Molecular study of *Mycobacterium tuberculosis* complex (MTBC) DNA from Port Elizabeth

Masters Dissertation

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DECLARATION

I hereby declare that the dissertation I have submitted to the University of Fort Hare for the degree of Master of Science in Microbiology, is original and has not been previously submitted for any degree at this institution or any other university.

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SUMMARY

Mycobacterium tuberculosis complex (MTBC) is a causative agent of tuberculosis (TB) in humans and animals. The burden of tuberculosis in South Africa is worsened by the concurrent epidemic of HIV. The dynamic of TB epidemics has been investigated and yet little data has been given about the Eastern Cape, particularly Port Elizabeth. The study aimed to investigate the prevalence of drug resistant MTBC and to determine the mutations causing resistance in Port Elizabeth. One hundred and ninety (190) DNA samples isolated from sputum specimen in humans suspected of having TB were amplified using the Seeplex® MTB Nested ACE detection assay. To differentiate *Mycobacterium tuberculosis* complex (MTBC) members for surveillance purposes a multiplex polymerase chain reaction (PCR) method was done based on genomic regions of differences such as RD1, RD1mic, RD2seal, RD4, RD9 and RD12. Target genes known to confer resistance to first and second-line drugs were amplified and the amplicons sequenced using Big Dye Terminator DNA sequencing kit v3.1 (Applied Biosystems, UK). The patient's demographic profiles were obtained from the National Health Laboratory Service (NHLS).

All hundred and ninety DNA samples tested positive for MTBC using the Seeplex® MTB Nested ACE assay. Results show a high prevalence of extensive drug resistant TB in Port Elizabeth, Eastern Cape Province. One hundred and eighty four (184) DNA isolates were used in the identification of different MTBC species. We ended up working with 184 DNA isolates because we ran out of DNA, and we could not go back to isolate DNA from the affected individuals due to the fact that some patients died, while some have been released to go to their homes. From the 184 DNA isolates 45 (24.5%) isolates were identified to be *M. tuberculosis,* 94 isolates (51.1%) to be *M. bovis* BCG and 3 isolates (1.6%) to be *M. cannetti*.

Sequencing results show the position of mutation in each DNA isolate; however in the study we got resistance to MDR to be 100% and 42% pre-XDR while 58% was XDR. These results raise an alarm for the prevalence MDR in MTBC from Port Elizabeth. This is a serious health concern which calls for a need to strategise on the identification of extensive drug resistant TB patients from multi-drug resistant TB patients and ensure monitoring of their treatment.

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LIST OF ABBREVIATIONS

AFB:	Acid Fast Bacilli
AIDS:	Acquired Immunodeficiency Syndrome
BCG:	Bacille Calmette-Guerin
bp:	Base Pair
BTB:	Bovine Tuberculosis
DNA:	Deoxy-Ribonucleic Acid
DOTS:	Directly Observed Treatment Strategy
DR:	Direct Repeat
DST:	Drug Susceptibility Testing
E.C:	Eastern Cape
EMB:	Ethambutol
ERDR:	Ethambutol Resistance Determining Region
HIV:	Human Immunodeficiency Virus
INH:	Isoniazid
IUATLD:	International Union Against Tuberculosis and Lung Disease

IS6110:	Insertion element 6110
L.J:	Lowenstein- Jensen
LTBI:	Latent Tuberculosis Infection
MDR:	Multi-Drug Resistant
MIRU:	Mycobacteria Interspersed Repetitive Units
MTBC:	*Mycobacterium Tuberculosis* Complex
NaCl:	Sodium Chloride
NaOH:	Sodium Hydroxide
NTM:	Non Tuberculous Mycobacteria
PCR:	Polymerase Chain Reaction
PGRS:	Polymorphic Guanine Cytosine Rich Sequences
PPD:	Purified Protein Derivative
PZN:	Pyrazinamide
RIF:	Rifampicin
RFLP:	Restriction Fragment Length Polymorphism
RRDR:	Rifampicin Resistance Determining Region
Spoligotyping:	Spacer Oligonucleotide Typing xi
TB:	Tuberculosis
TCH:	Thiophene-2-Carboxylic Hydrazide
TST:	Tuberculin Skin Testing
VNTR:	Variable Number of Tandem Repeat
W.H.O:	World Health Organization
XDR:	Extensively Drug Resistant
ZN:	Ziehl-Neelsen

CHAPTER 1

INTRODUCTION

1.1.Background of study

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is primarily transmitted by inhalation of aerosolised droplets containing the organism (Frieden *et al*., 2003). The tubercle bacillus is a Gram-positive bacterium that causes tuberculosis (Ducati *et al*., 2006). When turbercle bacilli gets to the body they can be coughed out without causing any damage, if swallowed they may be killed by stomach acid and if they get into the blood they can be destroyed by white blood cells (Ducati *et al*., 2006). The body, including the lungs have special ways of fighting germs such as tubercle bacilli. This happens in such a way that cells move to the spot where germs has found lodgement. In a short time they build a wall or shell, called a tubercle around them. Inside the tubercle the bacilli continues to multiply and to destroy the small amounts of lung tissue locked up within the tubercle (Frieden *et al*., 2003).

The organism may perish without causing any further harm or they may remain viable inside the tubercle for a long time. A gritty material (a calcium compound) slowly replaces the destroyed lung substance if the normal resistance and repair processes of the body are in action, and the little shell imprisons the germs (Ducati *et al*., 2006). Tuberculosis can also be transmitted through the consumption of raw and unpasteurised milk from an infected cow with tuberculosis. The bovine type has a predilection for the lymph nodes or bones other than the lungs which results to the development of extra-pulmonary TB (Todar, 2009).

1.2.Tuberculosis and Human Immunodeficiency Virus

Tuberculosis and Human Immunodeficiency Virus (HIV) infection have important and bio directional interaction that impact on the epidemiology, natural history, clinical presentation and management of each pathogen (Ahmed *et al.*, 2003; Alland *et al.*, 1994). TB and HIV form a lethal combination because they complement each other's progress (Mlambo *et al.*, 2008; Morozova *et al.*, 2003; Daley *et al.*, 1992). HIV infection increases the risk of latent TB infection reactivation. HIV also increased the risk of rapid progression to disease after infection or re-infection (Miriani *et al.*, 2001; Goletti *et al.*, 1996; Small *et al.*, 1993). TB disease rates remain at unpredicted high levels in countries with high prevalence of concomitant HIV and drug resistant epidemics (Ahmed *et al*., 2003).

In 2009 WHO (World Health Organization) gave a report which estimated that 10.4 million episodes of TB occurred during 2007. Among 9.3 million incidences of TB 1.4 million (15%) had HIV-co-infection and 79% of them originating from the African region and the 11% from South-East Asia (Ahmed *et al.*, 2003). In 2007, 456 000 deaths were estimated among HIV-infected TB patients, and this number of deaths equals a quarter of all HIV deaths for 2007. TB still remains the leading cause of death in HIV-infected adults in sub-Saharan Africa (Allix *et al.*, 2008). Treatment of HIV co-infected patients is challenged due to therapy for both diseases and there are severe side effects associated with these therapies (Dean *et al*., 2002). Also patients with TB-HIV co-infection have a high mortality risk (Ciglenecki *et al*., 2007; Haar *et al.*, 2007) and this is elevated when patients are infected with drug-resistant TB (Cox *et al*., 2008; Gandhi *et al*., 2006; Wells *et al.*, 2002).

1.3.Epidemiology of TB in the world

Epidemiologic information on TB has been recorded in detail since 1953 by the US Centres for Disease Control and Prevention (CDC) (CDC, 2011). Since the early 20th century, the incidence of TB started to decline due to various factors such as basic infection-control practices. However, in 1985, the incidence of TB was noted to resurge again especially in persons infected with HIV (Assam-Assam *et al*., 2010; Mlambo *et al*., 2008). As a result TB has since been associated with HIV infections. Worldwide, co-infection with HIV is highest in South Africa, India and Nigeria (Assam-Assam *et al*., 2010). Most TB cases have been found in Asia, however, the highest rates are in Africa where high rates of HIV and malnutrition weaken immune systems and speed up the spread of the disease (WHO, 2013; WHO, 2005).

Globally, TB is most common in Africa, West Pacific and Eastern Europe. All these three regions have been noted to have been overwhelmed with factors contributing to the spread of TB, which include limited resources, HIV infection, as well as multidrug-resistant (MDR) TB (WHO, 2010). Although international public health efforts have put a great control on the rate of increase of TB, these regions (Africa, West Pacific and Eastern Europe) still account for the continued increase in TB throughout the world (WHO, 2010). Since 1990, the number of new TB cases has more than quadrupled in most African countries, with 2.8 million new TB cases and approximately 735 thousand deaths every year (WHO, 2009). The socio-economic and hygienic conditions of human populations will always remain directly linked to the success of the propagation of TB (WHO, 2005), also drug resistance play a major role in the survival and spread of MTBC.

1.4. Epidemiology of TB in South Africa

South Africa has one of the highest rates of TB in the world; 389,000 people contract the disease each year (International Federation of Red Cross and Red Crescent Societies, 2013). In recent years, epidemiological situation of TB in South Africa has increased (WHO, 2006). One of the main causes is that infection by drug-resistant *M. tuberculosis* (DR-TB) strains, especially the multidrug-resistant *M. tuberculosis* (MDR-TB), is dominant. The introduction of GeneXpert has made TB to be easy due to its efficiency in the detection of both the organism and its resistance to rifampicin and isoniazid within 2 hours. Drug resistant TB (MDR and XDR-TB) has caused daunting challenges in TB management in the country.

