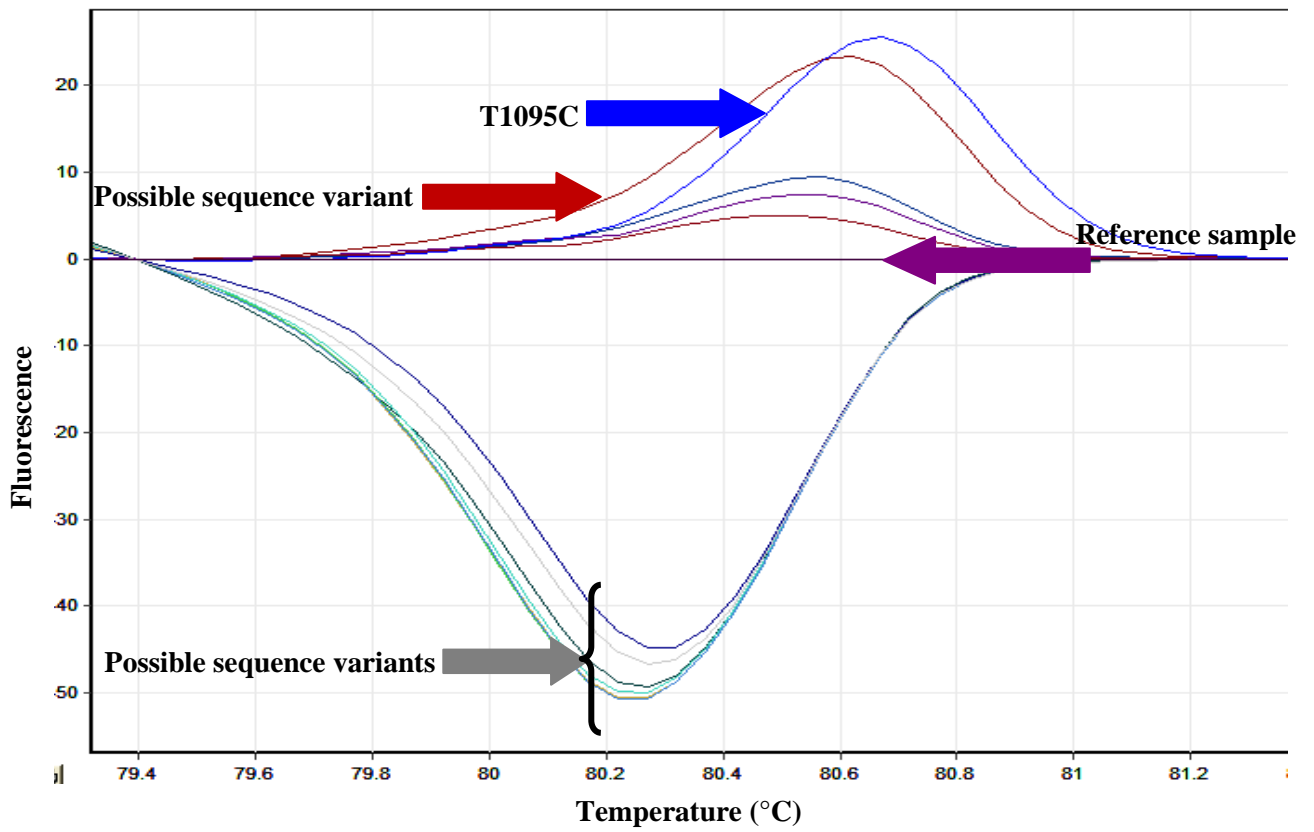
Figure 3.21: **Difference graph of fragment C.** This graph is a different representation of the differences between samples. Purple arrow: wild type; Blue arrow: positive control for T1095C. The gray arrow indicates a group of samples with a possible variant. The brown arrow indicates another sample with a possible variant.
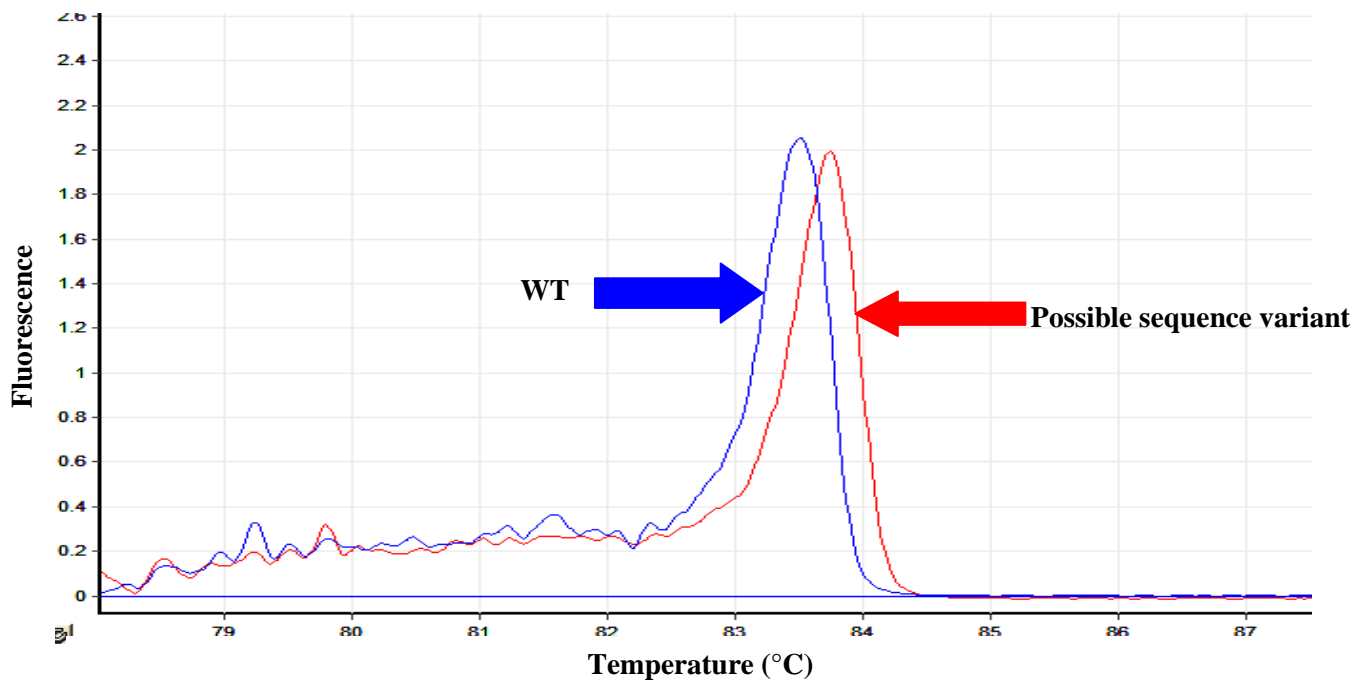
Figure 3.22: Melt curve for fragment D. Blue arrow: wild type sample. Another sample was included on the graph for a better overall picture. No positive controls were available for fragment D.
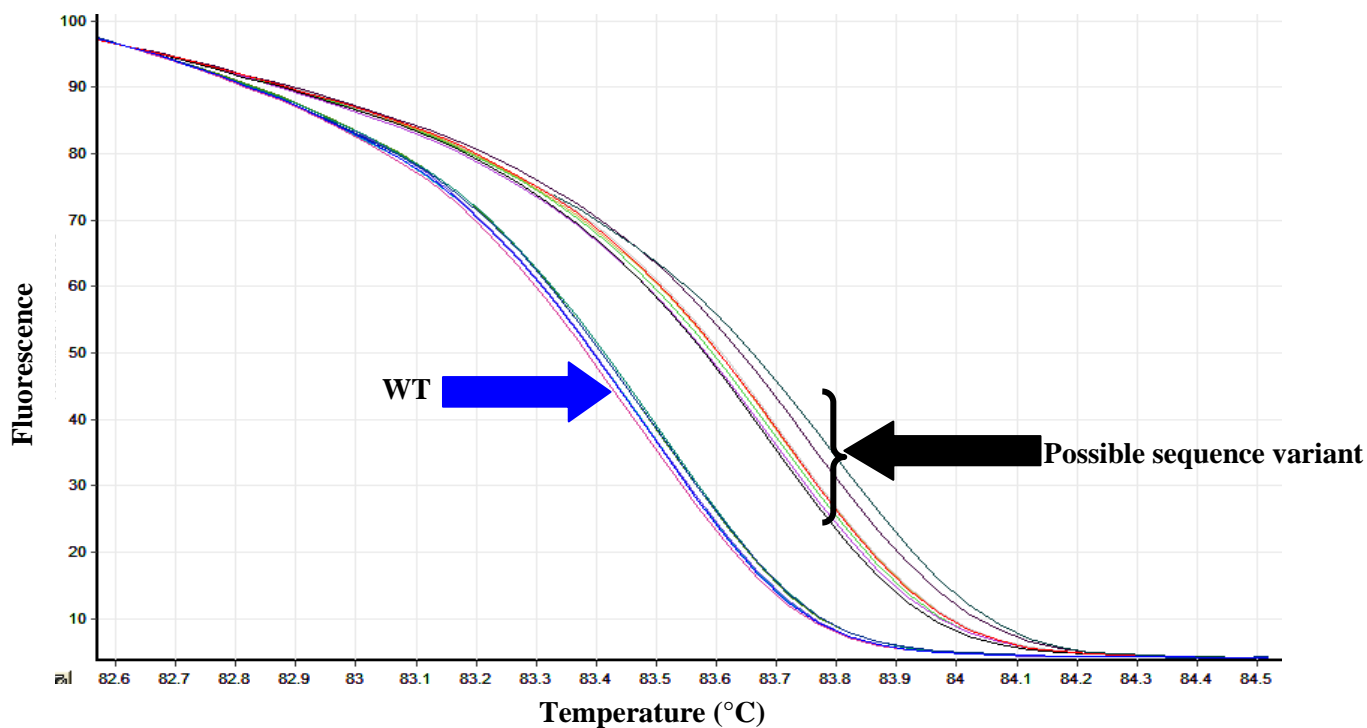
Figure 3.23: Normalised graph for fragment D. Blue arrow: wild type sample: Black arrow: a group of samples with a possible variant.
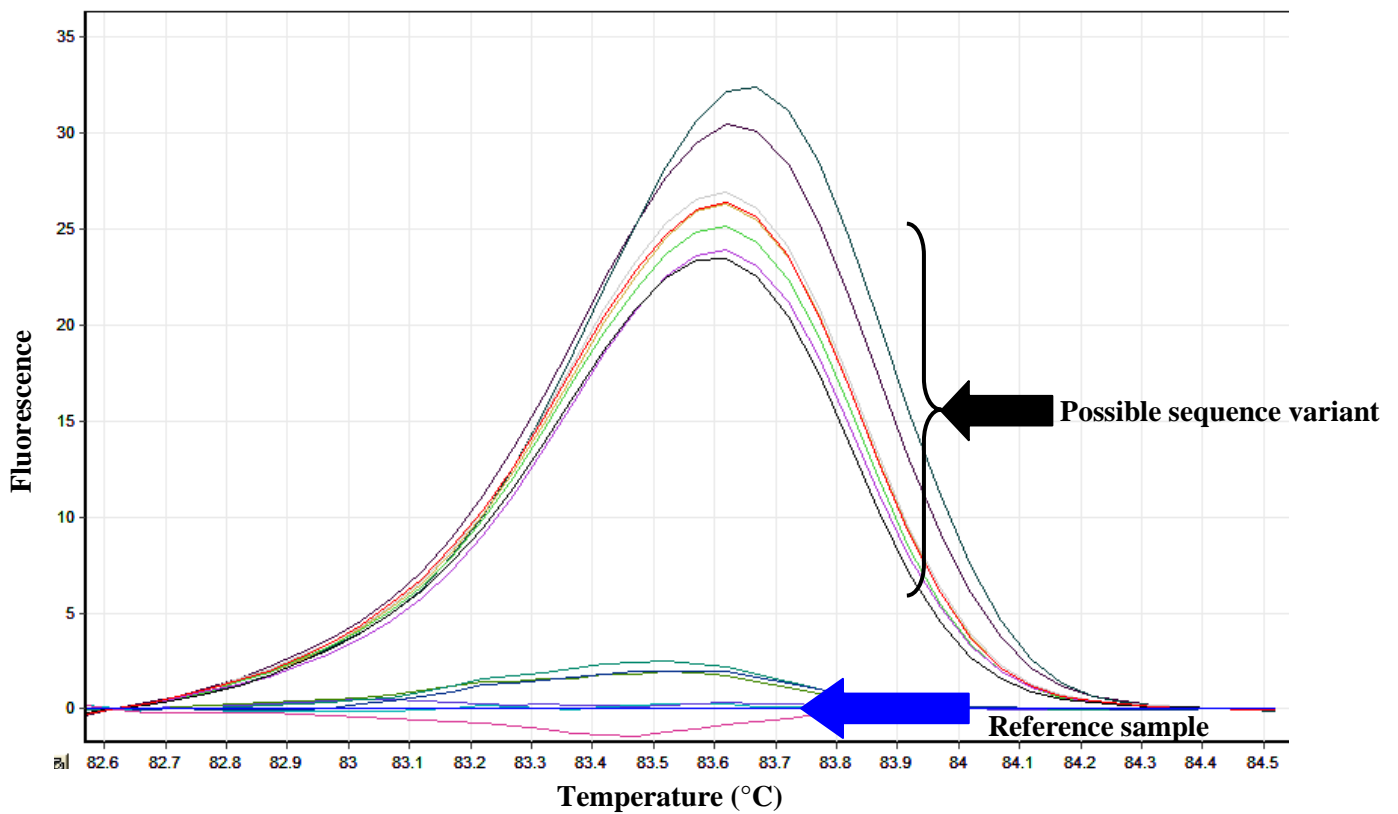
Figure 3.24: **Difference graph of fragment D.** This graph is a different representation of the differences between samples. Blue arrow: reference sample; Black arrow: a group of samples with a possible variant.
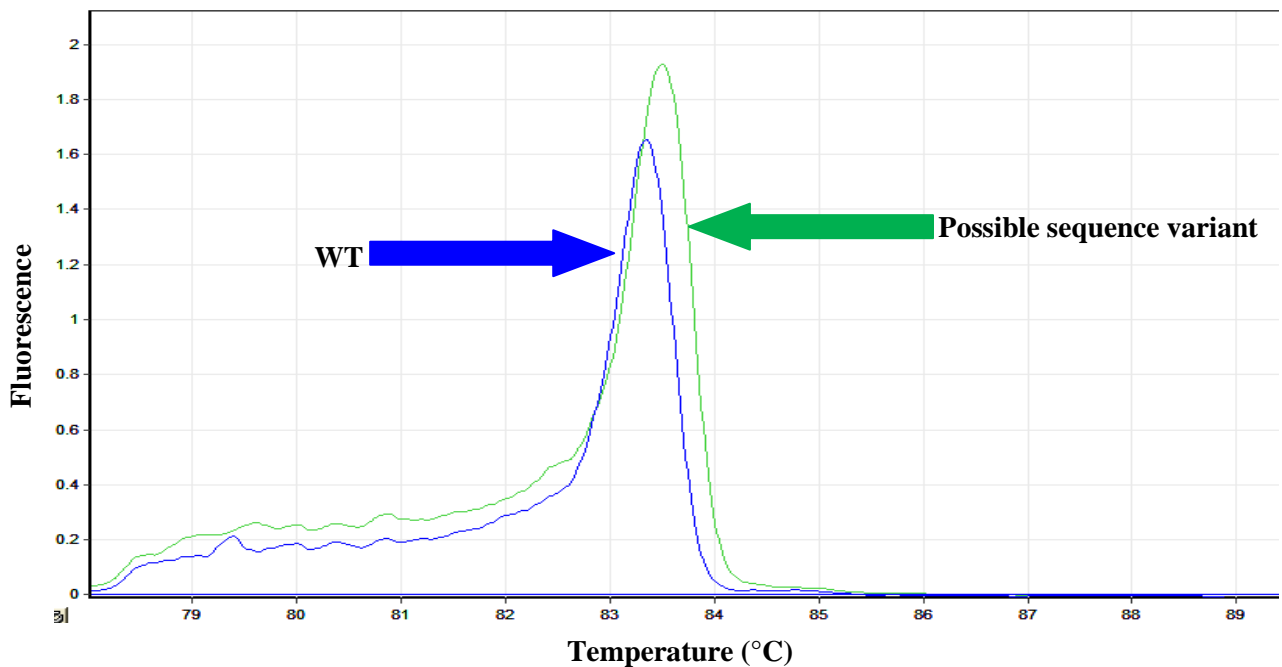
Figure 3.25: Melt curve of fragment E. Blue arrow: wild type; Green arrow: Samples with a possible sequence variant.
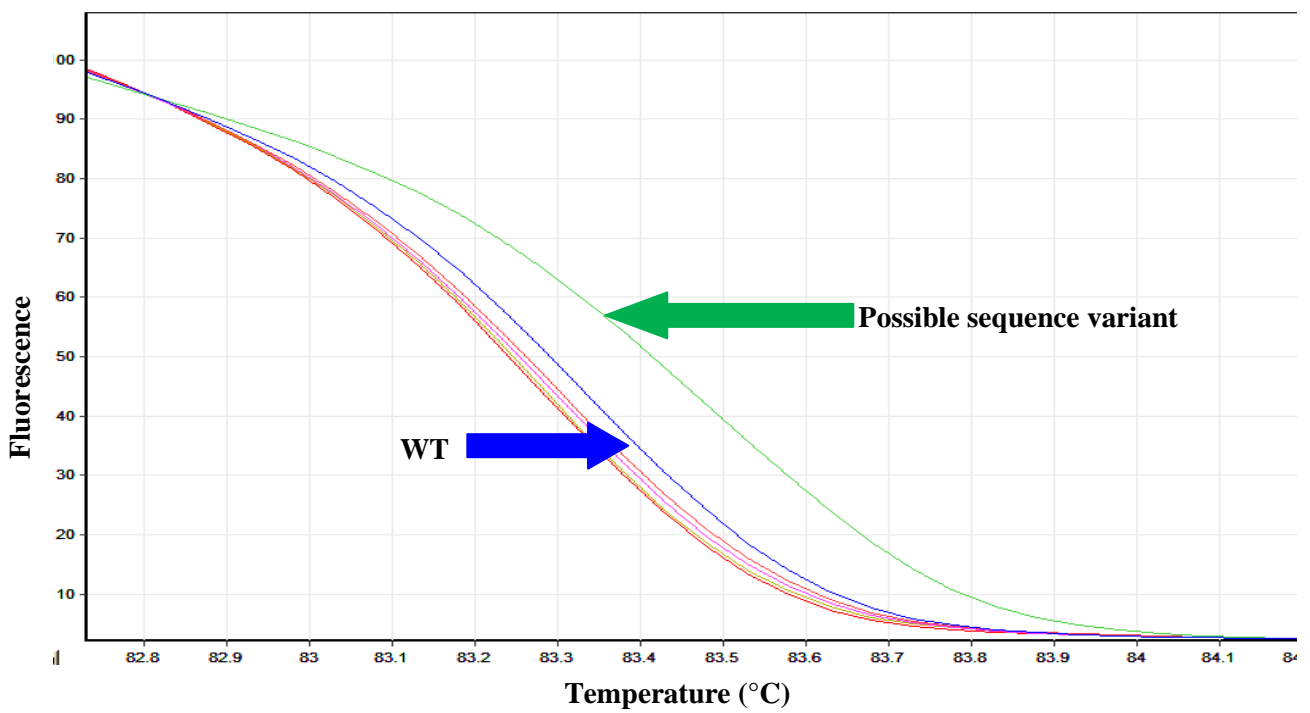
Figure 3.26: Normalised graph for fragment E. Blue arrow: wild type; Green arrow: Samples with a possible sequence variant.
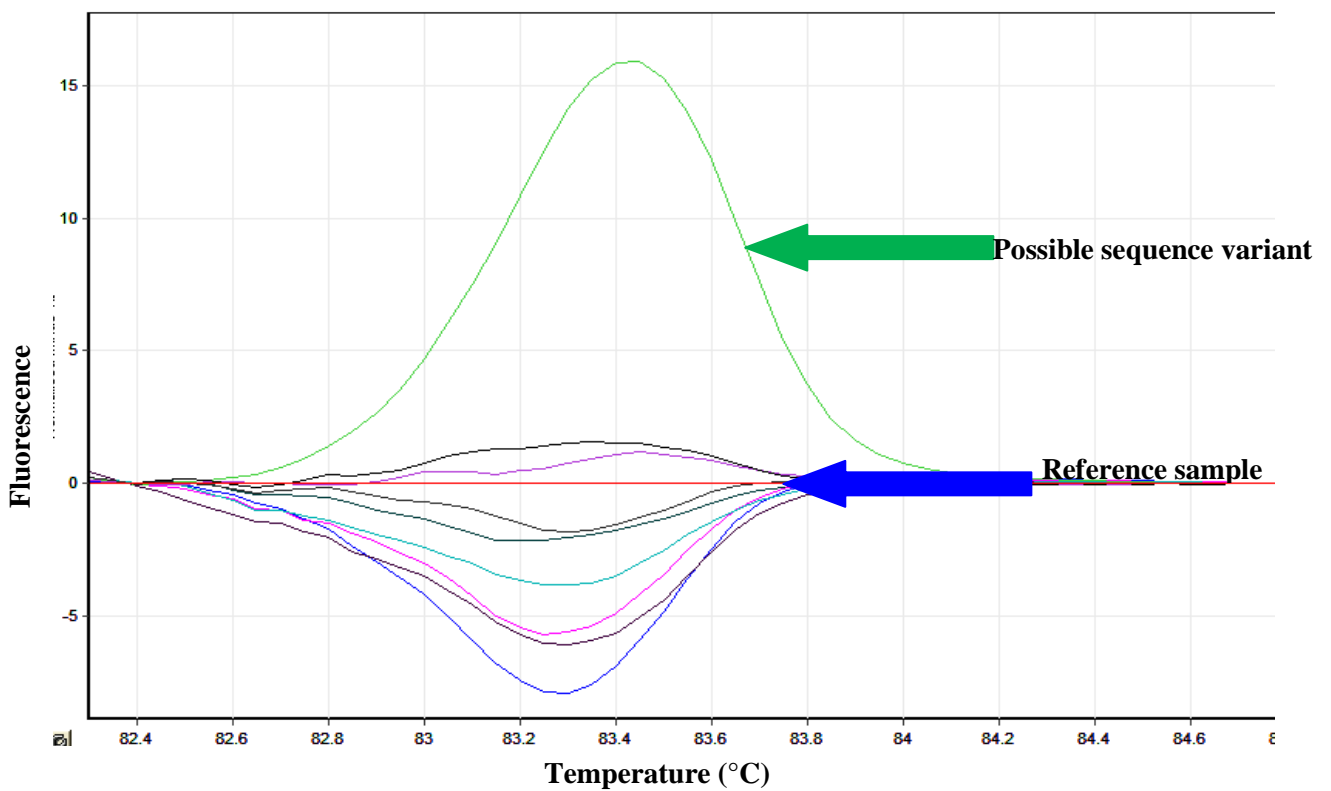
Figure 3.27: Difference graph of fragment E. This graph is a different representation of the differences between samples. Blue arrow: wild type; Green arrow: Samples with a possible sequence variant.