Eighty percent of estimated TB cases worldwide each year occur in only 22 countries, including India, Pakistan, South Africa, and Brazil, and are designated by the World Health Organization as high-burden countries (WHO, 2013). South Africa is ranked third of 22 tuberculosis high-burden countries in the world (USAID, 2009). According to WHO (2009), South Africa had nearly 460 000 new TB cases in 2007, with an incidence rate estimated at 948 cases per 100 000 population. In the year 2010 62 827 people died of TB, this number includes those people who were co-infected with HIV (Statistics South Africa, 2013). The World Health Organization gives a figure of 25 000 deaths from TB in South Africa in 2011, this figure is for only patients who had only TB (Statistics South Africa, 2013).

The four Provinces in South Africa with the highest burden of MDR and XDR-TB are the Eastern Cape, KwaZulu-Natal, Western Cape and Gauteng (Bateman, 2012). When extensive evolution studies were conducted it showed that the *M. tuberculosis* strain has been responsible for cases like MDR-TB since 1994 and XDR-TB from 2001 (Pillay and Strum 2010). In 2006 52 of 53 people contracted TB and died within months during the XDR-TB outbreak in KwaZulu-Natal. The strain that was responsible for the XDR outbreak in 2006 is F15Lam4KZN. It is estimated that 70% of XDR-TB patients die within a month of diagnosis (GATBDD, 2013). The discovery of XDR-TB in KwaZulu Natal posed serious challenges to the control of TB in South Africa and indicated a failure in control of MDR-TB (Green *et al.*, 2010; Gandhi *et al.*, 2006).

The Eastern Cape (E.C) Province is the second largest Province in the country with approximately 13.9% of the total land area of South Africa. Approximately 63% of the Province's population lives in rural areas (Bradshaw *et al.*, 2000). According to Bradshaw *et al.* (2000), E.C has the second worst burden of poverty in the country with approximately 47% of household living well below the poverty line. There is also a 55% rate of unemployment which worsens the poverty situation in this Province.

The E.C Province ranks as one of the high TB burdened Province in South Africa with incidence rates reacting approximately 662/100 000 population a year (Chabula, 2006). TB programmes such as the direct observed treatment strategy (DOTs) and the Bacille Calmette-Gueren (BCG) vaccine has been introduced and used but TB still remains an unsolved health

problem with the situation worsening each year (Karim *et al.,* 2009). Port Elizabeth has a potential in providing job opportunities especially to people from the Eastern Cape Province. The number of illegal immigrants from some of the African countries is increasing in the city. These people then reside in low-cost settlements that are overcrowded and poor. Therefore the chance of spreading the mycobacterial strains is very high.

1.5.Statement of research problem

The global HIV infection epidemic has caused explosive increase in TB incidence which is severe in South Africans and has nearly 20% of the worlds reported number of MDR-TB cases (WHO, 2013; Barnard *et al*., 2008; WHO, 2006). According to the latest statistics from the World Health Organisation, there were 335 974 cases in South Africa of TB in 2010, and those of 60 580 were due to re-treatments due to patient default and treatment failure (WHO, 2012). The number of deaths that were recorded by the end of the year 2012 were 579 711 patients who died due to TB in South Africa (WHO, 2013), this statistics show a rise in number of people who die because of the TB disease.

Differentiation of MTBC members has become particularly important in adult and paediatric patients with HIV related immune suppression. Polymerase chain reaction (PCR) based methods have been used extensively to differentiate TB from bacille Calmette-Guérin (BCG) disease in HIV-infected infants, where accurate diagnosis has implications for both clinical management and policy. This could serve an essential function in the differentiation of MTBC members and detection of transmission in captive and other animal populations and assist in curbing the disease. However, differentiation of MTBC is not done in the Eastern

Cape, particularly in Port Elizabeth. It is important to differentiate between the 9 MTBC members, this help in accurate diagnosis of TB and detection of TB transmission. Therefore, there is a problem in accurate diagnosis and tracing TB transmission in the Eastern Cape, hence differentiation using region of differences is not done.

TB is the fourth highest cause of death in children between 1 to 4 years of age (Mortality and cause of death in South Africa, 2010). The prevalence of XDR-TB in MDR-TB patients in South Africa is 90% (WHO 2009) which is in line with the global trend, but the South African XDR-TB patients are among the highest in the world (WHO 2009). Studies conducted in South Africa suggest that MDR-TB patients are not being effectively treated and cured (Holtz *et al.*, 2006); this is contributing to the development of XDR-TB (Mlambo *et al.*, 2008). XDR-TB is a problem that needs to be investigated to know the spread of such strains in the community. The most recent drug-resistance surveillance data issued by WHO estimates that an average of roughly 5 percent of MDR-TB cases are XDR-TB. Most laboratories are still ill-equipped which makes the estimation detection and diagnosis of drug resistant TB to be extremely difficult and it brings a thought such as that most of the XDR-TB cases go unnoticed (WHO, 2013).

The Eastern Cape region analysis of the host population has showed HIV co-infection to be a risk factor for the spread of the atypical Beijing strains (Strauss *et al.*, 2008). In contrast, the frequency of atypical Beijing strain was low in the Western Cape region, which in turn has a low incidence of HIV tuberculosis co-infection (Hanekom *et al.*, 2007); this raises concern on the spread of all drug-resistant strains in vulnerable population. Greater vigilance is required to stop the drug-resistant tuberculosis epidemic in high HIV prevalence settings.

The current form of management (centralised in specialised units) has been troubled with many challenges in the Eastern Cape Province and this include delays in treatment initiation which increase patients' suffering due to long waiting lists for admission of DR-TB (Lawn *et al.*, 2001). Refusal of hospitalization or absconding by some patients due to lengthy hospital stays, lack of recreational facilities in hospitals and some due to patients' responsibilities to attend to family needs and demands leads to drug resistant-TB and spread of drug resistant-TB in the Eastern Cape Province, particularly Port Elizabeth. There is also nosocomial transmission of MDR/XDR-TB in health facilities when infection control measures are not implemented adequately. There is substantial evidence that more than half of all XDR-TB infections are acquired in hospitals (Mitnick *et al.*, 2008; Basu *et al.*, 2007). The Eastern Cape is one of the poorest regions in South Africa and the community is aware of TB, but they lack knowledge on how to protect themselves from getting it, spreading it and managing their treatment which is a serious problem that lead to the high rate of infected people in this Province.

A study conducted in Pietermartizburg by Marra in 2009 indicated that 70% of MDR-TB patients' households are headed by females who cannot be admitted to hospital for a long period due to responsibilities such as caring for young children (Marra, 2009). Some patients feel that monthly follow up trips to the centralised hospitals for monitoring and medication are lengthy, arduous and unpleasant, which contributes to poor treatment. TB is consuming a lot of money hence the government gives free treatment and also it is responsible the patient's supervision to ensure that their treatment is taken correctly (NHLS, 2010).

There is a progression on uncontrolled contagious TB which is under the threat of drug resistance growth (WHO, 2013). Drug resistance arises due to improper use of antibiotics in chemotherapy of drug susceptible (WHO, 2013). There are several factors that contribute to the development of MDR-TB such as poor adherence of patients to first line anti-TB drugs (Olusoji *et al*., 2013). MDR strains of TB has been reported worldwide, with approximately 500 000 cases reported in 2007 (Green *et al*., 2010). Approximately 5–7% of MDR cases subsequently develop into XDR form of TB (Da Silva and Palomina, 2011). XDR-TB is a consequence of multiple acquisitions of mutation in specific resistance associated genes (Perdigão *et al*., 2012). These become a problem because such mutations may interfere with drug-target interactions or even cause overexpression of the protein target or drug modification enzymes (Perdigão *et al*., 2012). This mutations need to be delineated in order to know the type of mutation circulating in this region.

1.6. Hypothesis

Mutations in first and second drugs resistant TB strains are prevalent in reported TB cases among Port Elizabeth residents.

1.7.Aims and objectives

1.7.1. Overall objective

The study aims to assess the incidence of *Mycobacterium tuberculosis* complex in Port Elizabeth.

1.8.Specific objectives

1. To detect the presence of *Mycobacterium tuberculosis* complex (MTBC) in DNA samples from National Health Laboratory Services (NHLS) in Port Elisabeth.

2. To identify *Mycobacterium tuberculosis* species in the complex

3. To amplify and sequence the genes conferring first and second line drug resistance.

4. To analyse the frequency of gene mutations associated with resistance to INH, RIF and EMB for first line drugs and *rrs* and *gyrA* genes for second line drugs among DNA isolates received from NHLS in Port Elizabeth.

5. To define the epidemiology of *Mycobacterium tuberculosis* complex DNA isolates and phylogenetic exploration of the pathogen using the mutations from the sequenced genes.

REFERENCES

Ahmed N, Caviedes L, Alam M, Rao K.R, Sangal V, Sheen P. *et al.* (2003). Distinctiveness of *Mycobacterim tuberculosis* genotypes from human immunodeficiency virus type 1-seropositive and –seronegative patients in Lima, Peru. Journal of Clinical Microbiology. 41:1712-1716.

Alland D, Kalkut G.E, Moss A.R, McAdam R.A, Hahn J.A, BosworthW. *et al.* (1994). Transmission of tuberculosis in New York City. An analysis by DNA fingerprinting and conventional epidemiologic methods. North England Journal Medicine. 330:1710-1716.

Allix C, Supply P, & Fauville-Dufaux M (2008). Utility of fast mycobacterial interspersed repetitive unit-variable number tandem repeat genotyping in clinical mycobacteriological analysis. Clinical Infectious Disease 39:783-789.

Assam Assam J.P, Denlap V.B, Ngwa F.C, Tedom J.C, Anyangwe I.A & Titanji V.P (2010). *Mycobacterium tuberculosis* complex drug resistance pattern and identification of species causing tuberculosis in the west and centre regions of Cameroon. BMC Infectious Disease. 11:94-100.

Barnard M, Albert H, Coetzee G, O'Brien R & Bosman M.E (2008). Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. American Journal of Respiratory and Critical Care. 177:787-792.

Basu S, Andrews J.R, Poolman E.M, Gandhi N.R, Shah N.S, Moll A. *et al*. (2007). Prevention of nosocomial transmission of extensively drug-resistant tuberculosis in rural South African district hospitals: an epidemiological modelling study. Lancet. 370(9597):1500-1507.

Bateman (2012). The parted TB struggle-|SA ups the intensity SAMS. 100:207-209.

Bradshaw D, Nannan N, Laubscher R, Groenewald P, Joubert J, Nojilana B. *et al.* (2000). Mortality estimates for Eastern Cape Province. South African National Burden of Disease Study. 3-10.

CDC (2011). The difference between latent TB infection and active TB disease. www.cdc.gov/TB/publications/factsheets/general/LTBIandActiveTB.htm. (Accessed 8 January 2014).

Chabula M (2006). The burden of Tuberculosis in eastern Cape Province 1998-2006. 2-10.

Ciglenecki I, Glynn J.R, Mwinga A, Ngwira B, Zumla A, Fine P.E. *et al.* (2007). Population differences in death rates in HIV-positive patients with tuberculosis. International Journal of Tuberculosis and Lung Disease. 11:1121-1128.

Cox H.S, Kalon S, Allamuratova S, Sizaire V, Tigay Z.N, Rusch-Gerdes S. *et al.* (2008). Multidrug-resistant tuberculosis treatment outcomes in Karakalpakstan, Uzbekistan: treatment complexity and XDR-TB among treatment failures. PloS One. 2:e1126.

Daley C.L, Small P.M, Schecter G.F, Schoolnik G.K, McAdam R.A & Jacobs W.R.Jr (1992). An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus. An analysis using restriction-fragment-length polymorphisms. The New England Journal of Medicine. 326:231-235.

Da Silva P.E.A & Palomino J.C (2011). Molecular basis and mechanisms of drug resistance: strategies, sources and newmolecules. Curr Med Chem 16: 1898–1904.

Dean G.L, Edwards S.G, Ives N.J, Matthews G, Fox E.F, Navaratne L, Fisher M. *et al.* (2002). Treatment of tuberculosis in HIV-infected persons in the era of highly active antiretroviral therapy. AIDS. 16:75-83.

Ducati R.G, Ruffino-Netto A, Basso L.A & Santos D.S (2006). The resumption of consumption - a review on tuberculosis. Memorias Instituto Oswaldo Cruz. 101:697-714.

Frieden T.R, Sterling T.R, Munsiff S.S, Watt C.J & Dye C (2003). Tuberculosis. Lancet. 362:887-899.

Gandhi N.R, Moll A, Sturm A.W, Pawinski R, Govender T & Lalloo U (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 368:1575-1580.

Global Alliance for TB Drug Development (2013). http://www.tballiance.org/why/mdr-xdr.php (accessed 26 December 2013).

Goletti D, Weissman D, Jackson R.W, Graham N.M, Vlahov D, Klein R.S. *et al.* (1996). Effect of *Mycobacterium tuberculosis* on HIV replication. Role of immune activation. Journal of Immunology. 157:1271-1278.

Green E, Samie A, Obi L.C, Bessong O.P & Ndip R.N (2010). Inhibitory properties of selected South African medicinal plants against *Mycobacterium tuberculosis*. Journal of Ethnopharmacology. 130:151-157.

Haar C.H, Cobelens F.G, Kalisvaart N.A, van der Have J.J, van Gerven P.J & van Soolingen D (2007). Tuberculosis drug resistance and HIV infection, the Netherlands. Emerging Infectious Diseases. 13:776-778.

Hanekom M, van der Spuy G.D, Streicher E & Ndabambi S.L (2007). A recently evolved sublineage of the *Mycobacterium tuberculosis* Beijing strain family is associated with an

increased ability to spread and cause disease. Journal of Clinical Microbiology. 45:1483-1490.

Holtz T.H, Lancaster J, Laserson K.F, Wells C.D, Thorpe L & Weyer K (2006). Risk factors associated with default from multidrug-resistant tuberculosis treatment, South Africa. International Journal of Tuberculosis and Lung Diseases. 10(6):649-655.

International Federation of Red Cross and Red Crescent Societies (2013). Simple solution to stop the deadly spread of TB in South Africa. http://www.ifrc.org/en/news-and-media/news-stories/africa/south-africa/simple-solutions-to-stop-the-deadly-spread-of-tb-in-south-africa-61224/#sthash.Pu7GS1wY.dpuf (accessed 09 January 2014).

Karim S.A, Churchyard G, Karim Q.A & Lawn S.D (2009). HIV infection and tuberculosis in South Africa: an urgent need to escalate the public health response. Lancet. 374:921-933.

Lawn S & Griffin G (2001). The irreversible cost of delayed diagnosis of tuberculosis in HIV coinfected persons in sub-Saharan Africa. International Journal of Tuberculosis and Lung Diseases. 5(2):200-203.

Marra C (2009). Tuberculosis MDR/XDR: The Msinga Experience - Lessons learnt from South Africa 2005–2009. Pietermartizburg.

Miriani B.V, Alfredo P.L, Catalina A.H, Gilberto V.A, Midori K.M, Peter M.S. *et al*. (2001). *rpoB* Gene Mutations in Rifampin-Resistant Mycobacterium tuberculosis Identified by

Polymerase Chain Reaction Single- Stranded Conformational Polymorphism. Emerging Infectious Diseases. 7:1010-1013.

Mitnick C, Shin S, Seung K, Rich M, Atwood S, Furin J. *et al*. (2008). Comprehensive Treatment of Extensively Drug-Resistant Tuberculosis. New England Journal of Medicine. 359:563-574.

Mlambo C, Warren R, Poswa X, Victor T, Duse A & Marais E (2008). Genotypic diversity of extensively drug-resistant tuberculosis (XDR-TB) in South Africa. International Journal of Tuberculosis and Lung Diseases. 12(1): 99-104.

Morozova I, Riekstina V, Sture G, Wells C & Leimane V (2003). Impact of the growing HIV-1 epidemic on multidrug-resistant tuberculosis control in Latvia. International Journal of Tuberculosis and Lung Disease. 7: 903-906.

"Mortality and cause of death in South Africa, (2010): Findings from death notification'', Statistics South Africa 2013. www.health.ny.gov/diseases/chronic/basictat.htm. (Accessed 19 November 2013).

National Health Laboratory Service (NHLS), (2010). http://www.doh.gov.za/docs/policy/2011/policy_TB.pdf. (Accessed 18 October 2013).

Olusoji E.J, Abiodum O.A & Olu-Abiodum O (2013). An assessment of maternity care services in a semi-urban local government area in Nigeria. Journal of Medicine and Medical Science Research. 2(8):97-104.

Perdigão J, Macedo R, Silva C, Pinto C, Furtado C, Brum L. *et al*. (2012). Tuberculosis drug-resistance in Lisbon, Portugal: a 6-year overview. Clinical Microbiology and Infection. 17(9):1397-1402.

Pillay M & Sturm A.W (2010). Nosocomial transmission of the F15/LAM4/KZN genotype of *Mycobacterium tuberculosis* in patients on tuberculosis treatment. International Journal of Tuberculosis and Lung Disease. 14:223-230.

Small P.M, McClenny N.B, Singh S.P, Schoolnik G.K, Tompkins L.S & Mickelsen P.A (1993). Molecular strain typing of *Mycobacterium tuberculosis* to confirm cross-contamination in the mycobacteriology laboratory and modification of procedures to minimize occurrence of false-positive cultures. Journal of Clinical Microbiology. 31:1677-1682.

Statistics South Africa (2013) "Mortality and cause of death in South Africa: Findings from death notification''. www.health.ny.gov/diseases/chronic/basictat.htm. (Accessed 19 November 2013).

Strauss O.J, Warren R.M, Jordan A, Hayes C, Pittius N.C.G, Streicher E.M. *et al.* (2008). Spread of a low-fitness drug resistant *Mycobacterium tuberculosis* strain in a setting of high human immunodeficiency virus prevalence. Journal of Clinical Microbiology. 46:1514-1576.

Todar K (2009). Todar's Online Textbook of Bacteriology http://www.textbookofbacteriology.net/index.html.

United States Agency for International Development (2009). South Africa Tuberculosis profile. Pages 1-3.

Wells C.D, Cegielski J.P, Nelson L.J, Laserson K.F, Holtz T.H, Finlay A. *et al.* (2002). HIV infection and multidrug-resistant tuberculosis: the perfect storm. Journal of Infectious Diseases. 196:S86-S107.