The positive controls that were included in the analysis of the six fragments could all be detected during HRM analysis except for the A827G mutation. The positions of the primers relative to this mutation could have influenced the detection of this mutation using HRM analysis. The positive control for the A827G mutation was included in the HRM analysis for fragments A and B. In fragment A the A827G mutation lies within the reverse primer sequence and for fragment B the mutation lies only two base pairs away from the fragment B forward primer sequence. Analysis of the melt curves, normalised graphs and difference graphs indicated that particular samples had possible sequence variants and these samples were sequenced in order to characterise the variant. In some cases there was more than one sample that looked as if they all had the same variant. In such cases a representative sample from the group was chosen and was then subjected to direct sequencing.

3.3. DNA sequencing

Direct sequencing of different samples from each of the six sets of fragments spanning the entire *MT-RNR1* gene identified a total of ten variants (**Table 3.3**). In fragment A a total of five variants were identified of which only one was found to be a novel change according to the mitochondrial DNA database MITOMAP (MITOMAP: A Human Genome Database http://www.mitomap.org). Two variants were detected in the fragment C of which only one was found to be a novel change. Only one variant was detected in fragments D and E, respectively, both of which were not novel changes in the *MT-RNR1* gene.

Table 3.3: Variants identified in *MT-RNR1*.

Position of variant on mtDNA	Fragment	Change	Novel or Known	Sample
680	A	T>C	Known	72.85
709	A	G>A	Known	72.85
719	A	G>A	Novel	74.08
750	A	A>G	Known	72.85, 72.88, 74.08, 71.62, b.son
769	A	G>A	Known	72.88, 74.08, 72.85
1040	C	T>C	Novel	72.85
1048	C	C>T	Known	68.23
1243	D	T>C	Known	68.20, 69.07
1346	E	A>G Homoplasmic & Heteroplasmic	Known	72.16, 68.18

Novel variants are shaded in yellow. All variants detected were homoplasmic except where indicated otherwise.

Figure 3.28 and Figure 3.30 are representative chromatograms for the novel variants found in fragments A and C. The variant at position 719 (G719A) in *MT-RNR1* was found in one MDR-TB patient who is of Mixed Ancestry descent. The variant at position 1040 (T1040C) was found in a MDR-TB patient of black African descent. The frequencies of both these variants were determined in ethnic-matched control samples.
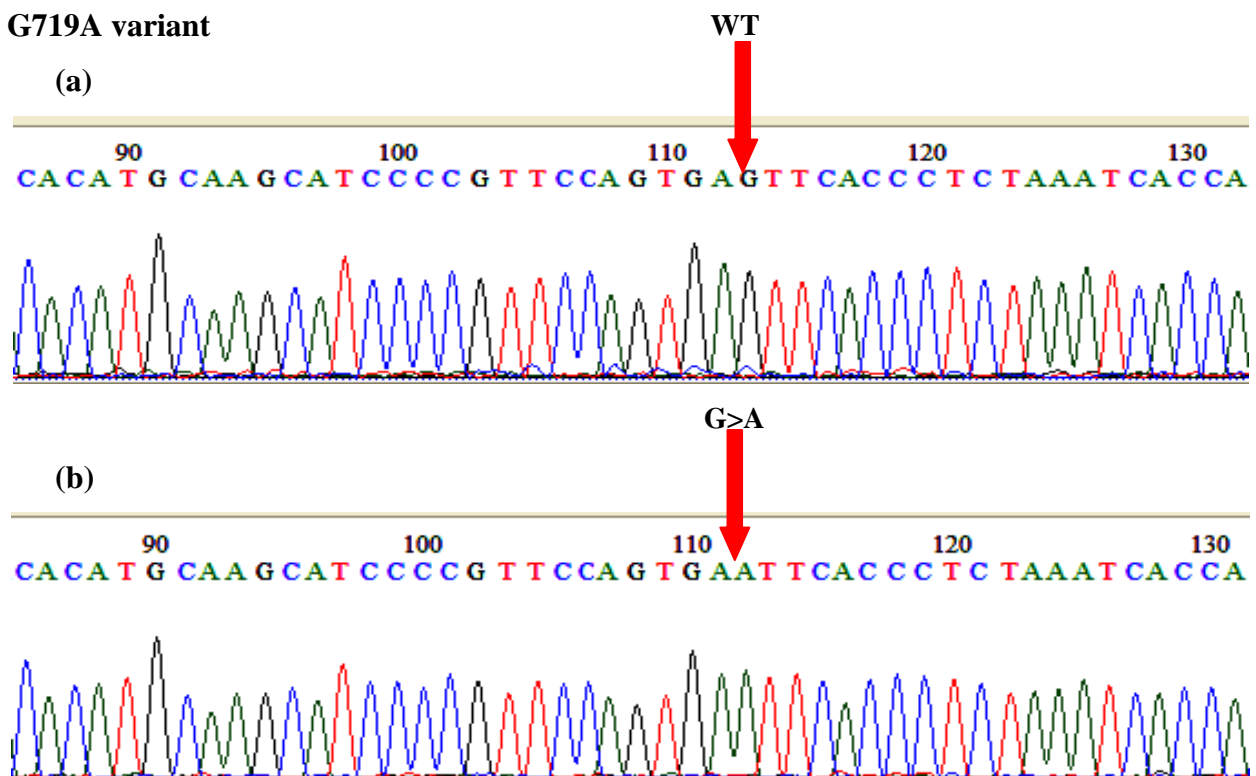
Figure 3.28: **Chromatogram of the novel variant identified in fragment A at position 719.** The position of the mutation is indicated with the red arrow. (a) Wild type and (b) G>A change at position 719 in *MT-RNR1*.

The frequency of the homoplasmic G719A variant was determined in 116 Mixed Ancestry controls by means of PCR-RFLP using EcoRI. The variant at position 719 is a G (wild type) to A (variant) change and the A allele creates a cutting site for the restriction enzyme EcoRI, resulting in the 245bp fragment being cut into two smaller fragments of 132bp and 113bp (**Figure 3.29**), while the G allele remains uncut.
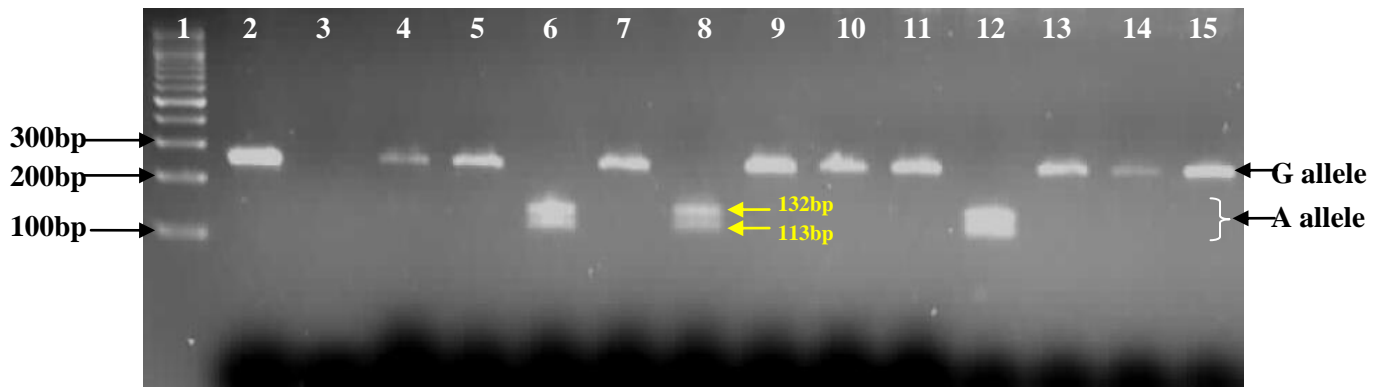
Figure 3.29: RFLP analysis (EcoRI) digest of Mixed Ancestry control samples for the G719A variant in *MT-RNR1*. Lane 1: 100bp molecular marker, 2: uncut sample (245bp) (sample that was not digested with EcoRI), lane 3: a non-template control, lanes 4-15: Digested PCR products, Lane 4: represents a sample with G allele, Lane 6: a sample with A allele.

The G719A variant was detected in 24/116 (20.7%) of the samples indicating that this variant is probably a common polymorphism in our South African Mixed Ancestry population.

T1040C variant

(a)

(b)
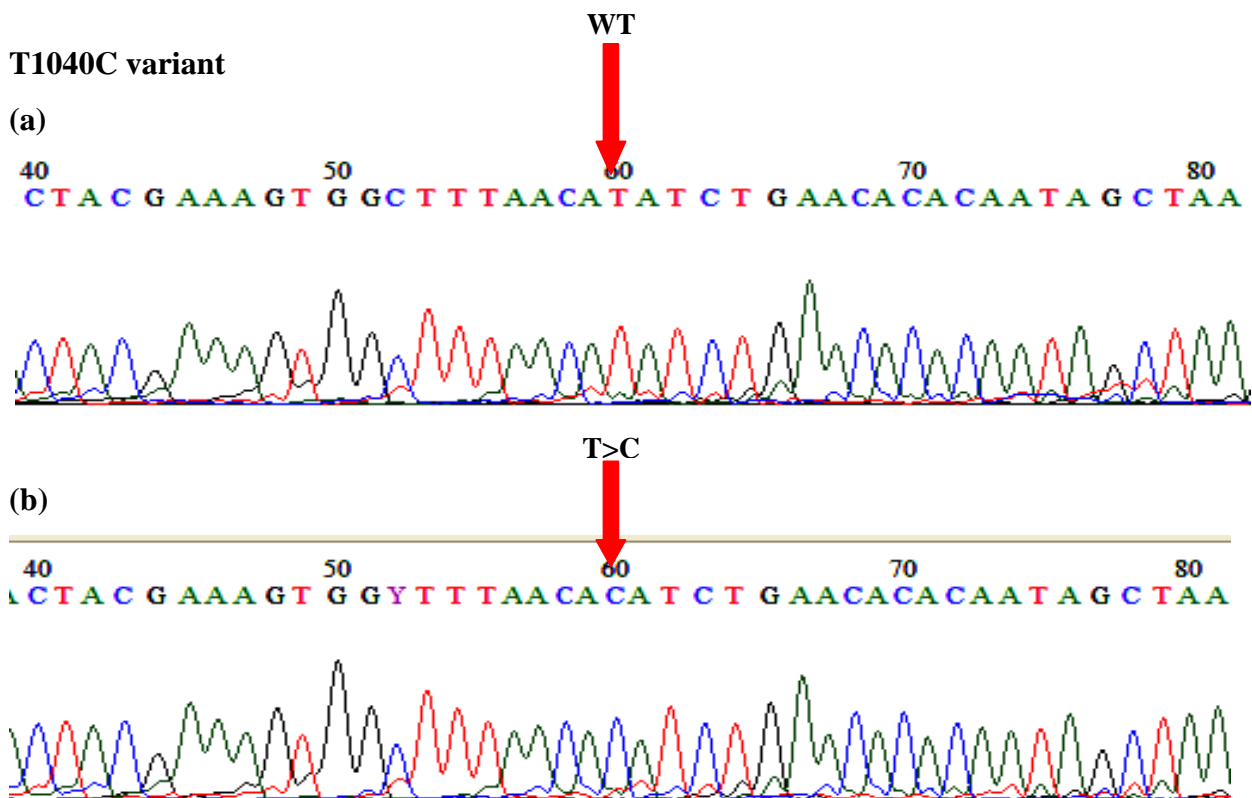
Figure 3.30: Chromatogram of the novel variant identified in fragment C at position 1040. The position of the mutation is indicated with the red arrow. (a) Wild type and (b) T>C change at position 1040 in *MT-RNR1*.

Since the T1040Cvariant does not create or destroy a restriction enzyme site, the frequency of this variant was determined in 112 black control samples by means of HRM analysis. In each HRM run a positive control for the T1040C variant and a wild type sample was included to which the melting profiles for the unknown samples were compared. The T1040C variant was detected in 2/112 (1.8%) of the samples indicating that this variant is probably a common polymorphism in our black South African population.

4. Sequencing of entire mitochondrial genome

The entire mitochondrial genome (16kb) was sequenced in eight MDR-TB samples. In these patients none of the known aminoglycoside-induced hearing loss mutations was detected but all were suffering from ototoxicity as determined by their audiological results. Due to the high cost involved in whole mitochondrial sequencing only eight samples were analyzed.

In these patients we detected a total of 50 novel variants **(Table 3.4)** that could possibly be associated with or play a role in aminoglycoside-induced ototoxicity. Some of the variants were present in more than one sample.

In the non-protein coding genes, one novel variant (C527T) was identified within the D-Loop region of the mitochondrial genome. One variant was identified in each of the two tRNA genes (*MT-TY* and *MT-TH*) (at position 5892 and 12142, respectively), in *MT-RNR1* (at position 719) and two novel variants were identified in the *MT-RNR2* gene (at positions 1694 and 2755).

The remaining variants were all identified within the following protein coding genes: **(Table 3.4)**

- NADH dehydrogenase 1 (*ND1*), four variants identified,

- NADH dehydrogenase 2 (*ND2),* four variants identified,

- Cytochrome c oxidase I (*COX1*), eight variants identified,

- Cytochrome c oxidase II (*COX2*), one variant identified,

- ATP synthase 8 *(ATP8*), two variants identified,

- ATP synthase 6 (*ATP6*), three variants identified,

- Cytochrome c oxidase III (*COX3*), six variants identified,

- NADH dehydrogenase 3 *(ND3)*, two variants identified,

- NADH dehydrogenase subunit 4L (*ND4L)*, one variant identified,

- NADH dehydrogenase 4 (*ND4*), three variants identified,

- NADH dehydrogenase 5 (*ND5*), four variants identified,

- NADH dehydrogenase 6 (*ND6*), two variants identified

- Cytochrome b (*CYTB*), four variants identified.

Each of the 50 novel variants identified were further evaluated for both nucleotide and protein conservation across five species: Human (*Homo sapiens*), Chimpanzee (*Pan troglodytes*), Mouse (*Mus musculus*), Bovine (*Bos taurus*) and Frog (*Xenopus laevis*). The variants identified in protein coding genes have potential important implications for the normal functioning of the mitochondrial oxidative phosphorylation system since all of the above mentioned protein genes code for some component of this system. The novel variants identified within the tRNA and mRNA genes may have an effect on protein decoding and translation, also affecting the normal functioning of the mitochondrial proteins.

Table 3.4: A total of 50 novel variants identified during whole mitochondrial genome sequencing of eight South African MDR-TB patients.

Gene	Position	Replacement *	Nucleotide		Protein		Number of individuals	Sample nr.
			Species Conservation ●(H/C/M/B/X)	Sequence Conservation	Species Conservation ●(H/C/M/B/X)	Amino Acid Conservation		
D-LOOP	527	C to T	C/A/A/T/T	N	-	-	2	68.43 , 69.49
MT-RNR1	719	G to A	G/G/A/G/-	N	-	-	2	70.69 , 68.43
MT-RNR2	1694	T to C	T/T/T/A/A	N	-	-	1	68.18
MT-RNR2	2755	A to G	A/A/A/C/A	N	-	-	1	69.07
ND1	3423	T to C	T/T/C/T/T	N	V/I/I/V/I	N	1	69.49
ND1	3756	A to G	A/A/G/T/C	N	L/L/L/L/L	Y	2	70.69 , 68.43
ND1	4215	A to G	A/A/A/A/A	Y	W/W/W/W/W	Y	1	69.49
ND1	4232	T to G (Ile-Ser)	T/C/T/T/T	N	I/T/I/I/I	N	3	70.69 , 68.43 , 69.07
ND2	4949	A to G	A/G/T/A/A	N	L/L/T/L/T	N	1	68.18
ND2	5090	T to C	T/T/C/C/T	N	I/I/I/I/I	Y	1	69.70
ND2	5111	C to T	C/C/C/C/C	Y	T/T/P/T/T	N	1	69.49
ND2	5483	T to G	T/C/C/A/A	N	P/P/P/P/P	Y	1	68.18
MT-TY	5892	T to C	T/-/-/-/-	N	-	-	1	69.49
COX1	6170	C to T	C/C/C/T/C	N	A/A/A/A/A	Y	1	69.07
COX1	6182	G to A	G/A/A/A/A	N	A/A/A/A/A	Y	1	69.49
COX1	6266	A to G	A/C/A/G/A	N	G/G/G/G/G	Y	2	70.69 , 68.43
COX1	6297	T to C	T/T/C/T/T	N	L/L/L/L/L	Y	1	69.49
COX1	6377	C to T	C/C/T/T/T	N	I/I/I/I/I	Y	1	69.07
COX1	6668	C to T	C/C/A/C/C	N	S/S/S/S/S	Y	1	70.69
COX1	6815	T to C	T/T/C/C/C	N	Y/Y/Y/Y/Y	Y	3	70.69 , 68.43 , 69.07
COX1	7283	T to C	T/C/T/T/T	N	I/I/I/I/I	Y	1	70.69
COX2	7972	A to G	A/A/A/G/C	N	E/E/K/K/T	N	1	69.49
ATP8	8478	C to T (Ser-Leu)	C/C/C/A/C	N	S/S/S/E/T	N	1	68.18
ATP8	8487	C to T (Phe-Leu)	C/C/C/C/C	Y	P/P/S/P/T	N	1	68.18
ATP6	8598	T to C	T/T/T/A/A	N	I/I/I/V/M	N	1	69.07
ATP6	9027	C to T	C/C/A/A/A	N	G/G/G/G/G	Y	1	69.07

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Gene	Position	Replacement *	Nucleotide		Protein		Number of individuals	Sample nr.
			Species Conservation ●(H/C/M/B/X)	Sequence Conservation	Species Conservation ●(H/C/M/B/X)	Amino Acid Conservation		
ATP6	9042	C to T	C/C/C/C/A	N	H/H/H/H/Q	N	3	70.69, 69.07, 68.43
COX3	9305	G to A (Met-Ile)	G/A/A/G/A	N	M/M/M/M/M	Y	1	69.49
COX3	9464	T to C	T/T/C/T/T	N	F/F/F/F/F	Y	1	68.18
COX3	9488	C to T	C/T/T/T/T	N	F/F/F/F/F	Y	1	69.07
COX3	9855	A to G (Ile-Val)	A/A/G/G/G	N	I/I/V/V/V	N	1	68.18
ND3	10114	T to C (Ile-Thr)	T/T/T/T/T	Y	I/I/L/I/I	N	1	69.07
ND3	10128	C to A (Leu-Met)	C/C/C/C/C	Y	L/L/L/L/L	Y	1	69.07
ND4L	10664	C to T	C/C/T/C/T	N	V/V/V/V/P	N	3	70.69 , 68.43 , 69.07
ND4	10783	A to C	A/A/A/A/A	Y	T/T/S/T/T	N	1	68.18
ND4	11318	T to C (Ser-Pro)	T/T/G/C/C	N	S/S/D/H/P	N	1	69.49
ND4	12022	C to T	C/C/C/C/A	N	H/H/N/Y/E	N	1	69.49
MT-TH	12142	A to G	A/A/A/A/A	Y	-	-	1	68.43
ND5	12432	C to T	C/C/C/-/T	N	Y/Y/F/Y/L	N	1	69.49
ND5	12609	T to C	T/C/G/G/A	N	P/P/S/P/P	N	1	68.18
ND5	12798	C to T	C/C/A/C/A	N	L/L/L/L/L	Y	1	68.43
ND5	13470	A to G	A/A/C/C/A	N	L/L/L/L/L	Y	1	68.18
ND6	14315	C to T (Ser-Ile)	C/C/C/C/T	N	S/S/G/G/E	N	1	70.69
ND6	14659	C to T (Leu-STOP)	C/T/A/T/C	N	L/L/I/V/V	N	1	70.69
CYTB	15312	T to C (Ile-Thr)	T/T/T/T/T	Y	I/I/I/I/I	Y	1	69.07
CYTB	15466	G to A	G/G/A/A/T	N	M/M/M/M/T	N	3	70.69 , 68.43 , 69.07
CYTB	15692	A to G (Met-Val)	T/T/T/T/T	Y	M/M/M/M/M	Y	1	70.69
CYTB	15735	C to T (Ala-Val)	C/C/C/C/C	Y	A/T/A/A/A	N	1	69.70

Novel variants that caused an amino acid change and showed amino acid conservation across all five species are shaded in yellow.

Novel variants that are in non-protein coding genes and are conserved across all five species are shaded in blue.

●(H): human; (C): Chimpanzee; (M): Mouse; (B): Bovine; (X): Frog

* Only changes that cause an amino acid replacement are indicated in brackets.

N= No;

Y=Yes

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Only one of the five novel variants identified within the non-protein coding genes (*MT-RNR1, MT-RNR2, MT-TY* and *MT-TH*) genes showed evolutionary conservation (A12142G in *MT-TH*). This variant was only present in one of the MDR-TB samples.

The A12142G variant does not occur in a well-conserved region of the *MT-TH* gene **(Figure 3.31),** compared to the A1555G mutation in *MT-RNR1*, and therefore might not be associated with aminoglycoside-induced hearing loss in our populations.
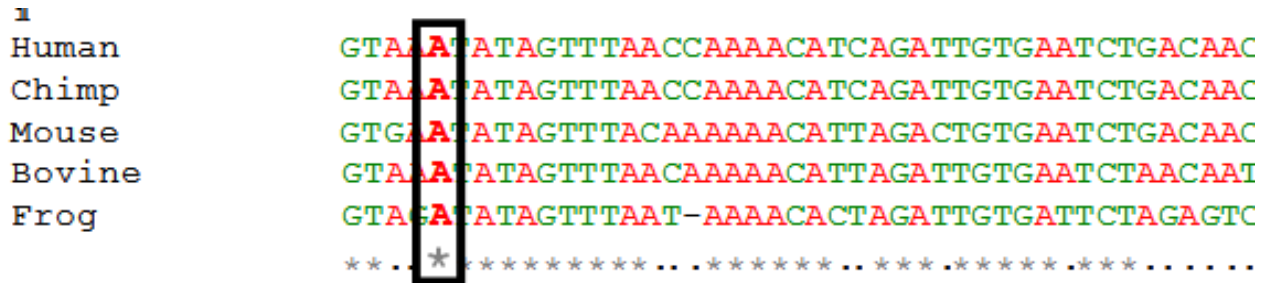
Figure 3.31: Nucleotide sequence alignment for the novel A12142G variant identified in the *MT-TH* gene.

From the 50 variants, only four caused an amino acid change in a protein coding gene and occur in evolutionary-conserved amino acids **(Table 3.4)**.

The position of the G9305A (M33I) variant in the *COX3* gene is evolutionary conserved across all five species only on the protein level (**Figure 3.32 a and b**). This variant was only found in one of the MDR-TB samples.

(a) Nucleotide sequence

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(b) Protein sequence

```
Human···········MTHQSHAYHMVKPSPWPLTGALSALLMTSGLA M WFHFHSMTLLMLGLLTNTLTMYQWWRD·
Chimp···········MTHQSHAYHMVKPSPWPLTGALSALLMTSGLA M WFHFYSTTLLTLGLLTNTLTMYQWWRD·
Mouse··········MTHQTHAYHMVNPSPWPLTGAFSALLLTSGLV M WFHYNSITLLTLGLLTNILTMYQWWRD·
Bovine·········MTHQTHAYHMVNPSPWPLTGALSALLMTSGLI M WFHFNSMTLLMIGLTTNMLTMYQWWRD·
Frog···········MAHQAHAYHMVDPSPWPLTGAVAALLLTSGLA M WFHFGSMILLTLGLITMVLTMIQWWRD·
            ·*;**;******.********* ;***;**** * ***;. *..**.;**.*...***.*****
```

Figure 3.32: **Sequence alignment for the novel G9305A (M33I) variant in the *COX3* gene.** (a) Nucleotide sequence alignment. Position 9305 is indicated by the box. (b) Protein alignment indicating the conserved Methionine amino acid at position 33.