World Health Organisation (2009). Global Tuberculosis Control: a short update to the 2009 report. WHO/HTM/TB/2009.426, WHO press, Geneva, Switzerland. (Accessed 19 September 2013).

World Health Organisation (2010). Tuberculosis fact sheet No.104, pages 1-4.

World Health Organization (2005). Tuberculosis control: The Situation in the African region Region. In the 55th session of the WHO original committee for Africa held in Maputo, Mozambique, August 2005, resolution AFR/RC55/R5. Geneva, Switzerland.

World Health Organization (2006). Geneva, Switzerland. http://www.who.int/mediacentre/news/notes/2006/np29/en/under.html. (Accessed 13 October 2013).

World health Organization (2012). Global Tuberculosis Control 2012, Geneva, 2012, 38 www.who.int/tb/publications/global_report/. (Accessed 27 August 2013).

World Health Organization (2013). http://www.foxnews.com/health/2013/10/23/who-tuberculosis-numbers-fall-but-drug-resistant-strains-on-rise/. (Accessed 23 September 2013).

CHAPTER 2

LITERATURE REVIEW

2.1. *Mycobacterium tuberculosis* complex

Mycobacterium tuberculosis complex are the aetiological agents that cause TB in both humans and animals (LoBue *et al*., 2010). The members of *Mycobacterium tuberculosis* complex (MTBC) includes *M. tuberculosis* the causative agent of human TB and *Mycobacterium africanum* (*M. africanum*) which is strictly found in humans (Brosch *et al*., 2002). This is the main causative cause of TB in West Africa and its predominant host is humans and primates (Kallenius *et al*., 1999). *Mycobacterium bovis* (*M. bovis*) (Alexander *et al*., 2010) was principally found in cattle but it can also infect humans. *M. bovis* is responsible for bovine TB and it includes *M. bovis* BCG which is a vaccine strain.

Mycobacterium microti (*M. microti*) is a pathogen of voles and it rarely infect humans (Brosch *et al.,* 2002), *Mycobacterium pinipedii* (*M. pinipedii*) found in seals (van Soonlingen *et al*., 1997) and *Mycobacterium caprae* (*M. caprae*) found in goats (Brosch *et al*., 2002), are non-human pathogens. The *Mycobacterium cannettii* (*M. cannettii*) predominant host is humans in Africa; it forms smooth colonies and showing characteristic phenotypic properties in a very limited geographical area (Salo *et al*., 1994). There is a recent proposed mycobacterium, *Mycobacterium mungi* which has Mangoose as its predominant host (Alexander *et al*., 2010). These organisms have genetic similarity of approximately 99.9%, but they differ in phenotypic traits such as the virulence, host preference and epidemiology

(Waters *et al*., 2010). The summary of the MTBC species and their host is shown in Table 2.1 below;

Table 2.1: MTBC species and their host

Species	Host	References
M. tuberculosis	Humans	(Brosch *et al*., 2002)
M. africanum	Humans	(Brosch *et al*., 2002)
M. bovis	Cattle	(Alexander *et al*., 2010)
M. microti	Vaccine strain	(Brosch *et al*., 2002)
M. pinipedii	Seals	(van Soonlingen *et al*., 1997)
M. caprae	Goats	(Brosch *et al*., 2002)
M. canettii	Humans	(Coros *et al*., 2008)
M. mungi	Mangoose	(Alexander *et al*., 2010)

2.1.1. *Mycobacterium tuberculosis*

M. tuberculosis is the causative agent of TB in humans. *M. tuberculosis* is a Gram-positive rod-shaped bacterium that is non-motile with no capsule and does not produce toxins neither form spores (Ducati *et al*., 2006). The aerobic bacterium has a unique cell wall structure which is needed for its survival (Allix *et al*., 2004). The cell wall contains fatty acids (60% of the cell wall is fatty acids), mycolic acid attached to the peptidoglycan polysaccharide arabinogalactan providing a lipid barrier that is extraordinary (Allix *et al*., 2004).

The *Mycobacterium tuberculosis* has medically challenging physiological characteristics which are caused by this extraordinary lipid barrier. They include resistance to antibiotics and host defence mechanisms (Mathema *et al*., 2006). The composition and quality of the cell

wall component also affect the bacteria's virulence and growth (Mathema *et al*., 2006). From the study of Demers *et al*. (2010) it can be concluded that *M. tuberculosis* is responsible for the majority of TB cases amongst the South African population.

It has been speculated that *M. tuberculosis* is from *M. bovis*, which it has evolved by adaptation of an animal pathogen to the human host (Stead *et al*., 1995). *M. tuberculosis* is considered as human pathogen, the study of Chen *et al.* (2009) has shown that *M. tuberculosis* can be transmitted from an infected human being to animals. Approximately 1% of bovine herds in Africa are infected by *M. tuberculosis*. The countries with a high prevalence of *M. tuberculosis* in bovine include Algeria and Sudan with an estimated at 6.2% and 7.4% respectively. *M. tuberculosis* is transmitted from one person to the other through close contact and it can also be transmitted from an unpasteurized milk of a bovine herd (Ameni *et al*., 2011).

2.1.2. *Mycobacterium bovis* and *Mycobacterium bovis* (BCG)

M. bovis is the causative agent of TB in cattle and other mammals. The disease in humans is clinically indistinguishable from infection with *M. tuberculosis* (Gibson *et al.,* 2004). *M. bovis* is one of the most recognized MTBC members which cause transmission of TB from animals to humans (zoonotic TB) (Angela *et al.,* 2006).

M. bovis can be transmitted from cattle to humans through the consumption of raw milk or milk products from cattle infected with TB or by inhalation of aerosols containing turbecle bacilli (Huard *et al*., 2003). Differentiation of the two species is very important for

epidemiological purpose and patient management; this is due to the fact that *M. bovis* is intrinsically resistant to the first line anti-TB drugs pyrazinamide (Waters *et al*., 2010). The prevalence of *M. bovis* in cattle is still high with more than 50 million cattle worldwide which are estimated to be infected by the pathogen (Lyaschrenko *et al*., 2004). Pasteurization of raw milk has reduced the transmission of *M. bovis* from cattle to human through milk consumption (Cockle *et al*., 2006; Gibson *et al*., 2004). Studies have concluded that there is still a high prevalence of *M. bovis* particularly in developing countries; although *M. bovis*'s contribution to the global prevalence of TB is still not clear (Waters *et al*., 2010; Cosivi *et al*., 1998).

According to Chen *at el*. (2009), TB incidences in humans due to *M. bovis* in developing countries is 0.5-1.5%. Jeon *et al.* (2010) estimated that in developing countries where there is no adherence to strict animal TB measures which includes the test and slaughter of infected cattle, *M. bovis* forms approximately 10% of human TB cases. *M. bovis* BCG (Bacillus Calmette-Guerin) has its origin from a virulent *M. bovis* strain. Calmette and Guerin performed 230 *in vitro* passages of *M. bovis* until the organism lost its virulence (Cadmus *et al*., 2006). This strain has been used as attenuated vaccine; it may cause disease but only after vaccination with BCG (Cadmus *et al*., 2006).

2.1.3. *Mycobacterium microti*

M. microti causes TB in rodents such as voles, mice and rats (Zanolari *et al*., 2009). *M. microti* has been reported to cause TB also in cats, humans and pigs (Zanolari *et al*., 2009; van Soolingen *et al*., 1998). *M. microti* had earlier been considered non-pathogenic for humans (Kremer *et al*., 1998). Considering the DNA sequences of the 16S rRNA gene and the 16S to 23S internal transcribed spacer region, *M. microti* proved to be a member of the MTBC. Differentiation and primary isolation using biochemical tests are complicated by the very slow growth of this species but Spoligotyping permits the simultaneous detection and typing of *M. microti* (van Soolingen *et al*., 1998). This technique has been applied in two retrospective studies of *M. microti* infections in the Netherlands and England which enable identification of cases in llamas, cats and ferrets and also including four humans (van Soolingen *et al*., 1998).

Genotyping and phenotyping studies have grouped *M. microti* isolated from a variety of animals into two main categories being the llama type and the vole's type (Rufenacht *et al*., 2011; Deforges *et al*., 2004). Both the llama type and vole type are *M. microti* and are involved in human infections (Kremer *et al*., 1998; van Soolingen *et al*., 1998). These *M. microti* genotypes differ from each other by spacer oligotyping (Spoligotyping). The llama-type (presence of spacers 4-7, 23, 24, 26, 37, 38) and the vole-type have only two spacers, 37 and 38). The international Spoligotyping database (SpolDB4) (Brudey *et al*., 2006) includes 40 *M. microti* strains, 37 from the United Kingdom and 3 from Scotland. A study has reported human TB due to both types, although the llama type it has been proved to be isolated in most cases of human TB (Deforges *et al*., 2004). Niemann *et al*. (1993) reported two cases of *M. microti* infection causing pulmonary TB in Germany. The isolates were

identified as *M. microti* of the llama and voles types, according to spoligotype patterns, which displayed reactions with only 2 of the 43 spacers (spacer 37 and 38) (van Soolingen *et al*., 1998). This was the first case of *M. microti* infection in humans.