The C10128A (L24M) variant in the *ND3* gene showed evolutionarily conservation across all of the species at both the nucleotide and amino acid level (**Figure 3.33 a and b**). This variant was only detected in one of the eight samples.

(a) Nucleotide sequence

```
Human··········TAATAATTATTACATTTTGA C TACCACAACTCAACGGCTACAT.
Chimp··········TGATAATTATCACATTCTGA C TACCACAACTCAACAGCTACAT.
Mouse·········TAATTCTAGTTGCATTCTGA C TCCCCCAAATAAATCTGTACTC.
Bovine········TCGTCATCATCGCATTCTGA C TTCCCCAACTAAATGTATACTC·
Frog··········TAGCAATCTTAAGTTTTTGA C TTCCCCAAATAACCCCTGATAT.
            ·....**.*****.**.***.. * .*.......*....**.***....
```

Figure 3.33: Sequence alignment for the novel C10128A (L24M) variant identified in the *ND3* gene. (a) Nucleotide sequence alignment. Position 10128 is indicated by the box. (b) Protein alignment indicating the conserved Leucine amino acid at position 24.

The T15312C (I189T) and A15692G (M316V) variants in the *CYTB* gene both showed evolutionary conservation across all of the species at both the nucleotide and amino acid level. Each variant was only detected in one MDR-TB sample (Table 3.4).

The *CYTB* gene is a highly conserved gene and both the T15312C (I189T) **(Figure 3.34 a and b)** and A15692G (M316V) **(Figure 3.35 a and b)** variants reside in regions of the gene that are evolutionarily conserved from human to frog. This indicates that these regions are important in the functioning of the protein.

(a) Nucleotide Sequence

(b) Protein Sequence

```
Human··········TFHFILPFI I AALAALHLLFLHETGSNNPLGITSHSDKITFHPYYTIKDALGLLLFLLSL
Chimp·········TFHFILPFI I TALTTLHLLFLHETGSNNPLGITSHSDKITFHPYYTIKDILGLFLFLLIL
Bovine········AFHFILPFI I MAIAMVHLLFLHETGSNNPTGISSDVDKIPFHPYYTIKDILGALLLILAL
Mouse·········AFHFILPFI I AALAIVHLLFLHETGSNNPTGLNSDADKIPFHPYYTIKDILGILIMFLIL
Frog··········AFHFLLPFI I AGASILHLLFLHETGSTNPTGLNSDPDKVPFHPYFSYKDLLGFLIMLTAL
              :***:***: *   .·:·:*********·**·*:·*·*·**:·****::·**·**·:::··*
```

Figure 3.34: Sequence alignment for the novel T15312C (I189T) variant identified in the *CYTB* gene. (a) Nucleotide sequence alignment. Position 15312 is indicated by the box. (b) Protein alignment indicating the conserved Isoleucine amino acid at position 189.

(a) Nucleotide Sequence

```
Human··········ACCTTTCACTTCATCTTGCCCTTCATTA T TGCAGCCCTAGCAACACTCCACCTCCTATTC
Chimp··········ACCTTCCACTTTATCTTACCCTTCATCA T CACAGCCCTAACAACACTTCATCTCCTATTC
Mouse··········GCTTTCCACTTCATCTTACCATTTATTA T CGCGGCCCTAGCAATCGTTCACCTCCTCTTC
Bovine·········GCTTTCCATTTTATCCTTCCATTTATCA T CATAGCAATTGCCATAGTCCACCTACTATTC
Frog···········GCATTTCACTTCCTCCTTCCTTTTATTA T TGCCGGAGCTAGCATTCTCCATCTTTTATTT
              ·*·**·**·**·**··**·*·**·**·**· *   ···*··········*····*·**·**··*·**·
```

(b) Protein Sequence

```
Human··········ILAMIPILHMSKQQSN M FRPLSQSLYWLLAADLLILTWIGGQPVSYPFTIIGQVASVLYF
Chimp··········ILTAIPVLHTSKQQSN M FRPLSQLLYWLLATDLLILTWIGGQPVSYPFITIGQMASVLYF
Bovine·········ILALIPLLHTSKQRSN M FRPLSQCLFWALVADLLTLTWIGGQPVEHPYITIGQLASVLYF
Mouse·········ILALMPFLHTSKQRSI M FRPITQILYWILVANLLILTWIGGQPVEHPFIIIGQLASISYF
Frog··········ILALMPLLHTSKQRSI M FRPFTQIMFWALVADTLILTWIGGQPVEDPYTMIGQLASVIYF
              **:··:*·**·***:*  *  **::*·.::*·*·::·*·********·.·*:··***:**:··**
```
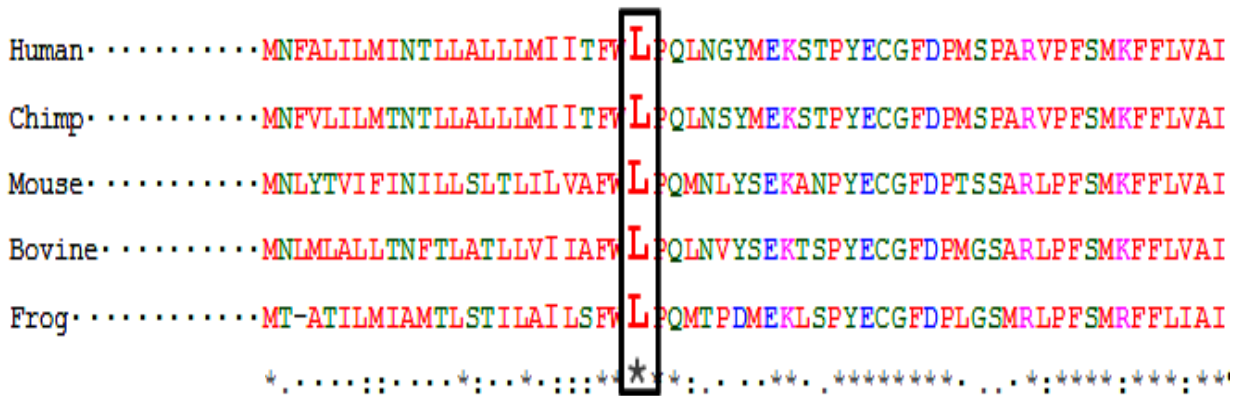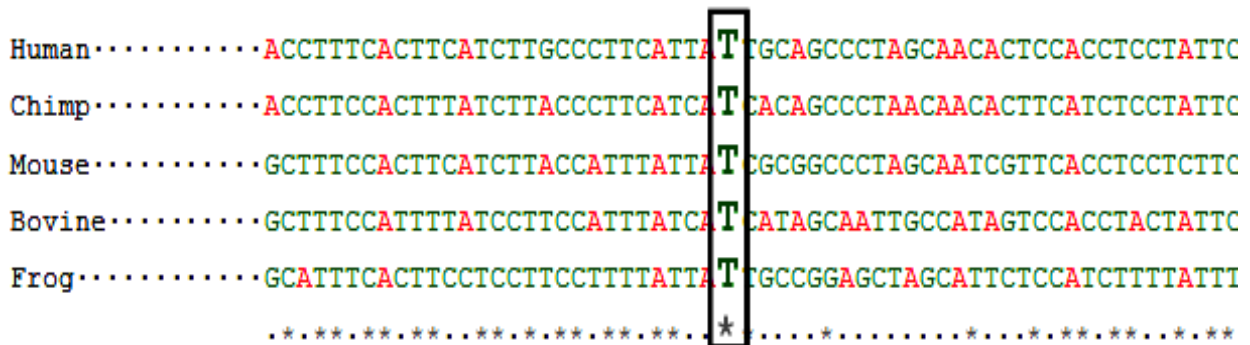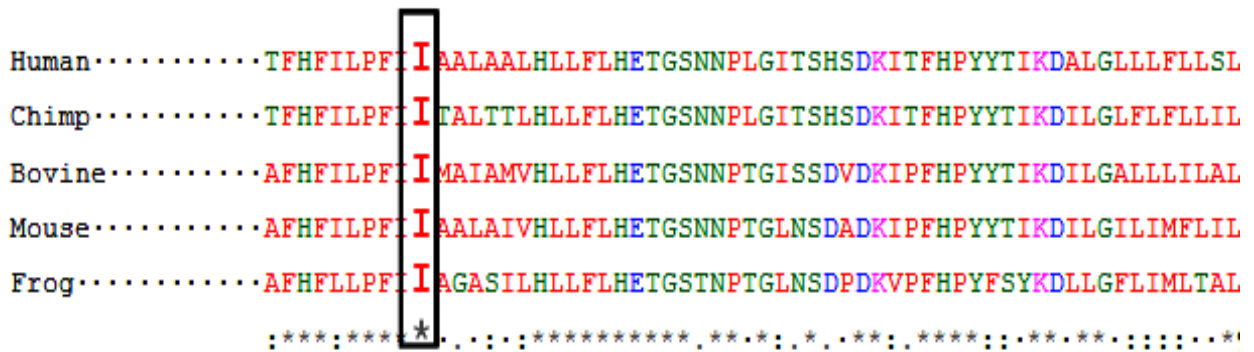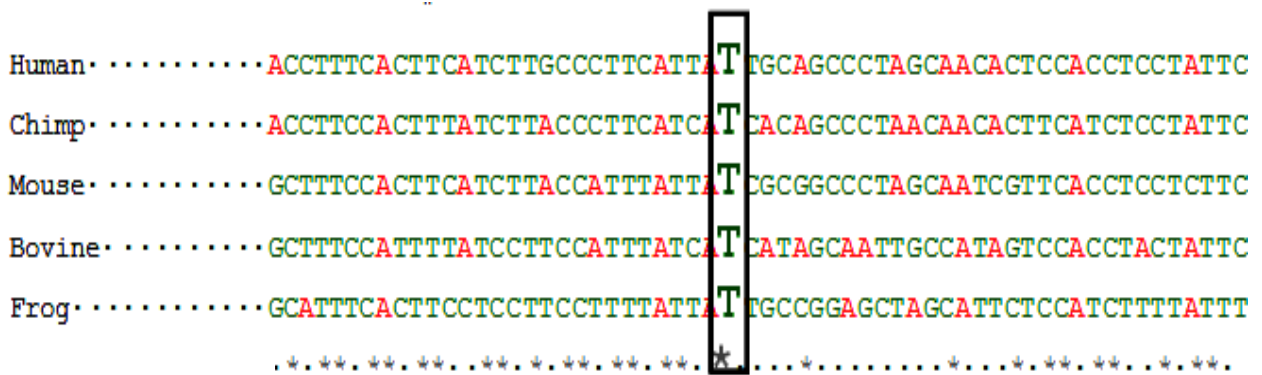
Figure 3.35: Sequence alignment for the novel A15692G (M316V) variant identified in the *CYTB* gene. (a) Nucleotide sequence alignment. Position 15692 is indicated by the box. (b) Protein alignment indicating the conserved Methionine amino acid at position 316.

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Four of the five novel variants were identified in protein coding genes and occur in regions that are evolutionarily-conserved and therefore it is possible that the variants identified could affect the normal functioning of these proteins. Thus further functional studies regarding these variants are needed. All these proteins are involved the mitochondrial respiratory chain and these variants may influence the normal functioning of this complex process. Altered functioning of the respiratory chain might lead to increased reactive oxygen species (ROS) formation in the mitochondrial thus possibly activating the apoptotic pathways (Chapter 1, Section 2.1.2) of the cell and causing cell death of the hair cells in the cochlea.

This is the first time that protein-coding genes are thought to be possibly involved in aminoglycoside-induced hearing loss.

5. Genetic analysis of the A1555G mutation-positive South African Family

In the present study we re-investigated the South African family with streptomycin-induced hearing loss known to harbour the A1555G mutation. This family had been first identified and investigated in 1983 after several members of the family had experienced hearing loss after treatment with streptomycin for TB. At the time it was believed that the ototoxicity was due to an autosomal dominant trait (Viljoen, Sellars, and Beighton 1983). After the discovery of the A1555G mutation and its association with aminoglycoside deafness (Prezant et al. 1993) molecular screening for the A1555G mutation was done on 18 family members. It was found that the mutation was present in all 18 of the members screened (Gardner et al. 1997).

5.1. Identification of mutation positive family members

We were successful in recruiting a total of 97 members from the family which included a number of small children under the age of 12 years. Genotyping was performed using SNaPshot analysis to identify A1555G-positive family members. As stated previously (Chapter 2 Section 5), for all children in this family, under the age of 12, DNA was extracted from buccal cells. Our results showed that the SNaPshot analysis method could be used successfully to genotype the DNA extracted from buccal cells (**Figure 3.36**). This DNA is usually of lower quality and concentration than DNA extracted from blood.
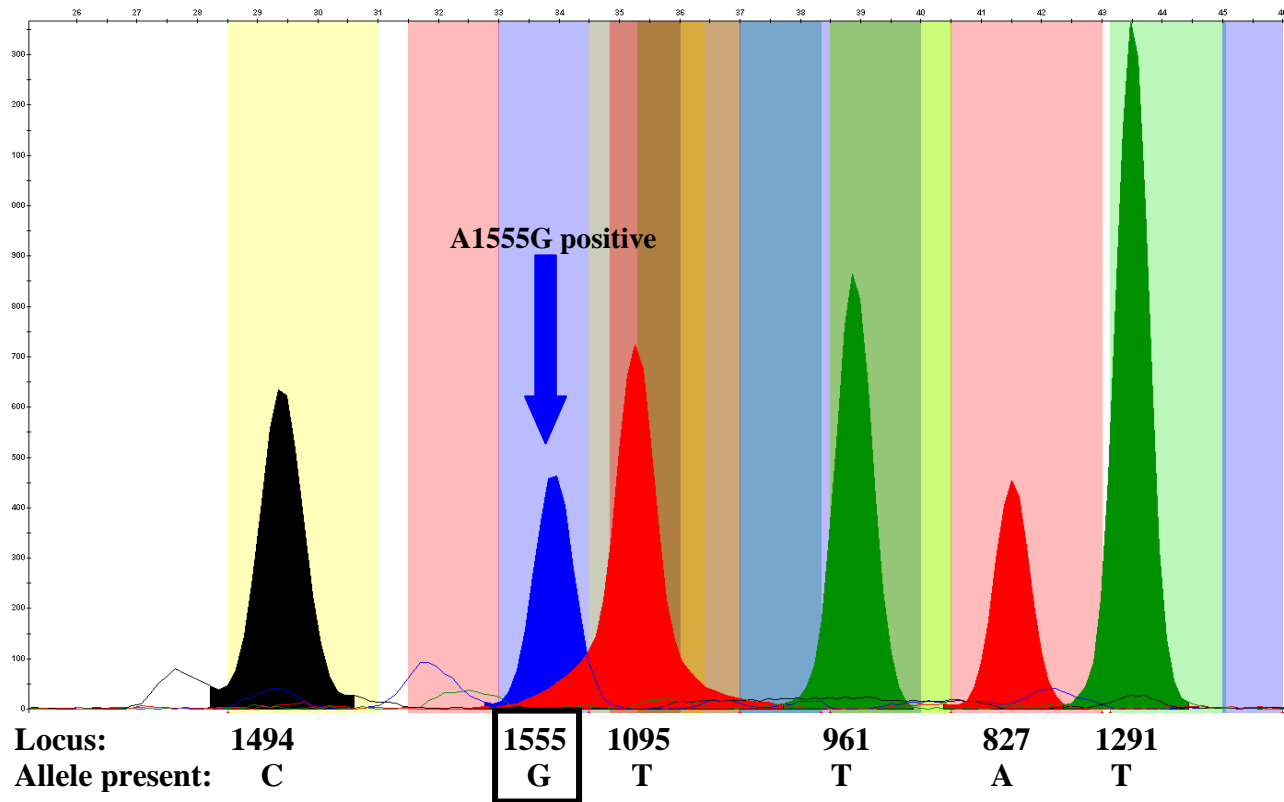
Figure 3.36: SNaPshot analysis of the six mutations associated with aminoglycoside-induced hearing loss (C1494T, T1095C, A1555G, 961delT +insC_{(n)}, A827G, T1291C) in a DNA sample extracted from buccal cells. The different peaks shown in this electropherogram represent wild type alleles, for one individual, at five loci (1494, 1095, 961, 827,1291) and a mutant allele at the 1555 locus (G allele; blue peak).

A total of 76 of the 97 individuals screened were shown to harbour the A1555G mutation (**Figure 3.37**). Of these, 7 individuals already have hearing impairment (Gardner et al. 1997) and 69 members are therefore at risk of developing hearing loss if treated with aminoglycosides. All 97 family members did not have C1494T, T1095C, 961delT + insC_{(n)}, A827G or T1291C.

All A1555G-positive members of this family were given genetic reports and counseled about their risk and that of their children of developing deafness as a result of aminoglycoside use. It is hoped that with this knowledge individuals can empower themselves to protect themselves from

the toxic side effects of these drugs. Knowledge about a patient's genetic status regarding these mutations can aid the clinician in the planning of the possible treatment regimes for that individual. Possible ways to preserve hearing in susceptible individuals include lowering the doses of aminoglycosides used, avoiding the use of other ototoxic drugs and motivation for regular

audiological monitoring. It is important to note that aminoglycosides are also used in pregnant women thereby placing the unborn child at risk of developing permanent deafness (Donald and Sellars 1981).   Aminoglycosides are often given to premature babies at birth to treat bacterial infections. In the present study, the A1555G-positive status of a baby who was born prematurely was reported to the medical personnel so that alternative drugs could be used for his treatment.
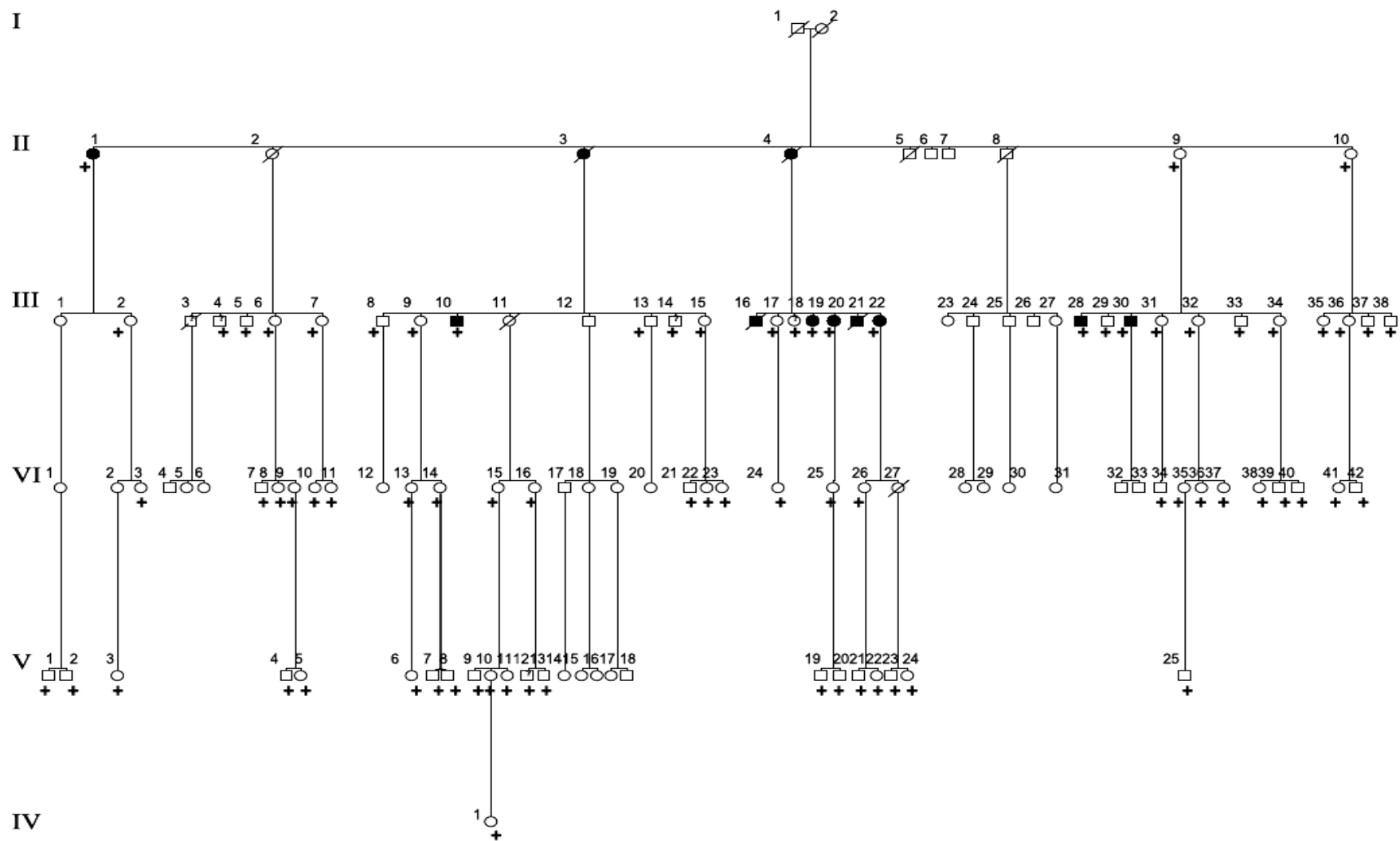
Figure 3.37: Pedigree of the South African family harbouring the A1555G mutation. Due to the large size of this family only members who were genotyped are included on the pedigree. Individuals found to be A1555G-positive are indicated by a +. Individuals with deafness are indicated by a filled in symbol.

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5.2. Mitochondrial haplogroup analysis

Mitochondrial haplogroup analysis was performed on a member of the family in order to determine to which haplogroup the family belongs to. It has been hypothesised that one of the factors that may contribute to the variability in the phenotypic expression is certain mitochondrial haplogroups (Guan et al. 1998; Li et al. 2004b; Pandya et al. 1999). However, to date no correlations have been made between certain mutations and mitochondrial haplotypes.

It was determined that the South African family belongs to the African L0d (L0d1b) previously known as L1d mitochondrial haplogroup (**Figure 3.38**). This is the first report of the A1555G mutation on haplogroup L, which is characterised by ancestral influences from the Khoi-San populations from South Africa as well as Bantu-speakers from Africa (Bandelt and Forster 1997; Salas et al. 2002). The mitochondrial DNA (mtDNA) types found in the African populations share certain common features and have been assigned to haplogroup L, which can be further sub-divided into L0, L1, L2, L3, L4, L5 and L6. The subgroup L0 includes the oldest common ancestor of human mtDNA.
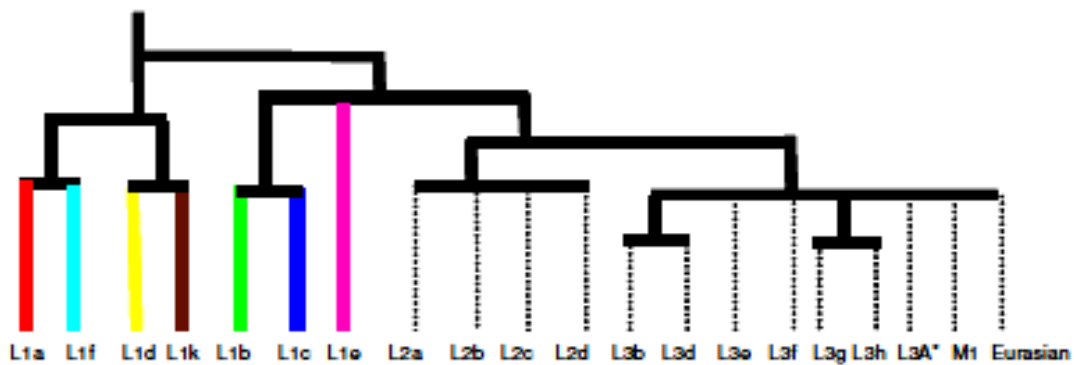
Figure 3.38: Phylogenetic representation of the different mitochondrial haplogroups. Haplogroup L0d (previously known as L1d) to which the South African family belongs is indicated in yellow (Taken from: Barkhan, 2004).

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5.3. Screening for genetic modifiers

Furthermore, we screened the family members who had hearing impairment for the presence of known genetic modifiers in tRNA$^{(Ser)(UCN)}$ and *TRMU*, and for the 35delG mutation in *GJB2*.

5.3.1. Analysis of the tRNA$^{(Ser)(UCN)}$

Mutations in the mitochondrial *MT-TS1* gene coding for the tRNA$^{(Ser)(UCN)}$ have been proposed to be genetic modifiers for the phenotypic expression of aminoglycoside-induced hearing loss (del Castillo et al. 2002; Jin et al. 2007). *MT-TS1* was screened to detect any of the known and novel variants.

5.3.1.1. Polymerase chain reaction (PCR) amplification

The entire *MT-TS1* was amplified in a 286bp fragment. Due to the small size of the tRNA genes in the mitochondrial genome and the absence of introns, the targeted fragment included another tRNA gene, *MT-TD* (tRNA$^{(Asp)}$) which lies directly adjacent to tRNA$^{(Ser)(UCN)}$. **Figure 3.39** is a representative gel of the amplified PCR products.
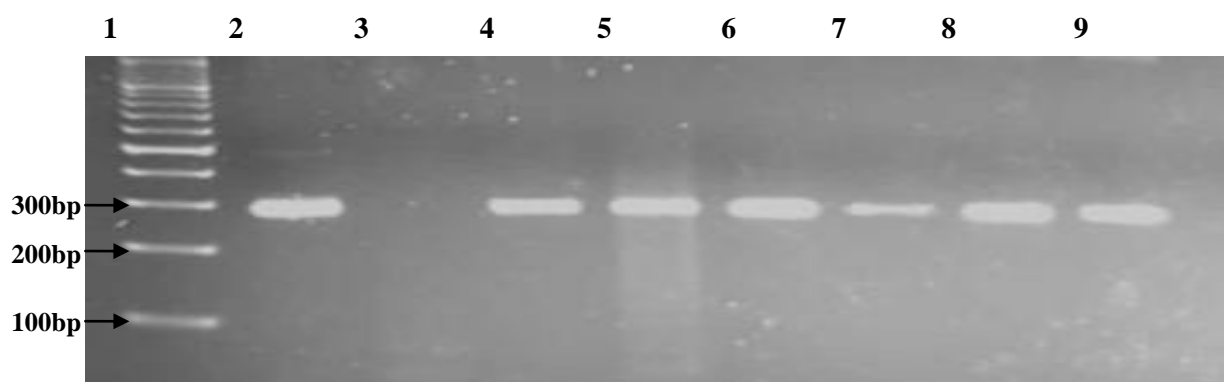
Figure 3.39: *MT-TS1* (tRNA$^{(ser)(UCN)}$) **PCR amplified products.** Lane 1: Molecular size marker; Lane 2: 280 PCR fragment; Lane 3: Non-template control; Lanes 4-9: PCR products. After amplification, the products were electrophoresed on a 2% Agarose gel and visualised under a UV light.

5.3.1.2. *DNA sequencing*

The PCR amplified products obtained from seven deaf members and the four possibly affected members of the family was subjected to direct sequencing analysis. Sequencing did not detect any sequence variants in *MT-TS1* in any of the affected or possibly affected family members.

5.3.2. **Analysis of A10S variant in the *TRMU***

The nuclear gene *TRMU* (tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase) has been hypothesized to be a modifier for the variable phenotypic expression found in individuals harbouring the A1555G mutation found in the mitochondrial *MT-RNR1* gene. The function of the TRMU protein is primarily 2-thiolytion of hypermodified nucleoside 5-methyl-aminomethy-2-thio-uridine, found in the wobble position of a number of bacterial and human mitochondrial tRNAs (tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}) and has a critical role in the structure and function of tRNAs. Recently, a missense mutation (A10S) was identified in a large Arab-Israeli, Italian and 6 Spanish families who harboured either the A1555G or the C1494T mutations. This mutation is located in the evolutionary conserved N-terminal region of *TRMU* (Guan et al. 2006). All members of the families who carried either the A1555G or C1494T in combination with the homozygous A10S mutation presented with profound deafness. Functional studies on the A10S mutation revealed that this mutation (in the homozygous form) results in a defect in the 2-thio modification of mitochondrial tRNAs, which lead to a decrease in the steady state levels of these tRNAs with subsequent impairment of mitochondrial translation, thus modulating the phenotypic manifestations of the deafness-associated *MT-RNR1* mutations (Guan et al. 2006). A total of 11 members (seven affected and four possibly affected) from the South African family with streptomycin –induced hearing loss were screened for the presence of the A10S mutation.

5.3.2.1. *Polymerase chain reaction (PCR) amplification*

A fragment of 165bp (within the first exon), containing the A10S (G28T) mutation, of *TRMU* was PCR amplified. After amplification the products were resolved on a 2% agarose gel to confirm the successful amplification of the fragment. A representative example of an agarose gel with the amplified product is shown in **Figure 3.40**. The correct size of the amplified product was examined by loading a 100bp molecular marker in the first lane of the gel. A negative control sample (containing no DNA) was always included to exclude any possible PCR contamination.
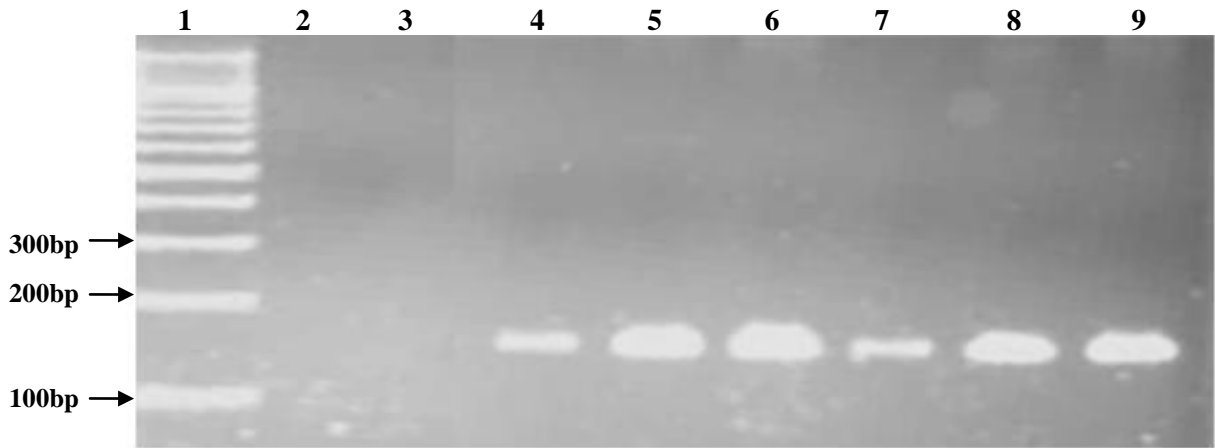
Figure 3.40: Amplified product of the fragment in exon one of the *TRMU*. Lane 1: Molecular size marker; Lanes 2-3: Non-template controls; Lanes 4-9: PCR products. After amplification, the products were electrophoresed on a 2% Agarose gel and visualised under a UV light.

5.3.2.2.   *DNA sequencing*

After successful amplification of the correctly sized fragment the PCR products of seven affected and four possibly affected members from the family were sequenced to determine if the A10S mutation was present. A representative chromatogram of the sequencing results are shown in **Figure 3.41 a and b**.
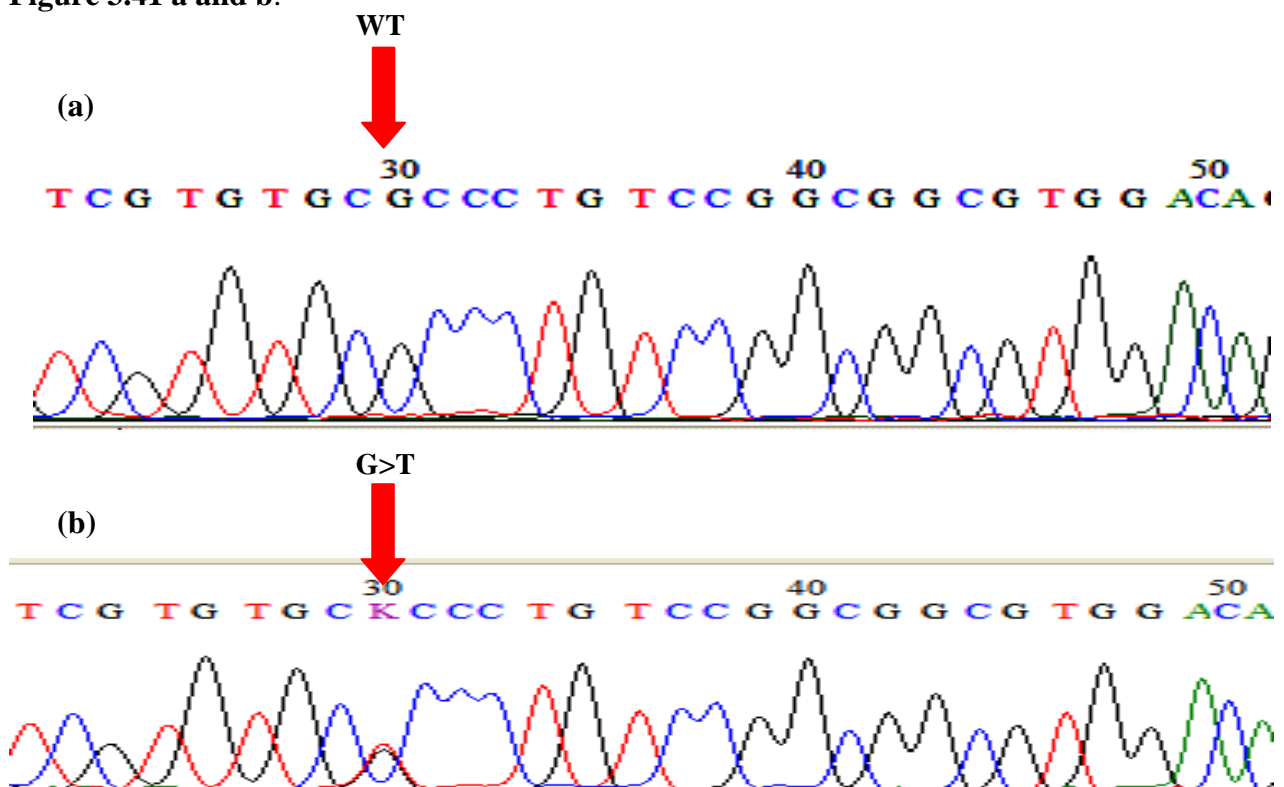
Figure 3.41: Chromatogram of the A10S (G28T) sequencing results. The position of the mutation is indicated with the red arrow. (a) Wild type and (b) Heterozygous for the A10S (G28T) mutation.

The A10S (G28T) mutation was only found in the heterozygous form in six of the 11 members screened for this mutation (**Table 3.5**). Of the six heterozygous individuals, three are affected and three are possibly affected members. The remaining five individuals were found to all be wild type for the A10S (G28T) mutation.

5.3.3. Analysis of 35delG mutation in the *GJB2*

The 35delG mutation in the *GJB2* (Chapter 3 Section 2) gene has been hypothesized to be a genetic modifier involved in the variable phenotypic expression in individuals harbouring the mutations associated with aminoglycoside-induced hearing loss.

5.3.3.1 Polymerase chain reaction (PCR) amplification and DNA sequencing

A region of 117bp, within the only coding exon (exon 2) of *GJB2* was PCR amplified and sequenced to determine if this mutation was present in this family. Sequencing did not detect the 35delG in any of the affected and possibly affected family members.

Therefore the absence of variants identified within the tRNA$^{(Ser)(UCN)}$ gene indicates that this gene does not act as a genetic modifier for the phenotypic expression of hearing loss in this South African family. Furthermore, the A10S variant (in homozygous form) in *TRMU* and the 35delG mutation in *GJB2* were not present and are therefore not considered to be contributing factors in the differential phenotypic expression seen in this family.

We only selected *MT-TS1, TRMU* and *GJB2* as candidate nuclear modifier genes since previous work has shown that specific variants in these genes are modifiers in A1555G positive families.

Table 3.5: Genotypes for the A10S (G28T) mutation in *TRMU* for members of the South African family with streptomycin-induced deafness.

Patient No.	*TRMU* (A10S)
II-1 (74.78)	-/-
III-10 (74.60)	-/+
III-19 (74.86)	-/-
III-20 (74.81)	-/+
III-22 (74.80)	-/+
III-28 (70.26)	-/-
III-30 (71.36)	-/-
III-4 (possibly affected) (77.83)	-/-
III-14 (possibly affected) (74.84)	-/+
III-18 (possibly affected) (74.77)	-/+
V-12 (possibly affected) (79.63)	-/+

Members heterozygous for the A10S mutation are shaded in yellow.

Pedigree numbers for each individual are indicated in *italics* (**Figure 3.7**).

+ = Mutant allele

- = Wild type allele

The family members labelled as 'possibly affected' apparently had hearing impairment but had not been clinically assessed by an audiologist.

6. Functional analysis of the A1555G mutation

For the present study the effect of streptomycin on the cell survival for both mutation positive (MT) and mutation negative (WT) cell lines for the A1555G mutation was tested using the MTT colorimetric assay. MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) is a water soluble tetrazolium salt and is used in assays to quantitatively measure cell survival or proliferation by means of a colour reaction. Dissolved MTT is converted to insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes and therefore measures the activity of these mitochondrial dehydrogenase enzymes (Slater et al., 1963). The insoluble formazan is solubilised by adding certain solvents to the reaction and the dissolved material can then be spectrophotometrically measured yielding absorbance as a function of the concentration of the converted dye. The tetrazolium ring is cleaved only in active mitochondria therefore the colour reaction can only take place in living cells with active dehydrogenase enzymes (Mosmann 1983). In cells that are dead, either due to necrosis or apoptosis, no colour reaction will take place due to the fact that there are no active mitochondria or metabolically active enzymes to reduce the MTT to purple formazan. This assay has also been used previously to determine the cytotoxicity of certain toxic medicinal agents (Carmichael et al. 1987; Mosmann 1983; Skehan et al. 1990; van de Loosdrecht et al. 1994).

For determining the effect of streptomycin on cell survival and proliferation, lymphoblast cells harbouring the wild type and mutant were cultured and treated with streptomycin at different concentrations ranging from 200µg/ml to 0.0002µg/ml. The preliminary results obtained showed that for streptomycin concentrations ranging from 2 to 0.0002 µg/ml there appeared to be a reduction in cell viability for the mutant cell line compared to wild type cells **(Figure 3.42)**. This difference was not however statistically significant (p-values ranging from: 0.0615-0.999) possibly due to the large error bars observed. This could be due to technical difficulties with the experiment since it was difficult to achieve complete solubilisation of the formazan. Our preliminary data supports previous studies that showed that A1555G affects mitochondrial function. Interestingly, at higher concentrations of streptomycin (200-20 µg/ml) no difference between the two cell lines was observed possibly due to the toxic effect of the drug on the cells irrespective of whether the A1555G mutation was present or not.

Absorbance (540nm) versus Streptomycin concentration.
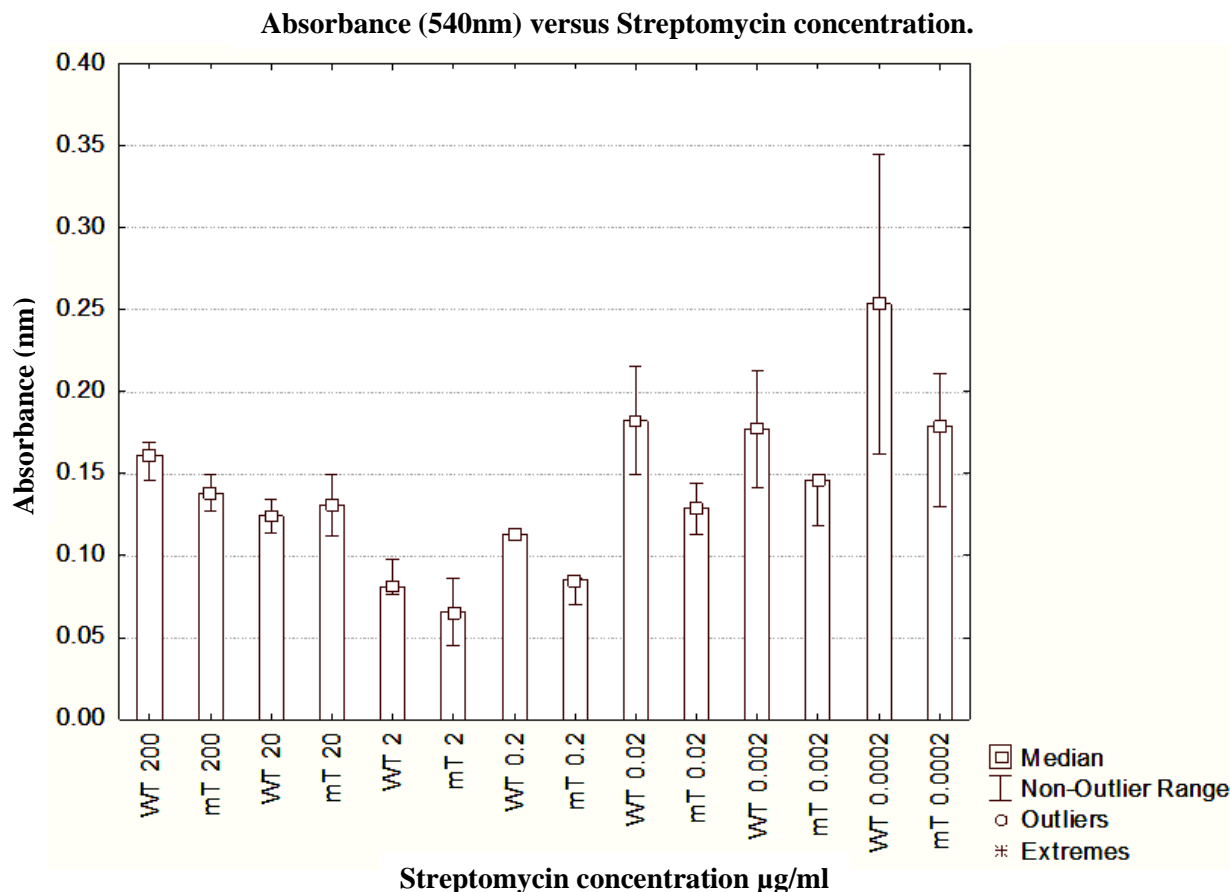
Figure 3.42: Assessment of mitochondrial function in wild type and mutation-positive cells treated with different Streptomycin concentrations. Each cell line was done in triplicate for each of the different drug concentrations.

7. Detection of heteroplasmic mutations

In the present study we aimed to develop a sensitive and robust method for the detection of the levels of heteroplasmy for mitochondrial mutations. This was performed using cloning and PCR-RFLP analysis, and analysing the results on either i) the ABI 3130xl Genetic analyzer or ii) on agarose gels with the UN-SCAN-IT software. In order to generate templates of varying mutant to wild type DNA ratios, PCR fragments of wild type and mutant samples were cloned into *E.coli*, the DNA was isolated and combined into the appropriate mutant:wild type ratios of 100%, 50%, 25%, 12.5%, 6.25% and 0%.

7.1. Cloning

Upon successful cloning of the fragment (Chapter 2 Section 6.3) of interest into a pGEM®-T Easy vector, the vector was transformed into live *E.coli* cells and these were plated onto LB/agar plates. Following incubation overnight the plates were inspected for colony growth. If colony growth was observed a number of white colonies **(Figure 3.43)** (believed to contain the PCR fragment of

interest as discussed in Chapter 2 section 8) were picked from the plates and subjected to PCR amplification to confirm the presence of the correct fragment size in the vector.

Figure 3.43: Transformed *E.coli* colonies. The multiple cloning region of the vector is located within the coding region of the enzyme ß-galactosidase, successful cloning of an insert into the pGEM®-T Easy vector disrupts the normal coding sequence of the ß-galactosidase gene resulting in clones containing the PCR insert producing white colonies (indicated by the blue circles) with clones containing no insert producing blue colonies when grown on agar plates containing XGAL.

The white colonies chosen from the LB/agar plates were examined using colony PCR to determine if the fragment containing the mutation of interest was successfully ligated into the pGEM®-T Easy vector. After PCR amplification the products were electrophoresed on a 2% agarose gel to confirm the successful amplification of the fragment. A representative example of an agarose gel with the amplified products from the wild type and mutant-positive colonies for both the A1555G and 961delT + insC$_{(n)}$ mutations is shown in **Figure 3.44 a and b**. The correct size of the amplified product was determined by loading a 100bp or a 200bp molecular marker in the first lane of the gel. A negative control sample (containing no DNA) was included in the PCR reaction to exclude any possible PCR contamination.