Other subsequent studies have reported sporadic cases of *M. microti* infection in both immune-competent and immune comprised individuals in Europe (Zanolari *et al*., 2009; Deforges *et al*., 2004). The use of DNA fingerprinting techniques such as the Spoligotyping and IS*6110-RFLP* can help to determine the accurate impact of *M. microti* on human TB burden due to its complicated diagnosis (Smith *et al*., 2009; Kremer *et al*., 1998). This technique can help overcome or improve the problem of conventional methods which have slow growth on LJ media and extremely selective in nature of the organism (Zanolari *et al*., 2009).

2.1.4. *Mycobacterium africanum*

This species was first isolated in 1968 from a Senegalese patient suffering from pulmonary TB and it has characteristics that appear to lie in between *M. bovis* and *M. tuberculosis* (Castets *et al*., 1968). The *M. africanum* species have been subdivided into two major subgroups based on geographic origin and biochemical properties. The two groups are subtype I, those from West Africa and more closer to *M. bovis* and subtype II which are from East Africa and closer to *M. tuberculosis* (Niemann *et al*., 2002). The subtypes can be differentiated based on biochemical tests such as nitratase activity which is negative and positive for subtype I and subtype II respectively (Ani *et al*., 2010). The two types of *M. africanum* have been further classified into distinct lineages using DNA fingerprinting techniques (de Jong *et al*., 2010). The lineages of *M. africanum* subtype I include West

African 1 (MAF1) genotype which is prevalent around the Gulf of Guinea and West African 2 (MAF2), which has been shown to be widespread in western parts of West Africa (de Jong *et al*., 2010).

The difference in distribution of *M. africanum* has been showed by surveys to be in various regions of Africa. Those regions included Ivory Coast, 5%; Guinea-Bissau, 60%; Burkina Faso, 18% and Uganda 49-67% (Huard *et al*., 2006; Niemann *et al*., 2002). *M. africanum* is predominant in west and central African countries and is not a common cause of human TB in other parts of the world (Cadmus *et al.*, 2010). According to Niemann *et al*. (2002), despite the high prevalence of *M. africanum* in Western African countries, there is a variability of its prevalence in other African countries. This variability is low in some countries like Ivory Coast (5%) and high in other countries like Gulnea Bissau (Niemann *et al*., 2002). *M. africanum* is not a predominant cause of pulmonary TB in South Africa, which is one of the leading countries in Africa with high TB burden (Demers *et al*., 2010). The TB epidemiology in countries such as Burkina Faso and South Africa which have high HIV/AIDS pandemic have indicated that *M. africanum* is the main cause of pulmonary TB in immune comprised individuals (Demers *et al*., 2010).

2.1.5. *Mycobacterium cannettii*

Mycobacterium cannetti (*M. cannettii*) which is a member of the MTBC has been reported to cause pulmonary TB in humans, the organism is said to form smooth, white glossy colonies on solid LJ media (Onipede *et al*., 2005). The organism was first isolated in 1969 from a TB patient in France (Fabre *et al*., 2010; van Soolingen *et al*., 1997). According to van Soolingen

et al. (1997), in 1993 the pathogen was isolated in the Netherlands from a Somali patient who had disseminated TB in the lymph. Subsequent studies have shown *M. cannettii* to be isolated from TB patients in Europe who has once been to Western Africa (Goh *et al*., 2006). *M. cannettii* has also been isolated from TB patients in countries such as Sudan, Kenya and Djibouti (Fabre *et al*., 2010). All known cases of TB caused by *M. cannettii* have been contracted in the Horn of Africa (Psyffer *et al*., 1998).

2.1.6. *Mycobacterium pinnipedii*

In 1993, tuberculosis was first diagnosed in seals (known as pinnipeds) in Australia (Cousins *et al*., 1993). *M. pinnipedii* can infect a wide variety of animals such as rabbits, cattle, guinea pigs and also humans (Bigi *et al*., 2005; Cousins *et al*., 2003). *M. pinnipedii* can cause TB in humans who are in close contact with infected animals (Bigi *et al*., 2005). This was demonstrated by isolation of the pathogen from a trainer who had a close contact with water seals, this trainer was a pulmonary TB patient (Kiers *et al*., 2008).

2.1.7. *Mycobacterium caprae*

M. caprae is the causative agent of TB in caprine herds (Aranaz *et al*., 2003). *M. caprae* was identified from caprine pathological samples in Spain which were biochemically and genetically characterized (Aranaz *et al*., 2003). It was first isolated from goats; it has also been isolated from cattle, pig's wildlife and humans in European countries (Rodriguez *et al*., 2009; Aranaz *et al*., 2003). *M. caprae* has been reported as the main cause of bovine TB in Crotia and Central European countries (Prodinger *et al*., 2005). European countries and Spain, may be a threat to public health due to the possibility animal and human transmission,

hence *M. caprae* is a significant zoonotic pathogen whose high prevalence is in goats, particularly in Spain and European countries (Bezos *et al*., 2010; Romeo *et al*., 2007). Studies have shown that the accuracy of human TB due to *M. caprae* is not a common case; it is rare (Rodriguez *et al*., 2009). *M. caprae* have a combination of *pcnA*, *katG*, *oxyR*, and *gryA* gene polymorphisms (Aranaz *et al*., 2003).

2.2. Anti-TB drug resistance

Drug resistance usually occurs mainly in where drug resistance follows sustained treatment failure. Treatment can be divided into first line and second line drugs (Johnson *et al*., 2009). For the first line drug resistance, a four-drug regiment is used consisting of isoniazid, rifampicin, pyrazinamide and ethambutol. Resistance to first line anti-TB drugs has been linked to mutations in at least 10 genes; *katG, inhA, ehpC, KasA and ndh* for INH resistance; *rpoB* for RIF resistance, *emb B* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for streptomycin resistance (Johnson *et al*., 2009).

Resistance associated point mutation deletion or insertions have been described for all first line drugs (Isoniazid, rifampicin, pyrazinamide and streptomycin), and for several line and newer drugs (ethionamide, fluoroquinolenes and macrolides) (Vester *et al*., 2001; Stover *et al*., 2000; Zhang *et al*., 2000). Second line drugs are classified as aminoglycosides (kanamycin and amikacin), polypeptides (capreomycin, viomycin and enviomycin), fluoroquinoles (ofloxacin, aprofloxacin and gatifloxacin), D-cycloserine and thionamides (ethionamide and prothonamide) (WHO, 2001). Amikacin, kanamycin and capreomycin inhibit protein synthesis by binding the ribosome (Johansen *et al*., 2006; Prammananan *et al*., 1998). Resistance to all these three drugs is associated with mutations in the 16S rRNA gene *rrs* (Engström *et al*., 2011; Maus *et al*., 2005). However mutations on the *tylA* gene are also

known to cause Cap resistance. Fluoroquinolones binds to DNA gyrase and inhibit proper regulation of supercoiling and cause chromosome double-strand breaks (Xu *et al.*, 1996) the DNA gyrase is encoded by the *gyrA* and *gyrB* genes (Piton *et al.*, 2010). Second line drugs are more toxic and less effective when compared with first line drugs and they are mostly used in the treatment of MDR-TB (Cheng *et al*., 2004).

2.2.1. Isoniazid (INH) Resistance

Isoniazid is a pro-drug that requires activation of isoniazid susceptible mycobabcterial species (Johnson and Schultz, 1994). INH resistance occurs as a result of different mutations in various genes such as *katG, inhA, kasA, ahpC* and *oxyR* (Aslan *et al.*, 2008). Isoniazid is a prodrug that requires activation by the enzyme catalase peroxidise which is produced by susceptible MTB species (Johnson and Schultz, 1994). The study that was conducted in 1990s when a primary mycobacterial catalase-peroxidase gene (*katG*) was cloned and sequenced revealed is found in 42%-58% of isoniazid-resistant clinical isolates (Ramaswamy and Musser 1998; Zhang *et al*., 1992). Although Cho *et al*. (2009) and Aslan *et al*. (2008) have reported about mutations on codon 315 of the *katG* gene being the most common in up to 95% of INH resistant strains and mutations in the regulations region of the *inhA* gene are responsible for INH resistance in approximately 35% of INH resistant strains (Aslan *et al*., 2008).

The Ser315Thr mutations found most often occurs in approximately 40% of isoniazid-resistant strains (Zhang *et al.*, 2000; Ramaswamy *et al*., 1998). This is the most common mutation associated with failure to active INH in approximately 40% INH resistant strains in Ser315Thr mutation (Somoskovi *et al*., 2001). Different experiments that have been

conducted have shown that there is failure to enzymatically activate INH by catalase-peroxidase; the pro-drug may also result in resistance. The failure to enzymatically active INH by catalase-peroxidase may be due to the fact that the Ser315Thr mutation results in an enzyme without the ability to activate isoniazid, but retains approximately 50% of its catalase peroxidise activity (Rouse *et al.*, 1996). The altered catalase-peroxidase provides high level resistance to isoniazid, while retaining a level of oxidative protection that is sufficient to enable the organism to maintain detoxifying activity against host antibacterial radicals (Somoskovi *et al*., 2001). Mutations in the promoter region of the *aphC* gene that encodes for alkyl hydroperoxidase, a detoxifying enzyme also show resistance to INH (Somoskovi *et al*., 2001). Studies show that mutations in the *oxyR*-aphaC gene are responsible for INH resistance for approximately 13% in South Africa (Valvatene *et al*., 2009).