(a) A1555G
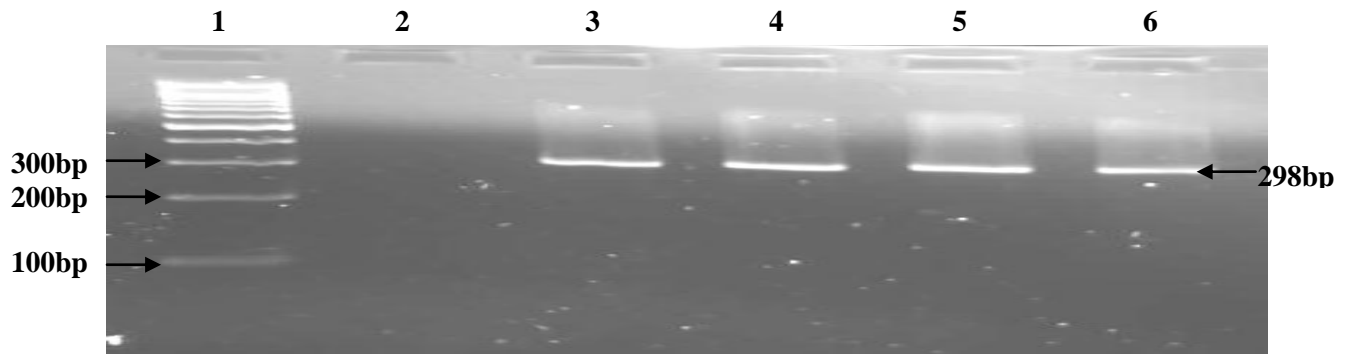
(a) 961delT + insC$_{(n)}$
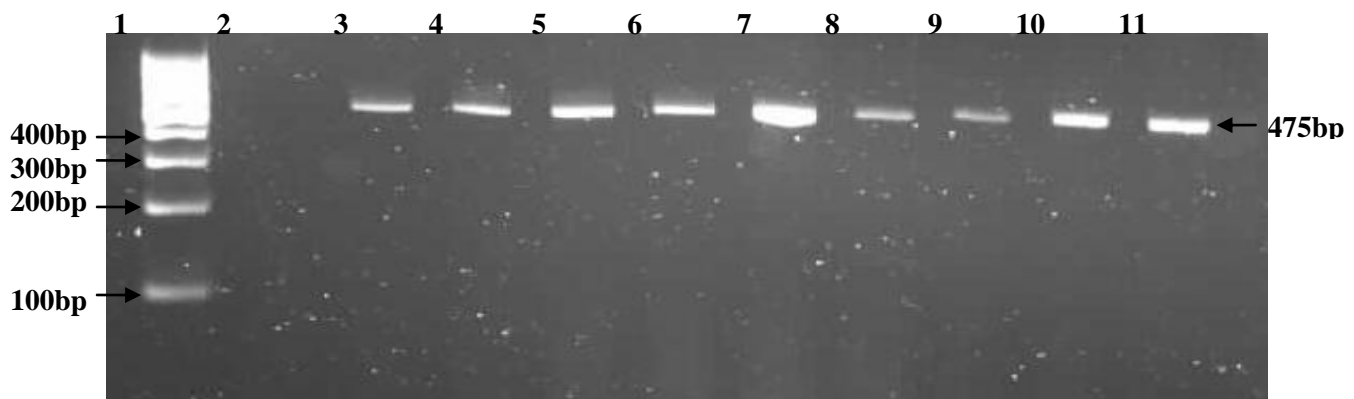
Figure 3.44: Colony PCR for A1555G and 961delT + insC$_{(n)}$. A total of nine wild type and nine mutant-positive colonies were picked from the LB/agar plates and PCR amplified to confirm that the correct size fragment was inserted into the vector and transformed into the *E.coli* cell for each mutation. (a) Lane 1: Molecular size marker; Lane 2: Non-template control; Lanes 3-4: PCR products with wild type insert; Lane 5; PCR products with mutant insert; Lane 6: PCR fragment of 298bp.

(b) Lane 1: Molecular size marker; Lane 2: Non-template control; Lanes 3-6: PCR products with wild type insert; Lanes 7-10: PCR products with mutant insert; Lane 11: PCR fragment of 475bp.

7.2. Generation of Wild type:mutant ratios and RFLP analysis

Colonies with the correct fragment size were inoculated overnight in LB media with subsequent isolation of the plasmid DNA. The isolated DNA was diluted into different ratios containing different percentages of wild type and mutant DNA in order to simulate different heteroplasmy percentages found in the human cell (100% mutant, 50% mutant, 25% mutant, 12.5% mutant, 6.25% mutant and 100% wild type). Each ratio was PCR amplified in triplicate and digested with an appropriate restriction enzyme (*Alw26I* for A1555G and *MnlI* and 961delT + insC$_{(n)}$) for each corresponding mutation.

7.3. Analysis of results

7.3.1. Analysis on the ABI Genetic Analyzer 3130xl

The digested products for the different ratios were electrophoresed on the ABI 3130xl Genetic Analyzer to test whether the different levels of heteroplasmy could be detected. For electrophoresis on the ABI 3130xl Genetic analyzer one of the PCR primers had to be labelled with a fluorescent dye (FAM) in order to visualise the different fragment sizes on the analyzer. **Figure 3.45 a and b** are representative examples of the results generated for each of the two mutations by the ABI 3130xl Genetic analyzer using the GeneMapper software version 3.7 (Applied Biosystems Inc, Foster City, CA, USA).

The G allele at position 1555 results in a loss of the cutting site for the enzyme *Alw26I*. Following *Alw26I* digestion, the expected fragment sizes for the A1555G mutation was 117bp and 181bp for the wild type allele (A allele) and 298bp for the mutant allele (G allele). In heteroplasmic subjects, the proportion of mutant copies was estimated from the peak areas of the fragments, in quantification experiments from three independent PCR amplifications.

The C allele at position 961 results in a loss of the cutting site for the enzyme *MnlI*. Following *MnlI* digestion, the expected fragment sizes for the 961delT + insC$_{(n)}$ mutation was 138bp and 234bp for the wild type allele (T allele) and 372bp for the mutant allele (C allele). In heteroplasmic subjects, the proportion of mutant copies was estimated from the peak areas of the fragments, in quantification experiments from three independent PCR amplifications.

(a) A1555G (75% mutant DNA)
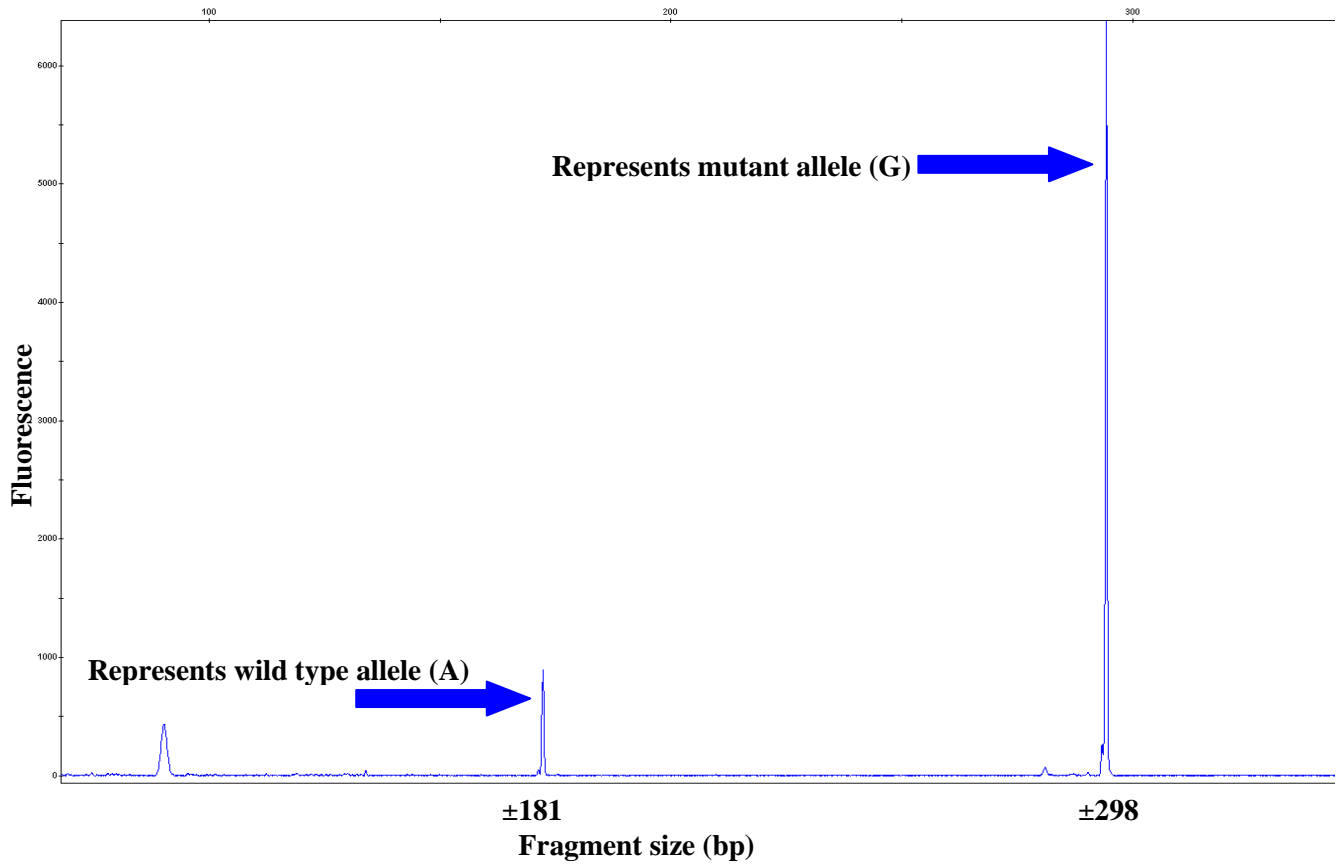
(a) 961delT + insC(n) (75% mutant DNA)

Fluorescence

±138   ±372
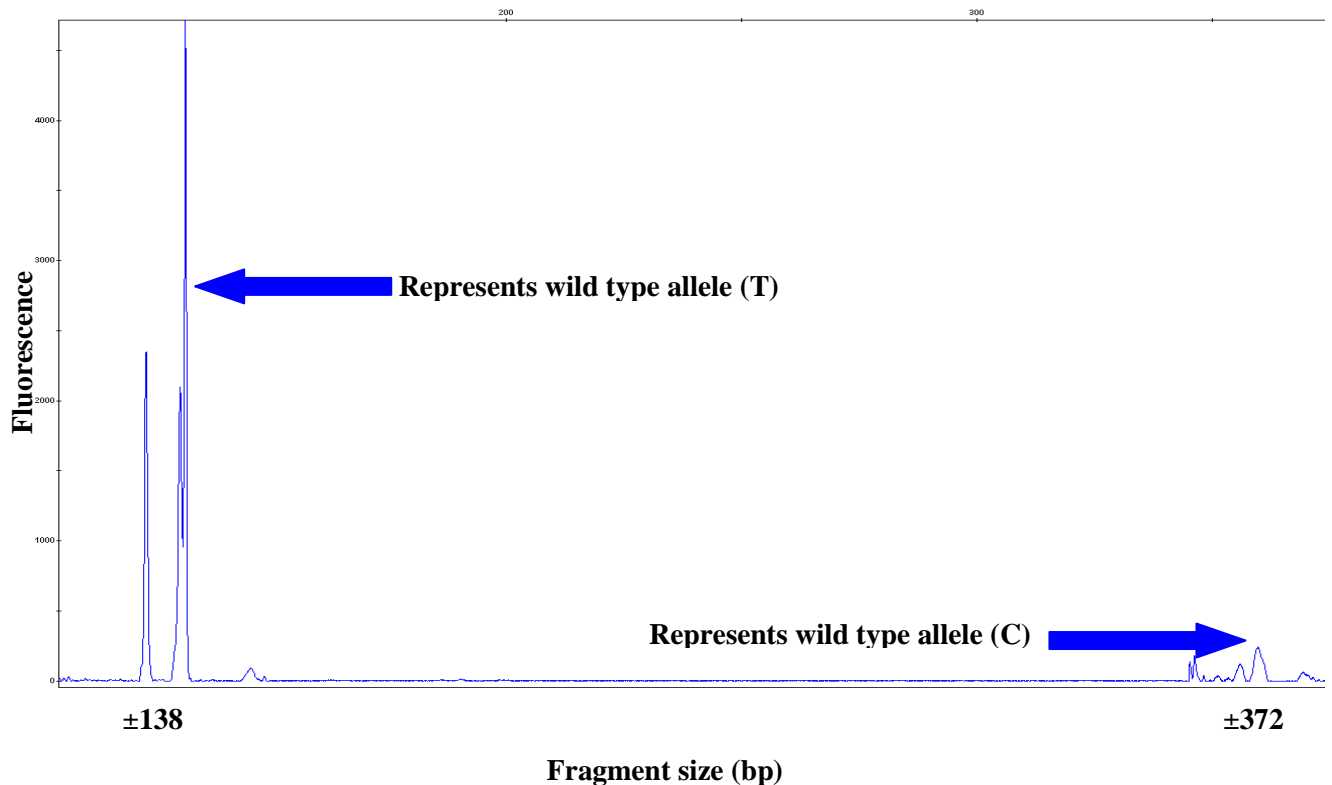
Fragment size (bp)

Figure 3.45: Heteroplasmy digest of 75% mutant for A1555G and 961delT + insC(n).
(a) Electropherogram for the 75% mutant to wild type DNA ratio for the A1555G mutation. The blue arrows indicate the fragments of 181bp and 298bp in size. (b) Electropherogram for the 75% mutant to wild type DNA ratio for the 961delT + insC(n) mutation. The blue arrows represent the fragments of 138bp and 372bp in size.

For the ABI results we expected to see different size peaks corresponding to the sizes of the digested fragments as well as differences in the intensities of the peaks as the ratio of wild type to mutant DNA changes. The area under each peak would then be calculated in order to determine the percentage heteroplasmy for each sample. However, upon calculation of the percentage heteroplasmy for each of the different ratios, inconsistent results were obtained. The many 'split peaks' that were seen on the ABI results are similar to the spurious bands that are often observed when digested PCR products are electrophoresed on polyacrylamide gels. This is not surprising since electrophoresis on the ABI 3130xl Genetic Analyzer is much more sensitive at detecting these spurious bands which complicates the calculations of our heteroplasmic ratios. After numerous repeated efforts and continued inconsistent results for both A1555G and 961delT + insC(n) it was decided to abandon this method for determining the levels of heteroplasmy.

As a result of the failure to detect the percentage of heteroplasmy on the ABI 3130xl an alternative method was chosen. It was decided to examine only the A1555G mutation digested products on an agarose gel and then determine the different intensities of the bands using the software programme UN-SCAN-IT version 5.1. This program compares the total pixel count of different bands on a gel with other bands in the same lane or in different lanes.

After the PCR products for A1555G ratios were digested with the restriction enzyme *Alw26I*, they were electrophoresed on a 2% agarose gel (**Figure 3.46**). The correct size of the amplified product was examined by loading a 100bp molecular marker in the first lane of the gel. For the different ratios of wild type to mutant, the 298bp fragment shows a reduction in the intensity of the band as the percentage of mutant DNA decreases, with the two representing wild type bands (117bp and 181bp) increasing in intensity as the ratio of the wild type allele in the different ratios increases, as one would expect.
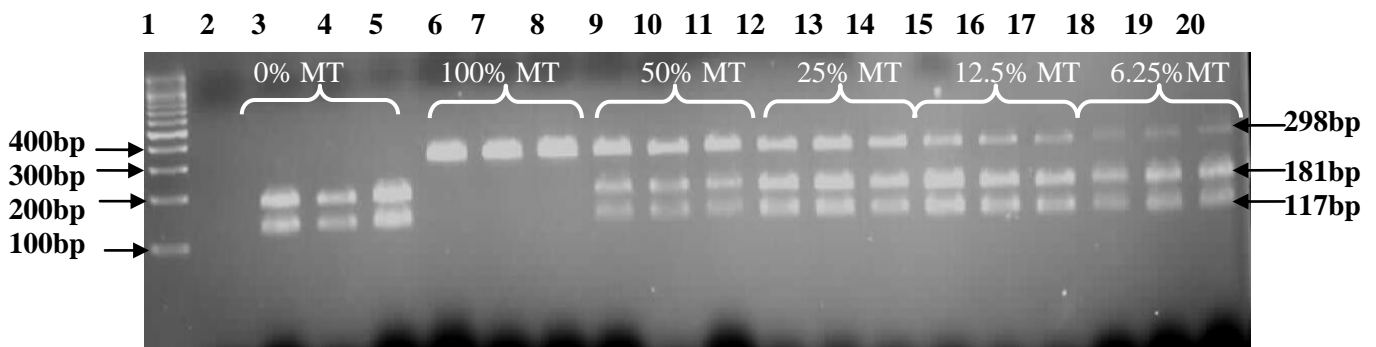
Figure 3.46: Restriction enzyme digest of A1555G in varying wild type to mutant ratios. Lane 1: Molecular size marker; Lane 2: non-template control; Lane 3-20: Six different ratios digested in triplicate with the restriction enzyme *Alw26I*.

The software program UN-SCAN-IT version 5.1 was used to calculate the intensity of a selected band or bands within a lane. The intensities of the bands are expressed as the total pixel percentage. The different total pixel percentage for each band in a lane is then exported into a excel worksheet where it may be compared to other band intensities from the same lane. **Figure 3.47** is a representation of the graph that is generated by the UN-SCAN-IT program for the different bands on the gel in lane 12 (25% mutant DNA).

Table 3.6 represents the calculated values of the total pixel percentage for each band of the different heteroplasmy ratios. The percentage calculated for each sample was roughly similar to the

expected heteroplasmy ratios. This indicates that the software programme UN-SCAN-IT version 5.1 can be used to detect different percentages of heteroplasmy in patient samples.
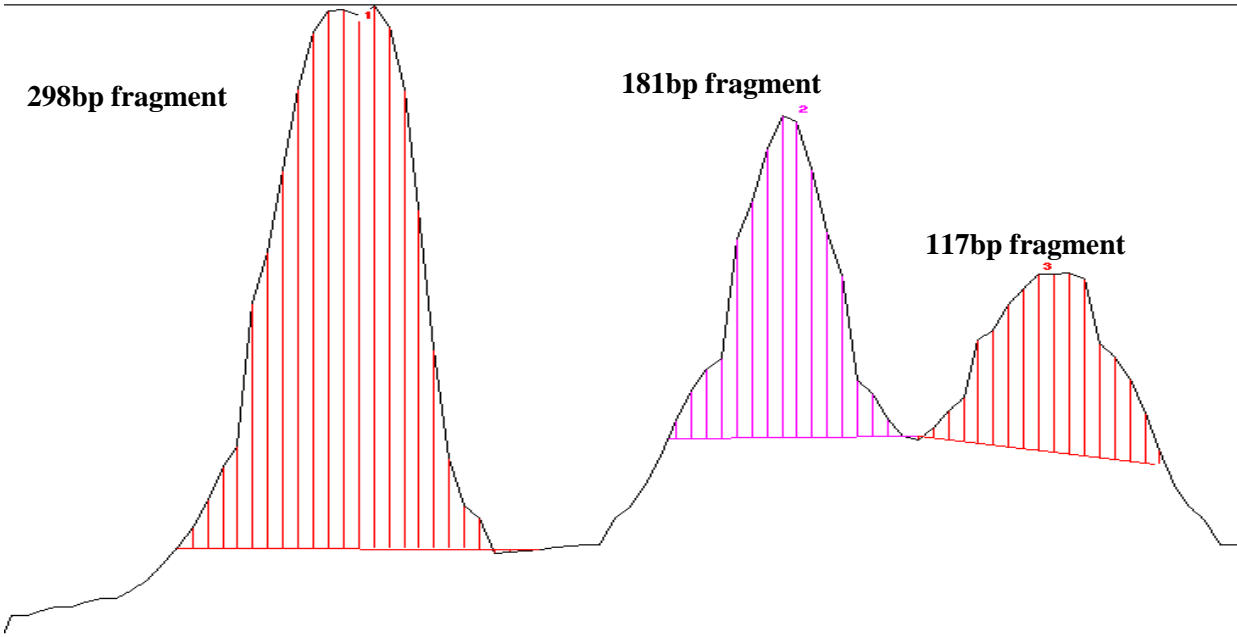
Figure 3.47: **UN-SCAN-IT graphical output for band intensities on an agarose gel.** Each peak is representative of a selected band on the gel following digestion of PCR products with *Alw26I*. The total pixel percentage for each band is calculated and used to determine the percentage heteroplasmy for the sample.

Table 3.6: Total pixel percentage calculated for each heteroplasmy ratio

Expected Heteroplasmy Ratio	Total Pixel Percentage of 298bp fragment	Mean ± SD
100% MT	100	
100% MT	100	100
100% MT	100	
50% MT	57.6	
50% MT	54.72	55.7 ± 1.6
50% MT	54.91	
25% MT	36.47	
25% MT	37.18	36.7 ± 0.4
25% MT	36.44	
12.5% MT	25.02	
12.5% MT	19.71	20.2 ± 4.6
12.5% MT	15.95	
6.25% MT	11.11	
6.25% MT	11.56	10.6 ± 1.3
6.25% MT	9.09	

The value of the total pixel percentage for the ratios only represents the intensity of the 298bp fragment (representing the mutant allele) because the intensity of this band should decrease as the amount of mutant DNA present in the sample decreases.

MT = mutant allele

These results show that UN-SCAN-IT software can successfully detect heteroplasmic mutations but that it is more accurate at higher ratios of the mutation (>25%) and less accurate at lower ratios.

7.3.3. Detection of levels of heteroplasmy with SNaPshot analysis

During the mutation screening using SNaPShot analysis of the control samples for the six mutations associated with aminoglycoside-induced hearing loss, an Afrikaner control sample was found to harbour the A1555G mutation in heteroplasmy. This indicates that SNaPshot can therefore be used to detect possible heteroplasmic samples (**Figure 3.48**) but cannot be used to determine the percentage of wild type to mutant DNA in the sample.

Locus: 1555 1555
Allele: G allele A allele
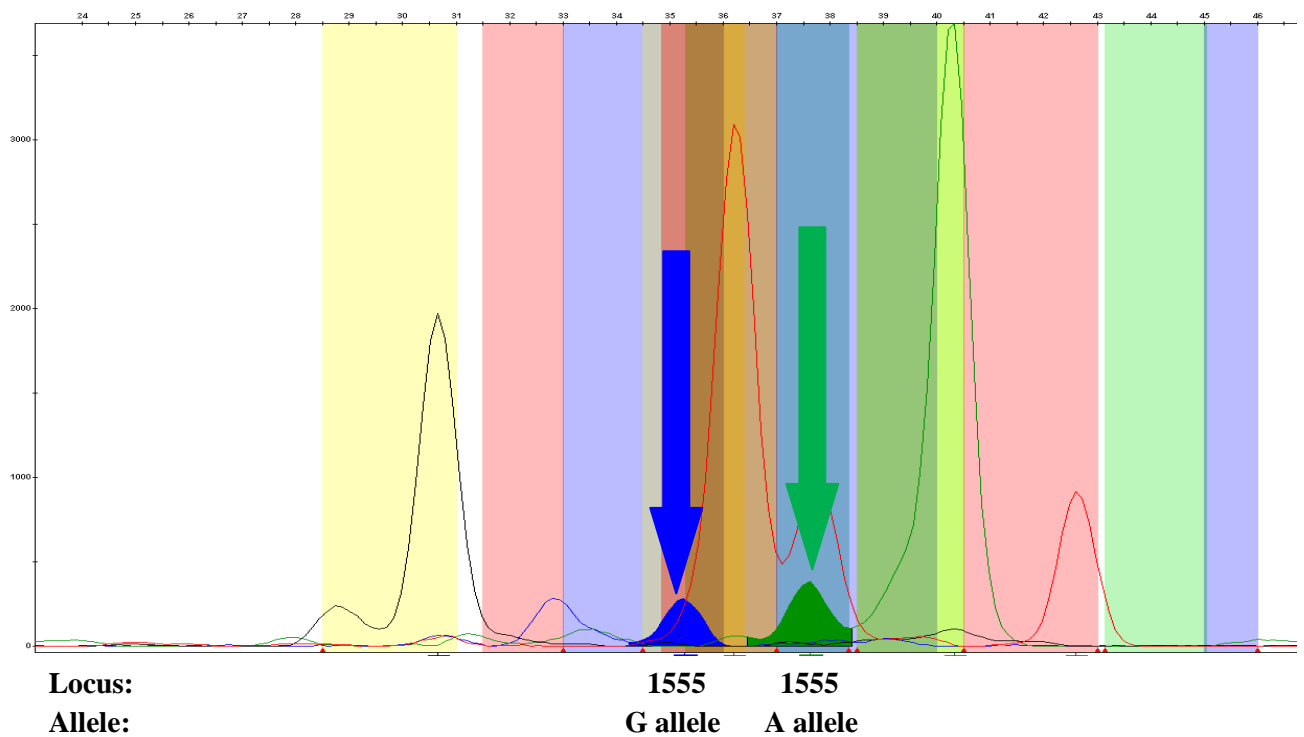
Figure 3.48: Detection of heteroplasmy with SNaPshot analysis. The blue and green arrows indicate the positions of the two alleles (G allele; A allele) found at position 1555 in *MT-RNR1*. Both alleles (A and G) are clearly present in the same sample.

The sample with possible heteroplasmy for the A1555G mutation was sequenced (**Figure 3.49**) for confirmation of the presence of the G1555 mutation as well as the presence of heteroplasmy. Furthermore, the PCR product was digested with restriction enzyme *Alw26I* (as described in Section 7.3) and analyzed with UN-SCAN-IT **(Figure 3.50)**. As expected, three bands were present for the heteroplasmic sample and using this method the ratio of mutant DNA was calculated as 35%.
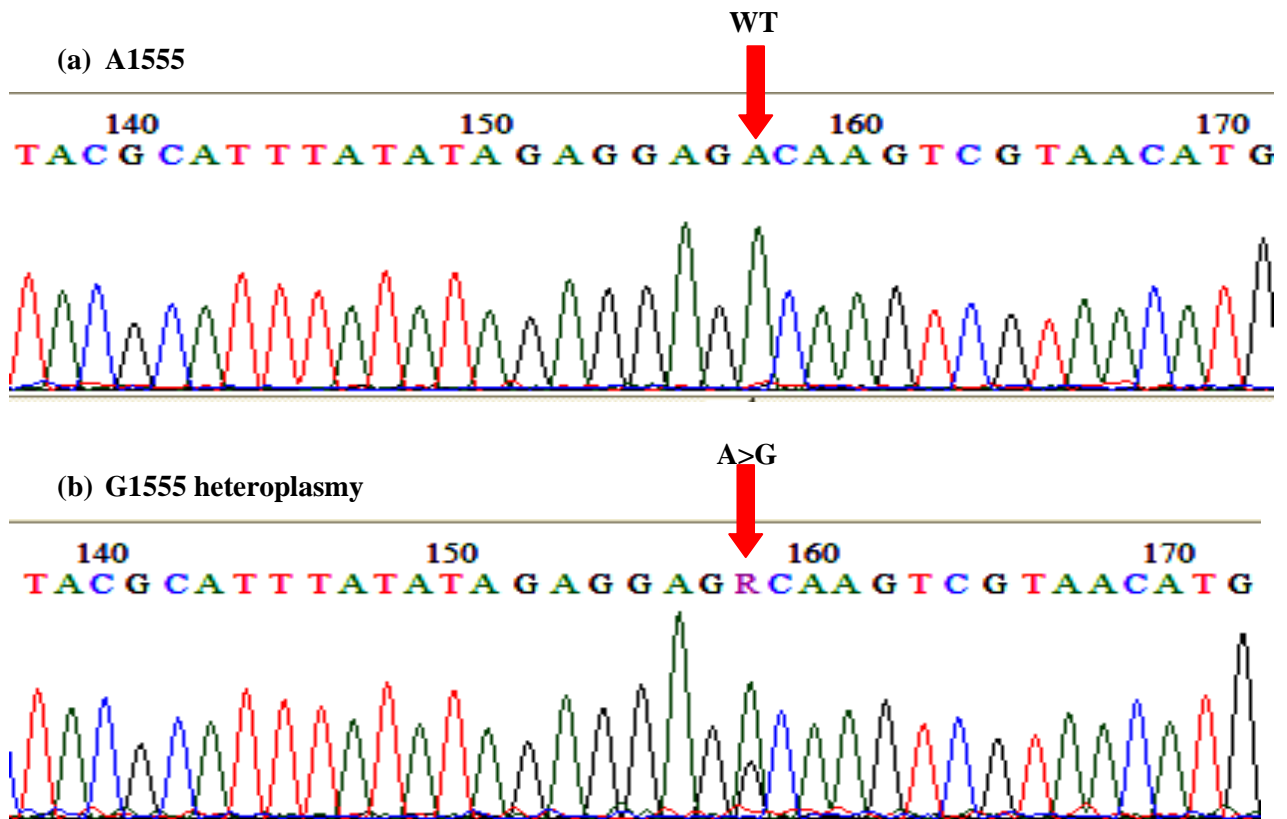
Figure 3.49: Chromatogram of sample with heteroplasmy at position 1555. (a) Wild type sample, (b) Heteroplasmic sample. The red arrow indicates the position of the A1555G mutation. Both the A and the G allele at this position are clearly visible.
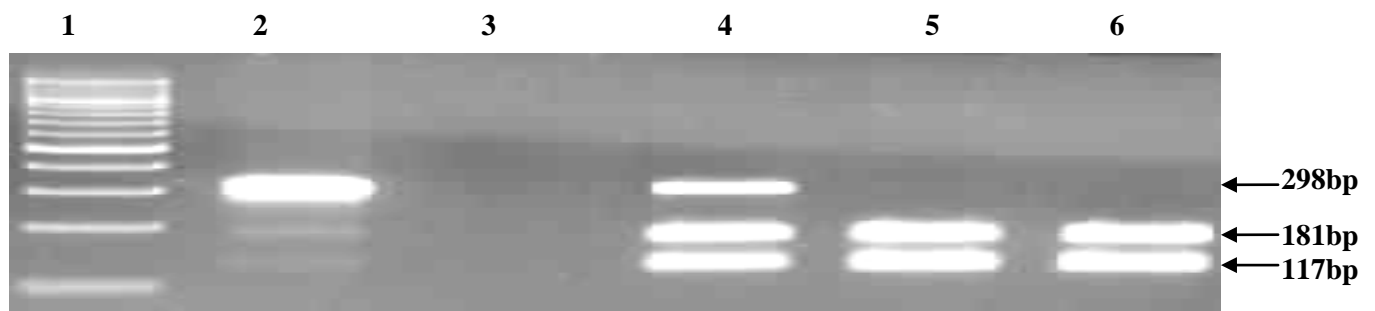
Figure 3.50: Restriction enzyme digest of A1555G heteroplasmic sample. Lane 1: 100bp Molecular size marker, Lane 2: 298bp PCR fragment, Lane 3: non template control, Lane 4: Afrikaner control sample displaying heteroplasmy with 3 bands sized 298bp (MT), 181bp and 117bp (WT), Lanes 5-6: wild type samples (A1555).

Chapter 4

DISCUSSION & CONCLUSIONS

All the objectives outlined in this study have been achieved. A robust and cost effective method has been designed to detect six of the known *MT-RNR1* mutations. Using this method we determined the frequency of these mutations in 115 MDR-TB patients as well as 439 control samples representative of four populations groups in the Western Cape region of South Africa. In one of the Afrikaner control samples, the A1555G mutation was detected in a heteroplasmic state and calculated to contain approximately 25% of mutant:wild type DNA using UN-SCAN-IT software.

Furthermore, we detected novel sequence variants (G719A, T1040C) in *MT-RNR1* in MDR-TB patients who were experiencing aminoglycoside-induced ototoxicity. A total of 50 novel variants of which five are evolutionarily-conserved, were also identified in a sub-group of MDR-TB patients who were experiencing ototoxicity but do not harbour any of the known mutations associated with aminoglycoside-induced hearing loss. One variant (A12142G) was identified in the *MT-TH* gene with the remaining four variants (G9305A, C10128A, T15312C and A15692G) all identified in protein coding genes (*ND3, COX3, CYTB*). This is the first time variants within protein coding genes have been identified that might possibly be associated with aminoglycoside-induced hearing loss.

In a South African family of Mixed Ancestry descent we determined that 76 of the 97 family members tested were A1555G mutation-positive and that the mutation was on the African haplogroup L0d. All mutation-positive family members were informed of their at-risk status and were provided with genetic reports that were placed in their medical records at their respective health care clinics.

Functional analysis of the A1555G mutation using the MTT colorimetric assay revealed possible differences between mitochondrial function of wild type cells compared to mutation-positive cells treated with different concentrations of streptomycin. However, these differences were not statistically significant and therefore further work is needed in this regard.

Currently, there are no known statistics on the frequency of the six mutations associated with aminoglycoside-induced hearing loss in South Africa (Chapter 1 Section 4.8). This country is currently facing an increasing tuberculosis (TB) epidemic, with the number of infections with the more resistant forms of TB (MDR-TB) increasing. Thus, due to this rise in MDR-TB infections and the concomitant increase in aminoglycoside use, more of the South African population will be at risk of developing aminoglycoside-induced hearing loss. We are therefore in a very unique position to study the short and long term effects of aminoglycosides on the auditory system. Knowledge about the frequency of these six mutations in our populations is imperative in understanding and determining whether certain ethnic or sub-population groups are at increased risk of developing hearing loss due to aminoglycoside exposure. Of the six mutations identified and associated with aminoglycoside-induced and non-syndromic hearing loss, only two mutations (A1555G and C1494T) have been proven through comprehensive functional studies to cause mitochondrial dysfunction (Guan, Fischel-Ghodsian, and Attardi 2000; Hobbie et al. 2008a; Zhao et al. 2005). The remaining mutations (T1095C, A827G, 961delT + insC$_{(n)}$, T961G, 961insC and T1291C) are thought to be pathogenic based on co-segregation of these mutations with a deafness phenotype in unrelated families, absence of these mutations in control populations, the evolutionary conservation of the nucleotide or the predicted effects of these variants on the secondary structure of the 12S rRNA ribosome.

The exact mechanism by which aminoglycosides induce hearing loss in individuals carrying one of the mutations is still unknown. However, what is known is that aminoglycosides exert their antibacterial effects (in bacteria) by binding to the Aminoacyl-tRNA binding site (A site) in the 16S rRNA subunit, which is the first attachment site in the ribosome for the Aminoacyl-tRNA, thereby inhibiting bacterial protein synthesis or inducing mistranslation of the mRNA (Cundliffe 1990; Moazed and Noller 1986). Human mitochondrial ribosomes have striking structural similarities to bacterial ribosomes **(Figure 1.13, pg 38)** and it is proposed that the 12S rRNA of the human mitochondrial ribosomes are the main target sites for the aminoglycosides (Xing et al. 2006b). The presence of the A1555G mutation alters the secondary structure in a highly conserved region of the 12S rRNA which is known from bacterial studies to be the decoding region (Cundliffe 1990; Noller 1991). When this site is disrupted it reduces the proof reading ability and ribosomal stability of the 12S rRNA (Hui, Eaton, and de Boer 1988; Noller 1991). It has been shown that both the A1555G and C1494T mutations increase the binding of the aminoglycosides to the human 12S rRNA leading to disruption of mitochondrial protein synthesis in the cell. All the proteins synthesized in the mitochondria are involved in oxidative phosphorylation (Chapter 1, Section 2.2.1), therefore

disruption of mitochondrial protein synthesis will reduce ATP production and this ultimately leads to an increase in reactive oxygen species (ROS) formation in the mitochondria **(Figure 4.1)**. The buildup of ROS in the cell activates the apoptotic pathway (Chapter 1, Section 2.1.2) ultimately leading to cell death (Guan, Fischel-Ghodsian, and Attardi 1996; Inoue et al. 1996).
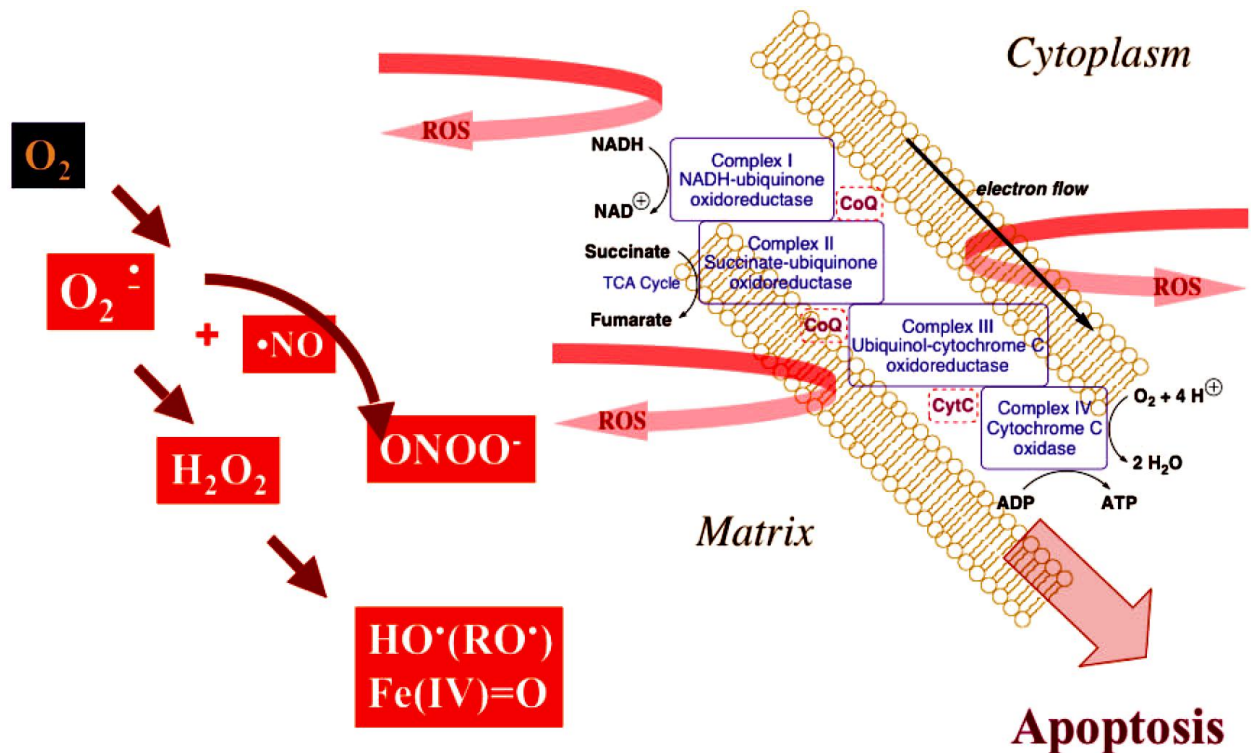
Figure 4.1: **Schematic model of ROS production in the mitochondria during oxidative phosphorylation.** The formation of the superoxide radical anion (O_2) initiates a cascade of events that triggers apoptosis of the cell (Taken from: Hoye et al., 2008).

The fact that mitochondrial mutations so frequently lead to hearing loss suggests that the cells of the inner ear are particularly dependent on mitochondrial function. What remains unclear is why some homoplasmic mutations only affect the ear despite being present in all other tissues of the human body. Histological studies show that advanced cochlear degeneration include the organ of Corti, stria vascularis and supporting cells (Lindsay and Hinojosa 1976).

The cells that are most likely to be affected by a mitochondrial defect due to their high energy requirement are the cells of the stria vascularis and the hair cells (both outer and inner) in the cochlea. The main pathogenic factor thought to be involved in the degeneration of these cells might be a deficiency in mitochondrial oxidative phosphorylation (caused by the increased binding of aminoglycosides to the A-site of the mitochondrial 12S rRNA subunit). Other factors may also

include ROS production as well as altered apoptotic signalling (McKenzie, Liolitsa, and Hanna 2004). There are different hypotheses to explain the manner in which mitochondrial mutations induce aminoglycoside and non-syndromic hearing loss. One hypothesis involves the energy dependence of the organ of Corti and the stria vascularis on mitochondrial oxidative phosphorylation. The presence of the mutant DNA (A1555G) reduces the oxidative phosphorylation capacity of the cell (as discussed in the previous paragraph). The decrease in oxidative phosphorylation reduces the ATP production in the cochlea influencing the ion transport in the hair cells, leading to a reduction in the efficiency of the transduction of acoustic signals to the brain. The stria vascularis is the most metabolically active site in the cochlea with its primary function to maintain the ionic environment of the hair cells (Steel and Kros 2001). Ion homeostasis in the inner ear requires the secretion of ions, particularly K^+, into the endolymph against the ionic gradient and in the process making use of an ATP-dependant pump. As a result, the most likely effect of a decrease in the ATP production caused by mitochondrial dysfunction (due to the A1555G and other mutations) is a slowing of the ATP-dependant pumps. The decreased functioning of these pumps disrupts the ionic balance in the inner ear, reducing the inner ear capacity to detect and transmit sound waves entering the ear (Steel and Kros 2001).

Complete and thorough functional studies on the exact mechanisms on how the mutations in *MT-RNR1* and the binding of the aminoglycosides affect the normal functioning of the hair cells are needed to test the various hypotheses.

Development of a rapid method to detect the six *MT-RNR1* mutations

For determining the frequency of the six mutations associated with aminoglycoside-induced hearing loss we aimed to develop a method that would be able to screen for all six mutations within a single reaction with minimal cost and labour. SNaPshot analysis is a particularly well-suited technique to use when screening mitochondrial DNA variants, since mitochondrial DNA are introns-less enabling the amplification of the entire gene in a single PCR fragment. In addition, homoplasmic mutations are represented as a 'single peak' at each locus simplifying the analysis of genotypes for each mutation. SNaPshot is also less labour intensive compared to other techniques that have been used to screen for these mutations. Other more conventional methods can only detect one mutation at a time such as PCR-RFLP with the enzyme *Alw26I* used to detect the A1555G mutation. The RFLP method is, however, prone to false positives as in the case of one study where a sample thought to be mutation positive by enzymatic digest was revealed to be positive for the T1556C

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variant and not the A1555G variant (Tanimoto et al. 2004). Other methods include allele-specific oligo-nucleotide hybridisation (ASO) or direct sequencing, however, the limitations of these methods are that they are costly or can only detect one mutation at a time. An estimated cost comparison of the SNaPshot method to PCR-RFLP, which is the method of choice in most cases, is R300 per sample per mutation compared to SNaPshot at approximately R170 per sample for all six mutations (Bardien et al. 2009).

We were also successful in genotyping children younger than 12 years of age by means of DNA extraction from buccal cells (Chapter 2, Section 4). Our SNaPshot method proved to be sufficiently sensitive to detect the six mutations in DNA from buccal swabs which is typically of lower concentrations and quality, enabling us to genotype children from as young as one month of age.

The application of this rapid screening test in clinical practice would aid in identifying susceptible individuals prior to the start of their aminoglycoside treatment therapy thereby potentially lowering incidence of aminoglycoside-induced hearing loss in South Africa. There are certain precautionary measures that can be applied to minimise the progression of cochlear and vestibular damage in mutation-positive individuals which include avoidance of drugs with synergistic ototoxic effects, reducing therapy time and regular audiological monitoring throughout treatment.

Frequency of the mutations in MDR-TB patients

This is the first report of the frequency of aminoglycoside-induced hearing loss mutations in South African populations. The A1555G, C1494T, T1095C and T1291C mutations were not detected in any of the MDR-TB patients tested.

The A827G mutation was detected in only one of the 115 (0.9%) patients who was, in fact, not South African but of Somalian descent. The frequency of the $961delT + insC_{(n)}$ mutation was detected in relatively high numbers (4/115) in this patient group (3.5%). The true pathogenicity of this variant have been under question for some time as position 961 in *MT-RNR1* is not evolutionary conserved (Bardien et al. 2009) and since its first discovery in 1999 (Casano et al. 1999) this variant have been detected in a number of control populations (Bae et al. 2008; Bardien et al. 2009; Kobayashi et al. 2005). It was initially thought to be pathogenic but no functional studies have been conducted on this variant to date. However, if proven to be pathogenic the presence of $961delT + insC_{(n)}$ in such high frequencies in the MDR-TB patient group is alarming since these individuals are exposed to very high concentrations of aminoglycosides.

The absence of the other mutations in the MDR-TB individuals is surprising since approximately 40% of these individuals were experiencing ototoxicity as a result of their aminoglycoside treatment. It is however possible that these individuals might harbour novel mutations in *MT-RNR1*.

In order to exclude the common 35delG mutation in *GJB2* as a possible cause of hearing loss in the MDR-TB patient group, mutation screening was performed. The 35delG mutation is the most common mutation in *GJB2* and accounts for up to 75-80% of all autosomal recessive deafness cases related to *GJB2* (Gasparini et al. 2000; Morell et al. 1998). Direct sequencing of all the MDR-TB patients did not detect the 35delG mutation in this group of samples.

Frequency of the mutations in control samples

The frequency of the six mutations associated with aminoglycoside-induced hearing loss was also determined within the general population of the Western Cape. Four different ethnic groups were screened for these mutations using SNaPshot analysis (Chapter 2, Section 3 and 8).

The A1555G mutation was detected in 0.9% of the Black control samples (1 in 112, in a homoplasmic state) and in 1% of the Afrikaner controls samples (1 in 93, in a 35% heteroplasmic state). This is the first time that the A1555G mutation has been detected in heteroplasmy in an African sample. The A1555G mutation has only been detected in heteroplasmy in the Spanish population in which it was shown that there is a correlation between the mutation load and the severity of the hearing loss in mutation positive individuals (del Castillo et al. 2003). The A1555G mutation, being the most common of the six mutations, has been found in relatively low frequencies in a range of different control populations worldwide. It was found in 2% (5/203) of Brazilian, 0.48% (1/206) of New Zealand and 0.09% (1/1,161) of American populations (Abreu-Silva et al. 2006b; Scrimshaw et al. 1999; Tang et al. 2002). The relatively high frequency of this well established mutation in the South African black population is alarming and holds important implications for this group since the incidence of TB and MDR-TB is very high in this particular ethnic group. However, further studies on larger numbers of individuals are needed to determine the true frequency of the A1555G mutation in South African populations.

The A827G mutation was detected at a frequency of 1% (1/93) in the Afrikaner control population and in none of the other control groups. This mutation has only been detected in 3 unrelated families with hearing loss in two other population groups, Chinese and Argentinean but was not

detected in any control individuals (Chaig et al. 2008; Xing et al. 2006b; Xing et al. 2006a). No functional studies have been conducted on this mutation but it has been generally accepted to be a pathogenic mitochondrial mutation that causes a genetic predisposition to non-syndromic hearing loss in the above mentioned populations. This is due to the fact that position 827 in *MT-RNR1* is in a highly conserved region within the gene and it is possible that the A827G mutation may cause certain alterations within the structure of the rRNA leading to mitochondrial dysfunction and ultimately hearing loss in a mutation positive individual (Xing et al. 2006a). The identification of the A827G mutation in unrelated families and the absence in control populations further point to its role in the pathogenicity of aminoglycoside-induced hearing loss.

The 961delT + insC$_{(n)}$ mutation was found in relatively high frequencies in a number of the control groups. It was detected at a frequency of 1.1% (1/93) in the Afrikaner controls, 1.5% (2/130) of the Mixed Ancestry controls and in 7.1% (8/112) of the Black controls. The true pathogenicity of this variant has been under question due to its identification in other control populations at frequencies of 2% in the Japanese and 7% in Korean control populations (Bae et al. 2008; Kobayashi et al. 2005). Our data supports the fact that 961delT + insC$_{(n)}$ is probably a common polymorphism.
The T961G mutation was detected only in our Caucasian and Afrikaner control groups at a frequency of 2.9% (3/104) and 3.2% (3/93), respectively. The frequency of the T961G mutation found in our control populations is comparable to the frequencies found in other control populations. This mutation have been previously detected in the Caucasian individuals with non-syndromic hearing loss at a frequency of 3% (5/164) (Li et al. 2004a). The T961G variant has also been identified in the German patients with non-syndromic hearing loss at a frequency of 3% (2/66). However this mutation was also detected at relatively high frequencies of 1.5% (5/320) in German control samples (Elstner et al. 2008).