2.2.2. Rifampicin (RIF) Resistance

Rifampicin is one of the crucial first line anti-TB drugs causing treatment failure (Aslan *et al*., 2008). The mechanism of action of RIF is to bind to the β-subunit of the targeted DNA-dependant RNA polymerase to inhibit mycobacterial transcription. Mutation in 81 bp region of *rpoB* has been found in more than 96% of RIF-resistant strains (Aslan *et al*., 2008). In Turkey, a study reported by Aslan *et al*. (2008) that mutations in codons 526 and 531 of the *rpoB* gene confer 36% and 43% respectively of RIF resistant strains.

Other reports from Mexico and Hungany show that mutation in the *rpoB* gene in codon 511 and 516 are the most common RIF's resistant strains (Nikolayesusky *et al*., 2004). Alternations in codon 511, 516, 518 and 522 result in low level RIF-resistance (Johnson *et al*., 2009). A study by Green *et al*. (2008) reported on 4.6% of isolates resistant to RIF alone

in Pretoria, South Africa. The most prevalent mutation among the RIF-resistant isolates of the study was at codon 516 with 42.2% (Green *et al.*, 2008).

2.2.3. Pyrazinamide (PZA) resistance

Pyrazinamide, a nicotinamide analog that targets an enzyme involved in fatty-acid synthesis (Zimhony *et al.*, 2000). It is a pro-drug that is converted by mycobacterial enzyme pyrazinamidase encoded by *pncA* to its active form (pyrazinoic acid [POA]). PZA has no effect on other mycobacteria; it is highly specific for *M. tuberculosis* (Johnson *et al*., 2009). *Mycobacterium bovis* has C-G point mutations in codon 169 of PZA in nature which causes *M. bovis* to be resistant to PZA. PZA is only active at acidic pH against *Mycobacterium tuberculosis* due to the accumulation of POA in the cytoplasm (Campbell *et al*., 2011; Zimhony *et al*., 2004).

A study by Louw *et al*. (2009) showed that PZA resistance is common around drug-resistant clinical *M. tuberculosis* isolates from South Arica. Resistance to PZA was shown to be strongly associated with MDR-TB and therefore it was concluded that PZA should not be relied on in managing patients with MDR-TB. Mutations in *pncA* gene correlate well with phenotypic resistance to PZA. Scorpio *et al*. (1996) reported that various *pncA* mutations have been identified in more than 70% of PZA resistant strains. The His71Asp mutation is most frequent in PZA resistant strains and is believed to play a major role in conferring PZA resistance (Jonmalung *et al*., 2010). It has been reported that 52% of MDR *M. tuberculosis* strain in South Africa were also PZA resistant (Mphahlele *et al*., 2008).

2.2.4. Streptomycin (SM) resistance

Streptomycin is an alternative first line anti-TB drug (Brzostek *et al*., 2004). The effect of SM is seen in the ribosomes where it binds to the 16S rRNA and 12S ribosomal protein (*rrs* and *rpsL*) (Sun *et al*., 2010; Abbadi *et al*., 2001), this includes ribosomal changes which cause inhibition of protein synthesis and misreading of mRNA. SM anti-TB drug is said to be less effective when compared to INH and RIF against *M. tuberculosis*. The mechanism of SM resistance is based on point mutations in different genes which include the *rrs* and *rpsL*, and it is reported in approximately 68% (Da Silva, 2011). Victor *et al*. (2001) demonstrated that in the *rrs* gene which is an A-C transversion at position 491, 512 and 516, the transition at codon 491 is not responsible for resistance to SM but it is strongly associated with the global spread of *M. tuberculosis* with a Western Cape F11 genotype.

The *rrs* encoding the 16S rRNA subunit once mutated they show low level streptomycin resistant (Sun *et al*., 2010). Mutations in the S30 loop and nucleotide region 912 of the *rrs* have been reported to confer low level SM resistance (Sreevatsan *et al*., 1997). Streptomycin resistance to *M. tuberculosis* is also caused by mutations in the *rpsL* gene, encoding S12 ribosomal protein (Sun *et al*., 2010; Cooksey *et al*., 1996).

It has been indicated that SM resistant isolates by MIC analysis that amino acid replacements in the *rpsL* genes correlate with an intermediate level of resistance while on the other hand *rrs* gene mutant correlate with an intermediate level of resistance (Meirer *et al*., 1996). It has been suggested that low level of SM resistance are also associated with altered cell permeability or rare mutations which lie outside of the *rrs* and *rspL* genes.

The most common type of mutation in the *rpsL* gene occurs at codon 43 and 88 which results in amino acid substitution of arginine for lysine in the S12 protein (Sun *et al*., 2010; Tudo *et al*., 2010). In several studies it has been reported that the occurrence of low SM strains and *M. tuberculosis* that do not harbour *rpsL* and *rrs* mutations, but postulates the other mechanisms of SM resistance (Da Silva and Palomino, 2011).

2.2.5. Fluoroquinolones resistance

Ciprofloxacin and ofloxacin which fall under fluoroquinolones are the two second line drugs in MDR-TB treatment (WHO, 2001). The quinolones target and inactivates the DNA gyrase, which is a type II DNA topoisomerase and is encoded by *gyrA* and *gyrB* (Ginsburg *et al*., 2003; Rattan *et al.*, 1998).

2.2.6. Aminoglucosides resistance

The two amino glycosides that inhibit protein synthesis are kanamycin and aminokacin. The mechanism of aminoglycosides binds to bacterial ribosomes and disturbs the elongation of the peptide chain in the bacteria (Suzuki *et al*., 1998). Mutations in the *rrs* gene encoding for 16S rRNA are associated with resistance to kanamycin and amikacin (Suzuki *et al*., 1998).

2.2.7. Peptides resistance

The basic peptide antibiotics that inhibit prokaryotic protein synthesis used as second line anti-TB drugs are viomycin and capreomycin (Taniguchi *et al*., 1997). Various studies have shown that resistance to viomycin in *M. tuberculosis* is caused by alterations in the 30S and 50S ribosomal subunits (Taniguchi *et al.*, 1997).

2. 3. Molecular epidemiology of TB

The resurgence of TB worldwide has renewed interest in understanding the epidemiology and pathogenesis of diseases (Mlambo *et al*., 2008). Genotyping of DNA fingerprinting made it possible by the discovery of various genetic markers in the early 90's, which has replaced phenotypic markers such as mycobacterial bacteriophage typing (Jones, 1978) which is the principal method for differentiating *M. tuberculosis* strains. Molecular techniques are being used worldwide in understanding the epidemiology of TB. The first reports in TB were published in 1990 using the insertion sequences (IS*986* or IS*6110*) to examine DNA polymorphism (Hermans *et al*., 1990; Mc Adam *et al.,* 1990).

Molecular tools have given insight into *M. tuberculosis* population diversity and the various risk factors associated with drug resistant TB. The global TB epidemic at molecular level is comprised of multiple genotype-specific sub-epidemics. IS*6110* is a well characterized repetitive sequence that can be used to identify the different MTBC genotypes (Mc Adam *et al.,* 1990). IS*6110* has become an important diagnostic tool in the identification of MTBC species because it is an insertion element found exclusively within the members of *Mycobacterium tuberculosis* Complex (Caros *et al*., 2008). Currently various genotyping techniques such as Spoligotyping has made a great impact in the understanding of *M. tuberculosis* epidemiology using the international databases to compare isolates from wide spread geographic areas (Brudey *et al*., 2006; Filliol *et al*., 2003; Filliol *et al*., 2002; Sola *et al*., 2001). There are three techniques that have been internationally standardized and are commonly used. This technique includes IS*6110*-Restriction Fragment Length Polymorphism analysis (IS*6110*-RFLP), Spacer Oligonucleotide typing (Spoligotyping) and Mycobacterial

Interspersed Repetitive Unit Variable Number Tandem Repeats (MIRI-VNTR) typing (Brudey *et al.,* 2006; Filliol *et al*., 2003; Filliol *et al*., 2002; Sola *et al*., 2001).

2.3.1. IS*6110*-RFLP analysis

Restriction Fragment Length Polymorphism is a technique used to show the variation of homologous DNA. This is one of the first groups of methods typing based which includes the presence of insertion sequences (IS). IS*6100*-Restrictiom Fragment Length Polymorphism analysis (IS*6110*-RFLP) is a standardized method for IS*6110* analysis proposed by van Embden in 1993 and it is gaining recognition as a gold standard for TB molecular typing (van Embden *et al*., 1993). The method uses insertion sequences (IS) and restriction fragments such as *pvuII* which cleaves IS*6110* at a single asymmetric site. Insertion sequences are mobile genetic elements that contain inverted DNA sequences at each end and translate transposase which make the sequence mobile (Mc Adam *et al*., 1990). There are more than 16 different IS in five IS families that has been reported in mycobacteria (Mc Adam *et al.*, 1990).

IS*6110* is specific to *M. tuberculosis* when analysed by southern blotting (Kremer *et al.*, 1999). The transposable elements in *M. tuberculosis* were discovered to have a great potential for use in strain differentiation (Eisenach *et al.,* 1988). The IS*6110*-RFLP method is convenient and reliable for the *M .tuberculosis* strains (van Embden *et al.,* 1993), but it has been reported that it lacks the sensitivity for majority of *M. bovis* strains because their genome contains only a few IS*6110* copies (van Soolingen *et al.,* 1994). The number of copies of one of the most widespread IS elements (IS*6110*) varies from 1 to 26 in different *M. tuberculosis* strains. The method uses common *Pvu*II restriction endonuclease that cuts DNA

into simple, asymmetric parts. These restriction fragments are used when organizing this method based on the Southern blot analysis. A DNA probe is denatured and undergoes hybridization of restriction fragments with an IS*6110* probe (kontsevaya *et al.,* 2011). The reaction results in the formation of a pattern, i.e., the sequence of bands corresponding to DNA fragments of different molecular weights (the molecular weight marker is used to estimate the lengths of fragments).