The 961insC variant was not detected in our South African populations however this variant has been detected in an Italian family with aminoglycoside-induced hearing loss but was not found in any control samples (Casano et al. 1999). Similar to the other variants found at position 961 the true pathogenicity of this variant remains unclear.

Even though the changes found at position 961 are rare and have only been identified within a small number of populations, the identification of these variants in control populations and the lack of evolutionary conservation strongly imply that these variants are rather common polymorphisms.

The C1494T, T1095C and T1291C mutations were not detected in any of the control populations. Further studies on larger sample numbers are needed to determine the true frequency of these mutations in South African populations.

Detection of heteroplasmic mutations

Heteroplasmy is a rare condition in normal individuals, and is often only associated with mitochondrial DNA disease. Heteroplasmy occurs within and between cells and also between tissue and organs (Naviaux 2000). The rate of heteroplasmy formation is a product of random segregation between wild type and mutant DNA occurring during early embryogenesis (Finsterer 2004). Most of the pathogenic mitochondrial mutations are usually heteroplasmic, yet few mitochondrial diseases are caused by homoplasmic mutations. As is the case with certain mutations associated with Leber's hereditary optic neuropathy (LHON) and the mutations associated with non-syndromic and aminoglycoside-induced hearing loss (Abreu-Silva et al. 2006b; Estivill et al. 1998b; Fischel-Ghodsian et al. 1997; Li et al. 2004b; Prezant et al. 1993). In mutations that exist in the heteroplasmic state there is a certain threshold level at which the deleterious consequences of the mutation is no longer compensated for by the effect of the wild type (Rossignol et al. 2003).

The A1555G mutation is mostly found in the homoplasmic form in most population groups, but has also been detected in heteroplasmy in seven unrelated Spanish families (Ballana et al. 2008; del Castillo et al. 2003). The existence of the A1555G mutation in heteroplasmy in the study by del Castillo, *et al*., (2003) indicated that individuals who carried less that 20% of the mutant copies had milder hearing loss compared to individuals that carried between 52% and 96% mutant copies. However, the hearing loss of the individuals with the A1555G mutation in the homoplasmic form (100% mutant) was more severe than in the individuals with the mutation in heteroplasmy. These results showed that there is a correlation between the mutation load and the severity of the disease phenotype (del Castillo et al. 2003).

In the present study we attempted to establish a method to detect heteroplasmic mitochondrial mutations. Different ratios of wild type to A1555G mutant DNA was digested with the restriction enzyme *Alw26I* and analysed using either the ABI 3130xl Genetic Analyzer or agarose gel electrophoresis. Analysis of the graphs from the ABI 3130xl Genetic Analyzer repeatedly gave conflicting results when the area under the peak was calculated and compared with the expected percentage of mutant DNA.

However, analysis of the digested products on a agarose gel using the software programme UN-SCAN-IT version 5.1 revealed more promising results. This program compares the intensities of different bands on a gel with other bands in the same lane or in different lanes. The intensities of the bands are expressed as the total pixel percentage. The different total pixel percentages for each band in a lane are then compared to other band intensities from within the same lane or across lanes. The percentage calculated for each sample was roughly similar to the expected heteroplasmy ratios. This indicates that the programme UN-SCAN-IT version 5.1 was sufficiently sensitive to detect different percentages of heteroplasmy in different samples.

One of the Afrikaner control samples was found to harbour the A1555G mutation in heteroplasmic form using the SNaPshot technique **(Figure 3.48, pg 149)**. However, SNaPshot is not able to determine the percentage of heteroplasmy (Chapter 3, Section 3.5). The presence of heteroplasmy in this sample was confirmed by direct sequencing restriction enzyme digest and gel analysis with the UN-SCAN-IT software. The total pixel percentage that was calculated was compared to the calculated values of the different ratios and a result of 35% heteroplasmy was determined for this sample.

Novel variants in the *MT-RNR1* gene

Only two (A827G, 961delT + insC$_{(n)}$) of the six mutations associated with aminoglycoside-induced hearing loss were detected in the MDR-TB patient group (Section 4.2). Out of the group of 115 patients approximately 40% of these patients experienced some form of ototoxicity, be it either mild or more severe. A total of 34 individuals who were experiencing ototoxicity were selected for screening for novel variants in the *MT-RNR1* gene. These patients were selected as they had clear signs of ototoxicity based on audiological results but do not harbour any of the known mutations. The entire *MT-RNR1* gene was screened for novel variants by dividing the gene up into six overlapping fragments of between 180 and 280bp. HRM analysis was performed for all 34 patients for each of the six fragments. Only two novel variants (not reported in the MITOMAP DNA database) (MITOMAP: A Human Genome Database http://www.mitomap.org) were detected at positions 719 (G/A) and 1040 (T/C). The frequency of the G719A was therefore determined in Mixed Ancestry controls using the PCR-RFLP technique and the restriction enzyme *EcoRI*. This variant was found in 20.7% (24/116) of the controls, which indicates that it is probably a common polymorphism in the Mixed Ancestry population. Sequence alignments across five species (Human, Chimp, Mouse, Bovine and Frog) also indicated that position 719 lies in a region of *MT-*

RNR1 that is not very well conserved indicating it does not play a important role in the normal functioning of the 12S rRNA.

The frequency of the T1040C variant was determined in the Black control population using HRM analysis. The T1040C variant was found in 1.8% (2/112) of the controls indicating that this variant might only be common polymorphism within the black control population of South Africa. Furthermore, the position of the T1040C variant within *MT-RNR1* is not well conserved also indicating that it does not play a role in the normal functioning of the 12S rRNA.

It remains unclear, although unlikely, whether these two variants are associated with aminoglycoside-induced hearing loss since it has not been found in any other population groups. It might be possible that these variants are unique to our South African populations.

Seven other variants were also detected **(Table 3.3, pg 118)**, however these variants have all been previously reported in other population groups predominantly Chinese (MITOMAP: A Human Genome Database http://www.mitomap.org). During the screening for novel variants in *MT-RNR1* positive controls for five of the six mutations associated with aminoglycoside-induced hearing loss were included in the screening. HRM analysis as a mutation detection method was sufficiently sensitive to detect four out of the five mutations (A1555G, C1494T, T1095C, 961delT + insC$_{(n)}$). However, detection of the A827G mutation was not successful and this mutation could not be distinguished from the wild type samples. The failure in detecting this mutation might be explained by the positions of the PCR primers relative to the mutation. The positive control for the A827G mutation was included in the HRM analysis for fragments A and B. In fragment A, the A827G mutation lies within the reverse primer sequence and for fragment B the mutation lies only two base pairs away from the fragment B forward primer sequence. We postulate that the close proximity of this variant to the end of the PCR fragment may not have a major effect on the melt curve of this fragment. The successful detection of the other known variants shows that HRM as a mutation detection method is sensitive enough to detect homoplasmic mitochondrial mutations.

HRM has certain limitations despite being a relatively cost-effective and rapid method. The success of the analysis depends on the quality of the starting DNA as well as a good quality PCR product with no primer dimers present. The type of intercalating dye used in the analysis has also been cited as a limitation for HRM. The type of analysis to be conducted will determine what dye to use. For example, LCGreen® Plus detects heterozygotes better than Syto® 9, which is better than dyes such as EvaGreen® and SYBRGreen I (Farrar, Reed, and Wittwer 2009). Other limitations of HRM include difficulty to detect smaller insertions and deletions, as was observed during our screening

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for the 35delG mutation in *GJB2*. Furthermore, the method may not detect sequence variants situated close to the ends of the PCR fragment as was observed for A827G in our screening. Future screening for this mutation using HRM as mutation detection method might be resolved by moving the primers further away from the position of the mutation.

Whole mitochondrial genome screening

Whole mitochondrial sequencing was performed on a group of eight MDR-TB patients who were experiencing ototoxicity. Only eight samples were chosen due to the relatively high costs involved for this type of analysis. A total of 50 novel variants (not previously reported in MITOMAP DNA database) **(Table 3.4, pg126)** were identified in the eight samples. Some of the variants were present in more than one sample. The presence of so many novel variants was not unexpected since the mutation rate in the mitochondrial genome is up to 10 fold higher than in the nuclear genome as a result of the virtual absence of any mitochondrial DNA repair system (Bachtrog 2003; Wallace et al. 1987). Furthermore, the mitochondrial genome is very susceptible to DNA mutational events due to environmental factors as well as oxidative stress. Each of the 50 novel variants identified were further evaluated for both nucleotide and amino acid conservation (for protein coding genes) across five species; Human (*Homo sapiens*), Chimpanzee (*Pan troglodytes*), Mouse (*Mus musculus*), Bovine (*Bos taurus*) and Frog (*Xenopus laevis*).

One variant (at position 527) was identified within the D-loop region of the mitochondrial genome. Two variants were identified in two tRNA genes (*MT-TY* and *MT-TH*) (at positions 5892 and 12142), respectively. One variant was identified in *MT-RNR1* (at position 719) and two novel variants identified in the *MT-RNR2* gene (at positions 1694 and 2755). Only the A12142G variant in *MT-TH* was evolutionarily conserved at that position. However, it does not occur in a well-conserved part of the gene. Further studies are needed to determine whether any of these five variants (not counting the D-loop region) possibly play a role in susceptibility to deafness in South African populations. The G719A variant which we also detected during our novel variant screening of *MT-RNR1* was present in 20.7% of the Mixed Ancestry controls and is therefore unlikely to be pathogenic.

The remaining variants were all identified within the following protein coding genes: NADH dehydrogenase 1 (*ND1*, 4 variants), NADH dehydrogenase 2 (*ND2*, 4 variants), Cytochrome c

oxidase I (*COX1*, 8 variants), Cytochrome c oxidase II (*COX2*, 1 variant), ATP synthase 8 *(ATP8*, 2 variants), ATP synthase 6 (*ATP6*, 3 variants), Cytochrome c oxidase III (*COX3*, 6 variants), NADH dehydrogenase 3 *(ND3*, 2 variants), NADH dehydrogenase subunit 4L (*ND4L*, 1 variant), NADH dehydrogenase 4 (*ND4*, 3 variants), NADH dehydrogenase 5 (*ND5*, 4 variants), NADH dehydrogenase 6 (*ND6*, 2 variants), Cytochrome b (*CYTB*, 4 variants), **(Table 3.4, pg 126)**. Each of these novel variants identified in protein coding genes have potential important implications for the normal functioning of the mitochondrial oxidative phosphorylation system since all of the above mentioned genes code for some component of this system. From the 50 variants only four (G9305A, C10128A, T15312C and A15692G) identified in protein coding genes, caused an amino acid change at that position and showed complete amino acid conservation across all five species.

The position of the G9305A variant (M33I) variant in the *COX3* gene is evolutionary conserved across all five species only on the protein level. This variant was also found in only one of the MDR-TB patient samples. The *COX3* gene encodes the Cytochrome c oxidase subunit III which is one of three mitochondrial DNA encoded subunits of the respiratory Complex IV. Complex IV is located in the inner mitochondrial membrane and is the last enzyme of the electron transport chain. Complex IV collects electrons from reduced cytochrome c and transfers them to oxygen to form water. The energy that is released from this transfer is used to pump protons across the mitochondrial inner membrane. Complex IV is composed of 13 polypeptides of which only subunits one to three are encoded by mitochondrial genes (Capaldi 1990). Variants identified within the *COX3* gene have been associated with disorders such as Leber optic atrophy and Lactic acidosis (Johns and Neufeld 1993; Seneca et al. 1996). The change at amino acid position 33 from Methionine to Isoleucine involves two very hydrophobic amino acids however it is possible that the more aliphatic side chain of Isoleucine may affect the folding of the protein. This might have an impact on the normal functioning of the *COX3* protein in Complex IV of the electron transport chain.

The C10128A variant (L24M) in the *ND3* gene showed evolutionary conservation across all of the species for both the nucleotide and protein sequences. This variant was detected in only one sample. The *ND3* gene encodes the subunit 3 that is one of the approximately 41 polypeptides that forms part of the respiratory Complex I in the mitochondrial electron transport chain. Complex I accepts electrons from NADH and transfers them to the ubiquinone (Coenzyme Q10) and in the

process utilizes the energy that is released from the protons that is pumped across the mitochondrial inner membrane (OMIM: Online Mendelian Inheritance in Man 2009, www.ncbi.nlm.nih.gov). The ND3 protein has been shown to be a hydrophobic protein fragment of Complex I (Ragan 1987). A number of variants have been identified in the *ND3* gene and associated with diseases that are linked to a Complex I deficiency, resistance to Parkinsons disease and Leigh syndrome (Kirby et al. 2004; Taylor et al. 2001; van der Walt et al. 2003). The change at amino acid position 24 from a Leucine to Methionine involves two highly hydrophobic amino acids, however, the longer and more complex side chain of methionine may affect the folding of the protein which might have an impact on the normal functioning of the *ND3* protein in Complex I of the electron transport chain.

The T15312C variant (I189T) and A15692G variant (M316V) in the *CYTB* gene both occur at evolutionary conserved positions at both the nucleotide and protein level. Each of these variants was only detected in one of the eight samples. The *CYTB* gene is the only mitochondrial DNA encoded subunit of the respiratory Complex III. Complex III is located within the inner membrane of the mitochondria and is the second enzyme in the electron transport chain. Complex III catalyzes the transport of electrons from ubiquinol (reduced Coenzyme Q10) to Cytochrome c and utilizes the energy that is released to translocate the protons from inside the mitochondrial inner membrane to outside the organelle. CYTB is a highly evolutionary conserved, hydrophobic protein consisting of nine transmembrane domains and two heme groups (OMIM: Online Mendelian Inheritance in Man 2009, www.ncbi.nlm.nih.gov). Variants identified within this gene have been associated with a range of different diseases such as Leber optic atrophy, colorectal cancer and exercise intolerance to name a few (Brown et al. 1992; Dumoulin et al. 1996; Polyak et al. 1998). The amino acid change at position 189 involves the change from Isoleucine to Threonine. Isoleucine is an uncharged highly hydrophobic amino acid and is most likely found in the interior of the protein. Threonine on the other hand is an uncharged amino acid with neither hydrophobic nor hydrophilic properties (neutral). This change in amino acid properties might have a impact on the folding of the *CYTB* protein affecting the normal functioning of this enzyme in the Complex III of the electron transport chain. The second variant identified within the *CYTB* gene causes an amino acid change at position 316 from Methionine to a Valine. Both Methionine and Valine are uncharged hydrophobic amino acids. Methionine has a longer and more complex side chain compared to Valine which might affect the folding property of the *CYTB* protein.

Further functional studies are needed to determine the effect of these variants (C10128A, G9305A, T15312C and A15692G) on the functioning of the *ND3, COX 3* and *CYTB* proteins. The roles of these variants in aminoglycoside-induced hearing loss are unlikely but warrant further investigation. Future functional studies on these variants would include assessing the activity of the above mentioned complexes in cell lines harbouring either one of these mutations to determine the resultant effect on normal activity. Muscle biopsies from patients with one of the above mentioned variants can also be assessed for functioning of Complexes I to V as has been done previously (Chen et al. 2006; Jackson et al. 1994).

South African A1555G mutation-positive family

In the present study, we re-investigated a South African family with streptomycin-induced hearing loss and recruited a total of 97 members. The family members were genotyped using the SNaPShot technique and a total of 76 individuals were shown to harbour the A1555G mutation. Of these, 7 individuals already have hearing impairment (Gardner et al. 1997) and 69 members are therefore at risk of developing hearing loss if treated with aminoglycoside antibiotics. The identification of A1555G-positive members of this South African family holds important implications for these individuals. These individuals are at increased risk of developing aminoglycoside-induced deafness if they receive prolonged aminoglycoside therapy for the treatment of MDR-TB. All A1555G-positive members of this family were given genetic reports and counseled about their risk and that of their children of developing deafness as a result of aminoglycoside use. It is hoped that with this knowledge individuals can empower themselves to protect themselves from the toxic side effects of these drugs. Knowledge about a patient's genetic status regarding these mutations can aid the clinician in the planning of the possible treatment regimes for that individual. Possible ways to preserve hearing in susceptible individuals include lowering the doses of aminoglycosides used, avoiding the use of other ototoxic drugs and motivation for regular audiological monitoring. It is important to note that aminoglycosides are also used in pregnant women thereby placing the unborn child at risk of developing permanent deafness (Donald and Sellars 1981). Aminoglycosides are often given to premature babies at birth to treat bacterial infections. In the present study, the A1555G-positive status of a baby, who is part of this family and was born prematurely, was reported to the medical personnel so that alternative drugs could be used for his treatment. Future generations of this South African family would benefit from this research by being informed of their risk of developing aminoglycoside-induced hearing loss, a potentially preventable disorder.

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It was determined that the South African family belongs to the African L0d (L0d1b) mitochondrial haplogroup. The A1555G mutation has been previously reported on East Asian haplogroups (B4, D4, D4a, D5, F1, N9a and R9a) and the European haplogroups H and V (Estivill et al. 1998b; Tang et al. 2007; Young et al. 2005). This is the first report of the A1555G mutation on an African haplogroup. There has only been one other report on A1555G in an African family from the Democratic Republic of the Congo (previously known as Zaire) (Matthijs et al. 1996). However it is quite unlikely that these two families share the same haplogroup which suggests that the A1555G mutation in the two African families arose through independent events.

Mitochondrial haplogroup analysis reconstructs the evolutionary history of all the mitochondrial DNA lineages, found in all living people, to a common ancestor. This common ancestor is sometimes referred to as the "Mitochondrial Eve". This ancestor lived in Africa, about 150 000 years ago and lies at the root of all the maternal ancestries of all 6 billion people on earth. Since mitochondrial DNA is maternally inherited, every person is a direct maternal descendant of this "Mitochondrial Eve". The hyper variable region (D-loop region) in the mitochondrial genome is the most polymorphic and it is thus this region that is focused on when constructing an individual's mitochondrial haplogroup.

The L0d haplogroup is characterised by ancestral influences from the Khoi-San populations from South Africa as well as Bantu-speakers from Africa (Bandelt and Forster 1997; Salas et al. 2002). The mitochondrial DNA (mtDNA) types found in the African populations share certain common features and have been assigned to haplogroup L. Haplogroup L can be further sub-divided into L0, L1, L2, L3, L4, L5 and L6. The subgroup L0 is the most recent common ancestor of human mtDNA.

It is thought that certain mitochondrial haplogroups might act as modifiers for the phenotypic expression of non-syndromic and aminoglycoside-induced hearing loss (Fischel-Ghodsian 2005; Guan, Fischel-Ghodsian, and Attardi 2000). The reason why mitochondrial haplotypes were implicated as modifiers was due to the different clinical expressions that were observed between mutation positive individuals of families from the same population group as well as different population groups. However, to date no correlation has been found between a certain mitochondrial haplogroup and a specific mutation. To date, the only known modifiers affecting A1555G and

other mutations are environmental factors such as aminoglycoside antibiotics and particular variants identified in nuclear genes.

Screening for genetic modifiers

Varying degrees of penetrance and severity of hearing loss have been described in a number of studies indicating that the A1555G mutation by itself is not sufficient to produce the deafness phenotype (Prezant et al. 1993; Young et al. 2005; Zhao et al. 2005). It has therefore been suggested that certain modifying factors such as the presence of aminoglycosides, nuclear modifier genes and certain mitochondrial haplogroups modulate the phenotypic expression of the A1555G mutation (Guan, Fischel-Ghodsian, and Attardi 1996; Guan, Fischel-Ghodsian, and Attardi 2000; Guan, Fischel-Ghodsian, and Attardi 2001; Guan et al. 2006). Therefore, to examine the role of some of the known genetic modifiers in the pathogenic manifestation of A1555G, we screened the seven affected members and four possibly affected members from the family for the A10S mutation in *TRMU* (Guan et al. 2006; Yan et al. 2006), 35delG in *GJB2* and various variants (C7471CC, A7472C, T7480G, G7497A, G7506A, T7510C, T7511C, T7512C) in the mitochondrial tRNA[Ser(UCN)] gene (Cardaioli et al. 2007; Chen et al. 2008; del Castillo et al. 2002; Hutchin et al. 2001; Li et al. 2004a; Yuan et al. 2005). Six individuals from the family were found to be heterozygous for the A10S mutation in *TRMU* and none were homozygous for this mutation. This mutation has been reported at frequencies of 10% in heterozygous form in the Jewish and white populations lacking the A1555G mutation indicating that the A10S mutation in the homozygous form is not sufficient to cause the deafness phenotype (Guan et al. 2006). The 35delG mutation was not found in any of the affected members. However, since we only screened for the one mutation in *GJB2* it might be possible that any of the other mutations in *GJB2* might be present in this family. None of the variants previously described in the tRNA[Ser(UCN)] gene were detected in any of the family members screened. This is unexpected due to the high mutation rate of mitochondrial DNA (Wallace et al. 1987) and the fact that mitochondria have a DNA repair mechanism that is not as well developed as that of the nuclear genome (Mason et al. 2003). In summary, none of the previously reported genetic modifiers were present in this South African family and further work is therefore needed to identify potential genetic modifiers in this family.

Functional analysis of the A1555G mutation

The A1555G mutation was the first mutation that was associated with non-syndromic and aminoglycoside-induced hearing loss. This mutation was first identified in a large Arab-Israeli family with maternally-inherited hearing loss (Prezant et al. 1993). Since then, this mutation has been identified in numerous families from diverse populations worldwide (del Castillo et al. 2003; Fischel-Ghodsian et al. 1997; Li et al. 2004b; Prezant et al. 1993). The frequency of A1555G in the general population is still unknown, however, this mutation still remains one of the most common causes of sensorineural non-syndromic hearing loss particularly in those individuals with hypersensitivity to aminoglycosides (Hutchin 1999). The frequency of A1555G is high among the hearing impaired with frequencies reported as high as 33% in some populations (Fischel-Ghodsian et al. 1993; Fischel-Ghodsian et al. 1997; Hutchin et al. 1993). In a district of Shanghai it was found that 22% of the hearing impaired could trace the cause of their hearing loss back to aminoglycoside exposure (Hu et al. 1991).

Biochemical studies have shown that cells harbouring the A1555G mutation had a decrease in both mitochondrial protein synthesis and mitochondrial oxygen consumption of up to 50% (Guan, Fischel-Ghodsian, and Attardi 1996) clearly demonstrating the deleterious effects of this mutation. It was further demonstrated in a study by Inoue et al that cells harbouring the A1555G mutation showed decreased mitochondrial protein synthesis and growth rates in the presence of streptomycin which had no affect on cells lacking this mutation (Inoue et al. 1996). It has been shown that the A1555G mutation by itself can be pathogenic even in the absence of aminoglycosides (Estivill et al. 1998b; Fischel-Ghodsian et al. 1997; Guan, Fischel-Ghodsian, and Attardi 1996; Hutchin 1999; Inoue et al. 1996).