A software is used to help cluster the strains under investigation and, subsequently, build dendograms and calculate genetic distances (Gel Compare and Bionumerics programs of Applied Maths Company, Belgium) which is usually developed in parallel with the IS*6100* RFLP method (Kontsevaya *et al.,* 2011). IS*6110*-RFLP analysis has some limitations compared with the PCR-based genotyping methods. The method cannot be used reliably to type isolates with less than 6 IS*6110* insertions, many of the sites of IS*6110* insertion are conserved in such strains (Soini *et al.,* 2001). The problem of IS*6110*-RFLP is usually overcome by using additional typing technique such as Spoligotyping and MIRU-VNTR typing (Goyal *et al.,* 1997; kemerbeek *et al.,* 1997).

2.3.2. Spacer Oligonucleotide typing (Spoligotyping)

Spoligotyping method is the bases for the creation of the largest genotype database for *M. tuberculosis,* containing a global distribution and phylogenic analysis for worldwide spoligotyping (Brudey, *et al.,* 2006). This is a typing method for *M. tuberculosis* strains that was first published in 1997 (Kemerbeek *et al.,* 1997). It simultaneously identifies and differentiates members of the *M. tuberculosis* complex. This method is based on a DNA polymorphism present at the Direct Repeat (DR) region, which is uniquely present in *M.*

tuberculosis strains (Kamerbeek *et al*., 1997). The DR region contain a variable number of 36 base pair direct repeats (DR), interspersed by non-repetitive spacers, each 35 – 45 base pairs in length (Hermans *et al.*, 1991).

The DR and adjacent spacer are known as direct variable repeat (DVR) (Groenen *et al*., 1993). Direct repeats are 36bp long and are interpreted by DNA spacers of 35bp to 41bp. Different MTBC strains vary in the presence or absence of the particular spacer among the 43 individual spacers of known sequence and in the number of RDs (Vitol *et al.*, 2006). The DR was first described by Hermans and colleagues who sequenced the region in *M. bovis* BCG (Hermans *et al*., 1992). Additional spacers have since been discovered in the DR region, and the order of the spacers is well conserved (van Embden *et al*., 2000). PCR analysis of the DR locus was originally by Direct Variable Repeat PCR with the technique enhanced and data simplified by the development of detection through reverse hybridization to probe against 43 selected individual spacer regions that are bound to nylon membrane. The variation in the absence or presence of spacer regions provides different hybridization patterns (Kamerbeek *et al*., 1997).

The mechanism of this method relies on the *in vitro* PCR amplification of the well conserved DR which are the target regions for amplification using primers DRa and DRb. The PCR products are then hybridized to a membrane which contains immobilized synthetic Oligonucleotide spacer sequences delivered to DR of *M. tuberculosis* H37Rv and *M. bovis* BCG. Spoligotyping can be applied directly to a variety of specimen including the cultured cells, non-viable samples and clinical samples (Kamerbeek *et al*., 1997).

Results can be readily digested and compared to an intermantioned database (Brudey *et al*., 2006; Dale *et al*., 2001). Spoligotyping has been used as a first-line screening technique supplemented by either IS*6110*-RFLP or MIRU-VNTR analysis to improve strain differentiation. The advantage of Spoligotyping is that it is a PCR-base, therefore small quantities of DNA are needed which can be isolated from clinical samples. This makes the method simple, rapid and economical (Singh *et al.*, 2007). Spoligotyping also has the ability to differentiate more strains when compared to RFLP methods (Singh *et al*., 2007). The disadvantage of this method is that it characterizes polymorphisms at a single genetic locus where IS*6110*-RFLP and MIRU-VNTR typing measure polymorphisms in the entire genome of *M. tuberculosis* (Warren *et al*., 2002).

The is an increasing number of the combination of the PCR-based methods Spoligotyping and MIRU VNTR used for molecular epidemiological studies as a preference over IS*6110*-RFLP (Godreuil *et al*., 2007) because they are fast and less technically demanding (Brudey *et al*., 2006). Spoligotyping may be a useful tool for monitoring global transmission of multi-drug resistant strains of *M. tuberculosis* and investigations of TB outbreaks.

2.3.3. MIRU-VNTR typing

Variable numbers of tandem repeat (VNTR) sequences are multiple independent genetic loci found in at least 40 locations across the genome of the *M. tuberculosis*. Each locus contains a repetitive DNA sequence that can be different in the number of repeats between strains (Supply *et al*., 2000). The MTBC genome consists of mycobacterial interspersed repetitive units (MIRU); these loci contains variable number of tandem repeats (Sola *et al*., 2003).

There are different types of VNTR. The *M. tuberculosis* H37Rv reference strain contains 41 MIRU loci (Neonakis *et al*., 2008). In 1998 first reports reported eleven initial VNTR loci comprising five major polymorphic tandem repeats (Frothinghnam & Meer-O'Connell 1998). Since then there has been an additional VNTR loci reported (Skuce *et al*., 2002; Roring *et al*., 2002). This technique is a PCR based method and it produces better resolution when compared to RFLP and Spoligotyping for MTBC isolates that can contain less than 6 IS*6110* copies (Sola *et al*., 2003). This means that more distinct patterns are produced by MIRU-VNTR for MTBC isolates with low copy number of the IS*6110*.

This method uses its specific primers for the flanking region of each VNTR flaking region (Collins *et al*., 2011). After amplification the amplicon size is determined through electrophoretic mobility of the PCR products, and the sizes represent the number of amplified VNTR copies (Supply *et al*., 2005). Size determination can also be determined by performing a capillary system (Kwara *et al*., 2003; Supply *et al.*, 2001). The MIRU-VNTR typing method currently employed in various laboratories worldwide has been standardized (Supply *et al*., 2006) and is based on variable numbers of tandem repeats of different class of interspaced genetic elements known as mycobacterial interspersed repetitive units (MIRU). The method evaluates the number of MIRUs distributed throughout the genome. It is first based of PCR amplification of multiple repeat loci using primers specific for flanking regions of each region locus, and on determination of sizes of the PCR products. This method is used as a secondary genotyping tool for TB epidemiological studies when IS*6110*-RFLP cannot discriminate MTBC isolates (Dou *et al*., 2008).

Various experimental studies have shown and from them it has been concluded that when MIRU-VNTR is used together with Spoligotyping, there is a higher discriminatory power which may even be greater than that of the IS*6110*-RFLP method (Christianson *et al.*, 2010). Genotyping of MTBC using MIRU-VNTR has a higher discriminatory power when compared to Spoligotyping (Dou *et al*., 2008). MIRU-VNTR can be used for the determination of phylogenetic relationships between strains. In addition they can be used to identify the genotype under which the strains can be classified (Kremer *et al*., 2005).

2.4. Epidemiology of drug-resistant TB

Drug resistant TB is more costly than normal TB to treat, and is more often fatal (WHO, 2012). There are two commercially available kits for drug resistant tuberculosis detection; the line probe assay (INNO-LIPA Rif. TB; inno-genetics, South Africa Pty. Ltd) and the GenoType® MTBDR*plus* kit (Hain Lifescience, Belgium) for rifampicin resistance only and simultaneous detection of rifampicin and isoniazid resistance respectively. The World Health Organization has approved the use of the Hain GenoType® MTBDR*plus* test for the rapid diagnosis of INH and RMP resistance, which also allows for the simultaneous identification of *M. tuberculosis* complex strains in clinical isolates (Warren *et al*., 2009; WHO, 2008).

Further investigations demonstrated the feasibility of the Hain MDRTB*plus* assay as an effective tool for MDRTB screening in high TB, and high MDR-TB incidence, region and good concor dance with phenotypic DST results (Barnard *et al*., 2008; Miotto *et al*., 2008; Hillemann *et al*., 2007). Reports demonstrated that application of this test reduces the

diagnostic delay to less than 48 hours, with high sensitivity and specificity to identify RMP resistance which is an important marker of MDR and XDR-TB (Barnard *et al*., 2008). Specificity was good for INH although sensitivity estimates were lower and variable because several genes are involved in conferring INH resistance and probes for these genes are not included in the Hain GenoType® MTBDR*plus* test kit (Warren *et al*., 2009).

Evaluation of the Hain GenoType® MTBDR*plus* test in the Western Cape Province demonstrated the presence of *inhA* gene or promoter mutation in 53.6% of INH mono-resistant strains and 38.2% of cases with MDR-TB strains (Barnard *et al*., 2008). Analysis of drug resistant TB cases collected during 2000-2006 from the Boland, Overberg, Karoo and Southern Cape regions of the Western Cape also showed high proportions of isolates with *inhA* promoter mutations; 24% among cases with INH mono-resistant TB and 29% among cases with MDR-TB (Warren *et al*., 2009).

However, there are no reports of the performance of Hain GenoType® MTBDR*plus* assay kit, for the screening of MDR-TB in the Port Elizabeth region as this kit was only implemented in December 2009 at the HNLS, PE. Molecular tests should not be applied alone and therefore they cannot totally replace culture methods for several reasons; (i) apart from rifampicin and isoniazid susceptibility testing, culture is needed for all other drug susceptibility tests; (ii) rifampicin and isoniazid susceptibility must be confirmed, since the possibility that a strain is resistant cannot be excluded for a strain with a wild type pattern by the MTBDR*plus* assay and (iii) in the case of mixed infection with an MTBC strain and a non-tuberculosis mycobacterium, interpretation may be difficult (Hillemann *et al*., 2007).