For the present study, the effect of streptomycin on the cell survival for both mutation positive (MT) and mutation negative (WT) cell lines for the A1555G mutation was tested using the MTT colorimetric assay. MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) is a water soluble tetrazolium salt and is used in assays to quantitatively measure cell survival or proliferation by means of a colour reaction. Dissolved MTT is converted to insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes and therefore measures the activity of these enzymes and indirectly measures mitochondrial function (Slater et al., 1963). The insoluble formazan is solubilised by adding certain solvents (eg. DMSO) to the reaction and the

dissolved material can then be spectrophotometrically measured yielding absorbance as a function of the concentration of the converted purple colour reaction. The tetrazolium ring is cleaved only in active mitochondria therefore the colour reaction can only takes place in living cells with active dehydrogenase enzymes (Mosmann 1983). However, in cells that are dead, either due to necrosis or apoptosis, no colour reaction will take place because there are no active mitochondria or metabolically active enzymes to reduce the MTT to purple formazan. This assay has also been used to determine the cytotoxicity of certain toxic medicinal agents (Carmichael et al. 1987; Mosmann 1983; Skehan et al. 1990; van de Loosdrecht et al. 1994).

For determining the effect of streptomycin on cell survival and proliferation, cells harbouring the wild type and mutant were cultured and treated with streptomycin at different concentrations ranging from 200µg/ml to 0.0002µg/ml. The results revealed a trend for lower cell viability in A1555G cells compared to wild type, however this difference was not statistically significant (p-values ranging between 0.0615-0.999. No difference was observed between cell lines at the higher drug concentrations. This might be explained by the fact that the higher concentrations of streptomycin were deleterious to both cell types irrespective of whether the A1555G mutation was present or not.

Certain limitations of this assay were evident during the course of the experiments. Firstly, the assay was designed for adherent cells thus as a result certain centrifugations steps needed to be implemented to compensate for the fact that we were using non-adherent lymphoblasts. Secondly, the purple crystals that form during the colour reaction were difficult to dissolve and repeated resuspension of the cells with DMSO caused foaming in the wells that might have influenced the absorbance reading of the cells. In addition, the concentrations of streptomycin used in the assay could be adjusted to exclude the higher concentrations of 200µg/ml and 20µg/ml which appeared to be toxic to the cells irrespective of the presence of the A1555G mutation.

Additional functional studies on the six mutations associated with aminoglycoside-induced hearing loss by means of the colorimetric assay would be important, however, other reagents such as WST-1 could be used instead of MTT. WST-1 is a reagent similar to MTT that is also used to assess the metabolic activity of viable cells by means of a colour reaction. WST-1, however, has a number of

advantages over MTT in that it is five times more sensitive, and is a ready-to-use reagent that can be directly added to the wells with the cultured cells. Therefore, this eliminates the need for solution preparation steps or solubilisation steps that are required when using MTT. There are a widerange of other functional assays that could also be applied to determine the effect of either the A1555G mutation or streptomycin on cell viability and cytotoxicity. The cell death detection ELISA^PLUS assay from Roche diagnostics can be used to distinguish between apoptotic and necrotic cells and is ideal to determine whether streptomycin causes apoptosis or necrosis in cells harbouring the A1555G mutation. Other assays include the Cytotoxicity detection kit^PLUS (LDH) (Roche Diagnostics, Basel, Switzerland) which can be used to quantify cell death and cell lysis by measuring lactate dehydrogenase activity (LDH), after LDH is released into the supernatant of damaged cells caused by cytotoxic agents such as streptomycin. Similarly, the Caspase 3 activity assay (Roche Diagnostics, Basel, Switzerland) can accurately detect early stages of apoptosis and could be useful in determining whether the A1555G mutation causes apoptosis in mutation-positive cells.

Conclusions

The development of a rapid and cost effective method to genotype individuals for the six mutations associated with aminoglycoside-induced hearing loss facilitates the identification of susceptible individuals prior to the start of aminoglycoside therapy. This could potentially lower the incidence of aminoglycoside induced deafness in South Africa. This test also proved to be sufficiently sensitive to detect the mutations in DNA of lower quality and concentrations from buccal swabs, therefore aiding in the genotyping of children as young as one month of age. The results from this study therefore can be directly applied to the South African population and used in clinical practice.

With this new method the frequencies of these mutations were determined within a group of MDR-TB patients and in four different control populations in the Western Cape. The frequencies identified within the respective groups gave an indication to which groups might be more susceptible to developing aminoglycoside-induced hearing loss. However, future studies are warranted to determine the true frequencies of the various aminoglycoside-induced hearing loss mutations in the general South African population and also whether certain aminoglycosides are more ototoxic than others. In developing countries like South Africa aminoglycoside antibiotics will be continually used for re-treatment TB and MDR-TB cases, despite their adverse side effects. South Africa is therefore an ideal setting to study the effect of these drugs on the auditory system.

The identification of the first novel variants within protein coding genes that could possibly be associated with aminoglycoside-induced hearing loss holds great possibilities with regards to the identification of a second gene involved in drug induced hearing loss. Thus, future studies where the possible effect of these variants on the normal functioning of these genes could be assessed are justified and would contribute greatly to this field of research.

The identification of 76 additional mutation-positive family members from the A1555G positive family holds important implications for these individuals. Their mutation-positive status puts them at increased risk of developing aminoglycoside-induced hearing loss if they receive prolonged aminoglycoside therapy for the treatment of MDR-TB or other bacterial infections. All A1555G-positive members of this family were given genetic reports and counseled about their risk and that of their children of developing deafness as a result of aminoglycoside use. It is hoped that with this knowledge, individuals can empower themselves to protect themselves from the toxic side effects of these drugs. The absence of any of the variants previously shown to act as modifiers in other

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families, indicate that our family might harbor unique genetic modifiers in different nuclear or mitochondrial genes. Thus, future work in screening the members for variants within other nuclear genes is warranted.

Determining the effect of streptomycin on the mitochondrial functioning of cells harbouring the A1555G mutation indicated a difference between mutant and wild type cell lines but this difference was not statistically significant. These results indicate that the A1555G mutation could affect mitochondrial functioning. Further studies using the same type of colorimetric assay with the reagent WST-1 might show more significant differences between the two cell lines. The effect of different aminoglycoside concentrations on the mitochondrial functioning of cells harbouring one of the other known mutations (A827G, T1095C, 961delT + insC$_{(n)}$ and T1291C) might indicate whether or not these mutations are truly pathogenic, and whether certain members of the aminoglycoside class of drugs are more ototoxic than others.

Aminoglycoside-induced hearing loss is a potentially preventable disorder. It is regrettable that the widespread and poorly controlled use of these drugs in South Africa, especially with the current MDR-TB epidemic, will lead to many individuals suffering from permanent deafness. More studies and public awareness is therefore needed to address this problem.

Appendix I

Consent form for recruitment of study participants

PARTICIPANT INFORMATION AND INFORMED CONSENT FORM

TITLE OF RESEARCH PROJECT:

An investigation of the role of pathogenic and disease predisposing genetic factors in mitochondrial DNA in different forms of hearing impairment.

ETHICS REFERENCE NUMBER: N05/09/165 (US)

INVESTIGATORS: Dr Soraya Bardien[1], Prof Greetje de Jong[1], Prof Simon Schaaf[1], Dr Tashneem Harris[2], Prof Johan Fagan[2], Ms Lucretia Petersen[2]

ADDRESSES:

[1] University of Stellenbosch, Tygerberg,

[2] University of Cape Town/ Groote Schuur Hospital, Observatory.

CONTACT NUMBER: Tel. Prof de Jong   021-938-4217
                       Tel: Sr Debbie Lombard   082 428 8693

Dear study participant/ legal guardian of the study participant

We would like to invite you/ your child to participate in a research study that involves DNA (genetic) analysis and possible long-term storage of blood or tissue specimens.

- Please take some time to read the information presented here which will explain the details of this project.
- Ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is important that you are fully satisfied that you clearly understand what this research entails and how you could be involved.
- Also, your/ your child's participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you/ your child negatively in any way whatsoever.
- You/ your child are also free to **withdraw from the study** at any point, even if you do agree to take part initially.
-

This research study has been approved by the ethics **Committees for Human Research at Stellenbosch University and at the University of Cape Town** and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki and the South African Medical Research Council's (MRC) Guidelines on Ethics for Medical and Genetic Research.

What is DNA analysis or genetic research?

Genetic material, also called DNA, is usually obtained from a small blood sample. Occasionally other tissues may be used. DNA consists of numerous genes, strung together in long strands and found in every cell in the human body. Genes are the "blueprint" that determines what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide research in this field is continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

What is this research study all about?

In most of your cells there are tiny structures called *mitochondria* that are responsible for producing energy needed for various processes in the cell. It has been shown that defects in the genes in the mitochondria can cause hearing loss. Either these defects result in hearing loss on their own or sometimes they need other factors such as certain types of antibiotics to result in hearing loss. This research project will investigate whether defects in mitochondrial genes account for a significant proportion of individuals with hearing loss in the South African population.

Why have you or your child been invited to participate?

We would like to establish whether the genes in your/ your child's mitochondria contain any defects that has resulted in hearing impairment or may predispose you/ your child to hearing loss when certain antibiotics are taken.

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What procedures will be involved?

We will need to take a small sample of blood (10-20 ml which is equivalent to 2–4 teaspoons) from one of your / your child's veins, usually in the arm. We will also need to test your / your child's hearing using a standard procedure.

Are there any risks involved in genetic research?

You/ your child may experience minor pain and/or bruising at the site where blood is taken. Some insurance companies may mistakenly assume that taking part in genetic research indicates a higher risk for disease. Thus no information about you or your family will be shared with such companies.

Are there benefits to taking part in this study & will you get told your results?

This research project is important as it may reveal some of the genetic factors involved in hearing impairment in South African populations. The results from this study may have important implications for your family members. Your/ your child's personal results will be made known to your clinician only if they indicate that you/ your child may have a genetic predisposition for developing hearing loss and therefore may need genetic counseling by a trained genetic counselor.

How long and where will you/ your child's blood be stored?

Your/ your child's blood and DNA will be stored for about 5-10 years in the Division of Molecular Biology and Human Genetics at the University of Stellenbosch, Faculty of Health Sciences.

Is there a chance that your blood will be used for other research?

Your/ your child's blood will only be used for genetic research that is directly related to studies on hearing impairment. Also if the researchers wish to use your/ your child's stored blood for **additional research in this field** they will be required to apply for permission to do so from the Human Research Ethics Committees at Stellenbosch University and the University of Cape Town.

If you do not wish your/ your child's blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

How will your confidentiality be protected?

Your/ your child's identity will be kept confidential and the research staff will use a numbered code for your DNA sample. Access to your personal information will be limited to authorised scientists and clinicians only. Any scientific publications, lectures or reports resulting from the study will not identify you by name.

Will you or the researchers benefit financially from this research?

You/ your child will not be paid to take part in this study and the researchers will not benefit financially by conducting this research.

In the unlikely event that the research leads to the development of a commercial application or patent, you or your family will not receive any profits or royalties. However profits will be reinvested to supporting the cause of further research, which may bring benefits to your family or community in the future.

Thank you for taking part in this research project!

Aminoglycoside-induced deafness study

Declaration by participant or legal guardian (in the case of a minor)

By signing below, I …………………………………..…………………………………. agree/ or give permission for my child to take part in a genetic research study entitled *"An investigation of the role of pathogenic and disease predisposing genetic factors in mitochondrial DNA in different forms of hearing impairment"*

I declare that:

☐   I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

☐   I have had a chance to ask questions and all my questions have been adequately answered.

☐   I understand that taking part in this study is voluntary and I / my child have not been pressurised to take part.

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Please tick the option you choose below

☐ I agree that my / my child's blood or tissue sample **can be stored**, but I can choose to request at any time that my stored sample be destroyed. I have the right to receive confirmation that my request has been carried out.

OR

☐ Please **destroy** my/ my child's blood sample as soon as the current research project has been completed.

Signed at (*place*) ....…........……………….. on (*date*) …………...……………..

....
**Signature/Thumbprint of participant
or legal guardian**

....
Signature of witness

Declaration by Investigator

I (*name*) ……………………………………………………….……………….. declare that:

☐ I explained the information in this document to ………………….………………...

☐ I encouraged him/her to ask questions and took adequate time to answer them.
☐ I am satisfied that he/she adequately understands all aspects of the research as discussed above.
☐ I did/did not use a translator. (*If a translator is used then the translator must sign the declaration below.*)
☐

Place		Signature of Investigator	
Date		Signature of Witness (and print name)	

Declaration by Translator

I (*name*) ………………………………………………… ……………………….declare that:

☐ I assisted the investigator (*name*) …………………………………………………. to explain the information in this document to (*name of participant*) …………………………………………………........………………………………using the language medium of Afrikaans/Xhosa.
☐ We encouraged him/her to ask questions and took adequate time to answer them.
☐ I conveyed a factually correct version of what was related to me.
☐ I am satisfied that the participant or the legal guardian fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

Place		Signature of Translator	
Date		Signature of Witness (and print name)	

Appendix II

DNA Extraction from Blood

The volume of approximately 20ml of blood collected were divided into half and added to 2×50ml sterile Falcon polypropylene tubes. The 50 ml tubes were filled up to 45ml with cold cell lysis buffer (Appendix III) and shaken. Tubes were left on ice for 10min, shaken and centrifuged at 3000rpm for 10min. After centrifugation, the supernatant was discarded and tubes were filled up to 45ml with cold lysis buffer and shaken. Tubes were left on ice for 10minutes, shaken and centrifuged at 3000rpm for 10min. 900µl of Sodium-ethylene diamine tetra-acetic acid (Na-EDTA) (Appendix II) and 100µl of 10% Sodium dodecyl sulfate (SDS) (Appendix II) was added to each pellet and vortexed. 100µl of Proteanase K (Roche Diagnostics, Basel, Switzerland) were added to each tube and incubated at 37ºC overnight. A total of 2ml distilled water (H$_2$O) and 500µl Na-acetate (sodium-acetate) (Appendix II) was added to each tube and mixed well. 2.5ml phenol-chloroform (Appendix II) was added to each tube and placed on a shaking incubator (lying half-flat) for 10min at a speed of 140rpm. The mixture was then transferred to a 10ml Corex glass tube and centrifuged at 7000rpm for 12min in a Sorval centrifuge with SS34 head at 4-10ºC. The supernatant was transferred to a clean Corex tube without disturbing the interphase using a 3ml plastic Pasteur pipet (Lasec Laboratory and Scientific Company Pty LTD, Cape Town South Africa). 2.5ml octonal-chloroform (Appendix II) were added to the supernatant, tubes were sealed and mixed slowly by inversion until milky. Tubes were again centrifuged at 7000rpm for 10min in a Sorval centrifuge with SS34 head at 4-10ºC. After centrifugation the supernatant was collected into a clean 12ml Falcon polypropylene tube. 5-7ml ice-cold 96% ethanol was then slowly added and mixed until the DNA precipitated. The DNA was transferred to a 1.5ml eppendorf tube that was filled with 70% ethanol. Tubes were centrifuged for 3 min at 14 000rpm and the supernatant discarded carefully in order not to lose the DNA pellet. The eppendorf tube was again filled with 70% ethanol and centrifuged for 3 min at 14 000rpm and the supernatant discarded. Tubes were then left open to dry the pellet at room temperature for 30min. After 30min 500µl of 1$\times$ Tris-EDTA (TE) (Appendix II) solution was added and tubes were incubated at 37ºC overnight. Tubes were then placed on a rotating wheel at 30rpm for 3 days. After 3 days the DNA concentrations were measured on the Nanodrop® ND1000-UV-Vis spectrophotometer (NanoDrop Technologies, Inc. USA) following the manufacturer's instructions and diluted to approximately 200ng/µl which was the working concentration for all later reactions.

DNA Extraction from Buccal Cells

Cotton swab was rubbed on the inside of the cheek for approximately 1 min a side. Immediately afterwards the swab was placed in a 2ml microcentrifuge tube with 400µl PBS (Phosphate Buffered Saline). The cotton swab was separated by the stick with scissors. 20µl QIAGEN Protease stock solution and 400µl buffer AL was added to the sample. The sample was immediately vortexed for 15 seconds and incubated for 10 min at 56ºC. 400µl of ethanol (96-100%) was added to the sample and mixed by vortexing. 600µl of the mixture in the 2ml tube was added to the QIAamp Spin Column and centrifuged at $6000 \times$ g (8000rpm) for 1 min. The spin column was then placed in a clean 2 ml collection tube, the supernatant discarded and remaining volume of the mixture added to a the column and centrifuged for 1 min at $6000 \times$ g (8000rpm). 500µl buffer AW1 was added to the spin column and centrifuged $6000 \times$ g (8000rpm) for 1 min. The QIAamp spin column was then placed in a new 2ml collection tube. 500µl of buffer AW2 was added to the column and centrifuged at $20,000 \times$ g (14 000rpm) for 3 min. Sample was subjected to and additional centrifugation step at $20,000 \times$ g (14 000rpm) for 1 min. The QIAamp spin column was then placed in a clean 1.5ml microcentrifuge tube and 150µl AE buffer was added to the column. The sample was incubated at room temperature for 2.5min and then for 2.5min in at 56ºC. After incubation, the sample was centrifuged for 2 min at $6000 \times$ g (8000rpm). Sample was then placed at -20ºC until further use.

DNA extraction solutions

Cell Lysis Buffer
109.5g Sucrose
10ml 100% Triton X
5ml 1M $MgCl_2$
10ml 1M Tris HCl
1L H_2O

Na-EDTA
18.75ml 4M NaCl
250ml 100mM EDTA
Made up to 1L

10% SDS
10g SDS
Dissolved in 100ml autoclaved distilled H_2O incubated at 37ºC to dissolve

Na-acetate
3M Sodium
Dissolved in H_2O
Add acetic acid till pH6

Phenol-Chloroform
The phenol-chloroform ratio was set up 1:1 depending on the volume needed for the extractions

Octonol-chloroform
480ml Chloroform
20ml Octonol
Add together and mix well

1×TE
10µM TrisHCl pH8
1µM EDTA pH8

PBS
1 tablet dissolved in 200µl of ddH_2O

Electrophoresis solutions, loading dyes, agarose and polyacrylamide gels

Sodium Borate (SB) buffer (20×stock)
38.137g $Na_2B_4O_7$ (di-sodium tetraborate decahydrate) dissolved in 1L distilled water

For 1×SB running buffer for gel electrophoresis:
    Dilute 100ml of the 20×SB buffer in 2L distilled water

Bromophenol Blue Loading Dye

0.2% (w/v) bromophenol blue
50% glycerol
10mM TRIS

Add all of above and stir gradually until properly mixed

Agarose gel solutions

Concentrations	1%	2%
Agarose (g)	1	2
1×SB stock (ml)	100	100

Agarose was melted in SB solution, in the microwave oven until completely dissolved. A total of 8μl ethidium bromide was added to the melted agarose after it was allowed to cool down for a few minutesw and poured into a gel try.

10×TBE

216g Trizma base
110g Boric Acid
18.6g Sodium-EDTA

12% Polyacrylamide gel

6ml 40% Acrylamide
1.5ml 10×TBE
120μl 10% APS (Ammonium-Perox-Disulfate)
45μl TEMED (Tetra-methyl-ethylene-diamine)
7.5ml H_2O
Let gel set for 30min

Gel staining solutions

Staining solution 0.1% AgNO$_3$ (Solution 1)

1g $AgNO_3$
Dissolved in 1L H_2O

Developing Solution (Solution 2)

15g NaOH
0.1g $NaBH_4$
4ml Formaldehyde

Add the 15g NaOH to 1L of H_2O and dissolve
Then add the remaining components

Solutions for the preparation of bacterial competent cells

CAP Buffer

60mM $CaCL_2$	2.21g
15% Glycerol	37.5ml
10mM PIPES	0.76g
4M NaOH	1ml (for PH)

Make up to 250ml with sterile H_2O
Keep at 4°C until ready to use

Bacterial Media

Luria-Bertani (LB) Media
Bacto Tryptone   10g
Yeast Extract   5g
NaCl   10g
Make up to 1 L (500ml in $2\times$ 1L Flasks)
Autoclave bottles with media

Agar for plates (500ml)
Bacto Tryptone   5g
Yeast Extract   2.5g
NaCl   5g
Bacto Agar   7.5g
Add H_2O up to 500ml
Bottle was autoclaved and left to cool down to 55°C or cool to the touch.

Bacterial Ligation reactions

Ligations using pGEM Teasy vector
After PCR amplification, the fragment containing the mutation of interest a total of 3 ligation reactions were set up: 1:1, 1:3 and 1:5
The following was added to each ligation reaction:
1µl Ligase buffer
1µ T_4 Ligase
1µl pGEM Teasy vector
1µl DNA (PCR product) (the amount of DNA added depends on the ligation reaction: 3µl for 1:3, 5µl for1:5)
Water was added to a final volume of 10µl.

Cell culture growth media

RPMI 1640 growth media
The RPMI 1640 growth media is supplemented with:
15% Fetal Calf Serum
1% Pen/Strep (Penicillin-Streptomycin)

Appendix IV

Lymphocyte separation and transformation

Peripheral blood samples were collected in Heparin coated tubes. All the lymphocyte separation and transformations was done by Mrs. Ina Le Roux. The protocol for the lymphocyte separation and transformations is as follows: RPMI 1640 growth media (Appendix Ш) and Epstein-Barr-virus medium (EBV) was heated to 37ºC as well as the Histopaque 1077 to 22 ºC before the start of the protocol (Sigma, St Louis, MO, USA). 3ml Histopaque was syringed into a 12ml sterile Greiner tube (Greiner Bio-one, Frickenhausen, Germany) and half the volume of the heparin blood (4-5ml) was slowly added on top of the histopaque. Tubes were centrifuged for 20min at 1800 r.p.m using a Sorval® GLC-4 general laboratory centrifuge (Seperations Scientific, Johannesburg, South Africa). After centrifugation, the white blood cell layer was removed with a sterile Pasteur pipet (Lasec Laboratory and Scientific Company Pty Ltd, Cape Town, RSA) and placed into a clean 12ml sterile Greiner tube. 3ml RPMI 1640 growth media was added to the white blood cells and centrifuged for 30 seconds at 1000 r.p.m. After the centrifugation, the white blood cells were washed once more with 3ml RPMI 1640 growth media and centrifuged for 30 seconds at 1000 r.p.m. The pellet was then resuspended in 0.3ml RPMI 1640 growth media and transferred to a small cell culture flask (25cm^3) (Sigma, St Louis, MO, USA) that contained 1.5ml EBV medium. The cells were then incubated in a Ferma termosteri-cycle 5% carbon dioxide (CO_2) incubator (Farma International, Miami, Florida, USA) for 1 week in an upright position at 37ºC, 5% CO_2 and 90% humidity. After 1 week, the cells were examined under a Nikon TMS light microscope (Nikon, Tokyo, Japan). If transformed cells are observed 0.5ml RPMI 1640 growth media was added and the cell culture flask was incubated again in a horizontal position and fed twice weekly with 0.5ml RPMI 1640 growth media until the total volume in the flask reached 5ml. The 5ml culture was centrifuged for 30 seconds at 1000 r.p.m afterwards the cells were allowed to grown again to a volume of 5ml in order to concentrate the cells. When the volume of 5 ml is reached the cell culture is transferred to a large cell culture flask (75cm^3) and fed with 10ml RPMI 1640 growth media twice weekly. When a total volume of 50ml is reached in the flask the cells were frozen in liquid nitrogen (N_2), 10% Dimethyl sulfoxide (DMSO) (Merck, Darmstadt Germany) and RPMI 1640 growth media in 2 cryovials (Sigma, St Louis, MO, USA).

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