Furthermore, the Hain GenoType® MTBDR*plus* assay kit does not detect the full spectrum of mutations conferring resistance in *M. tuberculosis*. Amplification and sequencing of target genes can be restricted to the investigation of less frequent mutations or when the interpretation to the hybridization pattern is not straight enough (Hauck *et al*., 2009). Understanding the mechanism of action of actimycobacterial agents and the basis of resistance to these compounds have led to the possibility of predicting drug resistance in *M. tuberculosis* (Hauck *et al*., 2009).

Another assay that is being used is the GenoType MTBC (Hai LifeScience GmbH, Nehrem, Germany) which is a commercially available DNA strip assay for rapid identification of the members of *M. tuberculosis* complex. It was evaluated by use of well characterized collection of *M. tuberculosis* compex isolates and was proven to be useful for species differentiation (Richter *et al*., 2003). The GenoType MTBC assay is based on an *M. tuberculosis* complex specific 23S ribosomal DNA fragment, *gyrB* DNAsequence polymorphisms and RD1 deletion of *M. bovis* BCG (Kasai *et al*., 2000; Niemann *et al*., 2000; Rüsch-Gerdes *et al*., 1999). The GenoType MTBC assay can anable a very rapid identification of *M. tuberculosis* complex species and species differentiation can be performed efficiently with liquid medium in order to speed up mycobacterial diagnostics. It fits easily into the work flow of a routine laboratory and can be conducted in laboratories that do not carry out shophisticated biochemical tests for differentiation (Richter *et al*., 2004).

There is a currently Gold standard molecular assay for discrimination of MTBC which is the application of Genetic Sequencing. DNA sequencing enables scientists to determine genome sequence. The next-generation technologies have been used for standard sequencing applications, such as genome sequencingand resequencing and for novel applications previously unexplored by Sanger sequencing. There are three commercially available next-generation sequencing technologies in comparison to state-of-the-art Sanger sequence (Hall, 2007). The Sanger method has been partially supplanted by several next generation sequencing technologies that offer dramatic increases in cost effective sequence throughput, albeit at the expense of read lengths. The next generation technologies commercially available today include the 454 GS20 pyrosequencing based instrument (Roche Applied Science) the Solexa 1G analyze (Illumina, inc) the SOLiD instrument from Applied Biosystems, and the Heliscope from Helicos, Inc. (Huttchinson III, 2007).

2.5. Multidrug-resistance and extensive drug resistant tuberculosis (MDR-TB and XDR-TB).

The drug resistance of *M. tuberculosis* strains was discovered in the KwaZulu Natal (KZN) Province of South Africa in 2005 (Gandhi *et al*., 2006). MDR-TB and XDR-TB are fast becoming progressively more significant. Genotype studies have shown that between 63% and 75% of XDR-TB cases progress through acquisition of resistance (Mlambo *et al*., 2008). Not only do MDR-TB and XDR-TB produce fulminant and incurable disease in people with HIV, but they are also very infectious, with conversion rates of as much as 50% in exposed health care workers. XDR-TB has been reported to be in all the country's provinces (WHO, 2008) and it is composed of different strain families (Mlambo *et al*., 2008).

Drug resistant is categorised into initial or primary resistance and acquired resistance. Initial resistance is whereby a patient who has no history of TB treatment develops resistance to anti-TB drugs (Campbell *et al*., 2011; Green *et al*., 2010). This means the person would have been infected by a resistant strain transmitted by another person. Acquired resistance occurs when a patient who has been treated for TB develops resistance to one or more of the drugs previously used (Green *et al*., 2010). MDR and XDR-TB treatment involves using second-line anti-TB drugs which are less potent, more toxic and more expensive compared with the first-line TB drugs used to treat drug-susceptible TB (WHO, 2012). These factors contribute to the low cure and treatment completion rates as well as increased mortality seen when comparing outcomes from MDR and XDR-TB patients with patients with drug-susceptible TB (Mlambo *et al*., 2008).

Treatment for MDR and XDR patients are poor compared with patients with drug susceptible TB, positive outcomes have been reported in predominant HIV negative population with both MDR and XDR-TB (Mlambo *et al*., 2008). In a study in Latvia, treatment of MDR-TB patients using information from regional drug resistance patterns or individualized drug susceptibility patterns for patients with DST data resulted in cure or treatment completion in 66% of HIV negative patients (Leimane *et al*., 2005). When comparing MDR-TB and XDR-TB treatment outcomes; XDR-TB patients have been reported to have poorer outcomes, especially with HIV-infected patients, where XDR-TB infection is usually associated with rapid death (Dheda *et al*., 2010; Kim *et al*., 2008; Migliori *et al*., 2007; Gandhi *et al*., 2006).

The use of fluroquinolones in the treatment of MDR and XDR-TB as well as the integration of TB and HIV therapy seems to improve successful outcomes in MDR and XDR-TB

patients (Dheda *et al.*, 2010; O'Donnell *et al.*, 2009; Padayatchi *et al.*, 2009; Friedland *et al.*, 2007; Torun *et al.*, 2007; Chan *et al.*, 2004; Leimane *et al.*, 2005; Yew *et al.*, 2000). First line drugs treatment takes the duration of six months. Many patients fail to complete their treatment correctly; this is the factor that has fuelled a rise in the drug-resistant forms. Researchers who studied rates of the disease (TB) in South Africa found that almost 44% of cases of MDR-TB were also resistant to at least one second line drug. Treatment options for XDR-TB patients are limited and toxic (Evans, 2012).

2.6. *Mycobacterium tuberculosis* complex diagnosis

The diagnosis of pulmonary TB depends upon accurate detection of *M. tuberculosis* in clinical samples, which is mostly sputum from TB suspected patients (WHO/EMC/ZOO/96.4). Accurate diagnosis of TB is the major method used to control the disease from being further transmitted (Yam, 2006). The diagnosis of suspected TB cases depends on physical examination of clinical and radiographic features as well as the medical history which includes any exposure to TB and also previous TB treatment (Yam, 2006). The most used confirmative diagnosis is achieved through sputum microscopy and mycobacterial culture (Sun *et al.*, 2009). Biochemical tests and conventional methods for identifying *M. bovis* in clinical samples is time consuming and require more labour (Jeon *et al.*, 2010).

2.6.1. Imaging

Imaging plays a significant role in the diagnosis of TB. A multivariable analysis showed that upper lobe disease on a chest radiograph was positively associated with culture proven tuberculosis (Wisnivesky *et al.*, 2000). Chest radiographs do not detect all cases of pulmonary TB. Pepper and colleagues in the study they conducted 2008 found that 9% of

patients with culture-confirmed pulmonary TB had a normal chest X-ray (Pepper *et al*., 2008). NICE guidance states that patients with suspected pulmonary TB should undergo a posterior-anterior chest x-ray. All patients will non-respiratory TB should have a chest x-ray to exclude or confirm co-existing respiratory TB (NICE, 2006).

2.6.2. Tuberculin Skin Test (Intradermal Mantoux test)

The tuberculin skin test (TST) is an *in vivo* assay that is used to detect latent TB infection. This test is also known as the Intradermal Mantoux test, is one of the oldest diagnostic tests still used in modern medical practise (Richeldi, 2006). The method uses tuberculin, which is a glycerol extract from *M. tuberculosis* or purified protein derivative (PPD) to induce a delayed type hypersensitivity reaction on the skin, resulting in a swelling which indicates infection with *M. tuberculosis* (Arend *et al*., 2008). TST have limitations such as low sensitivity and the possibility of yielding false positive results due to the BCG vaccination and also because of infection with NTM from environment (Kong *et al.*, 2005).

Improvements on the TST are two commercially available immunological assays that detect exposure to *M. Mycobacterium*. The Quanti FERON-TB Gold (QFT-G, Celleshs, Carnegi, Australia and the T-SPOT-TB (Oxford Immunotec, Oxford, UK) are *ex vivo* assays that measure the interferon gamma (IFN-γ) (Lalvani, 2007).

2.6.3. Microscopy

The early diagnosis of TB is central to its control. In the poorest parts of the world, where majority of the disease burden lies, the main stay of diagnosis is microscopy. Microscopy of respiratory samples may be performed using either light with a Ziehl-Neelsen or kinyoun stain or fluorescent microscopy with stain such as auramine-O (Mizuno *et al*., 2009). Light

microscopy is easier and cheaper and is used in the diagnosis of TB in many resource poor settings. In addition, it may be performed in areas with no electricity using reflected sunlight. Fluorescent microscopy is more sensitive but conventionally requires an expensive fluorescent microscopy and a dark room. The introduction of low cost fluorescent LED technology has enabled this more sensitive diagnostic tool to be introduced into the areas where it is most needed (Mizuno *et al*., 2009). The sputum microscopy still forms an integral part of the cornerstone for TB control in most low resource high burden TB countries where specialized laboratories for culturing may not be available (Palomino, 2009; Aftab *et al*., 2008). Fluorescence microscopy using auramine-rhodamine staining or the Ziehl-Neelsen (ZN) is the most used approach (Sun *et al*., 2009). Smear microscopy is widely used as a diagnostic test for pulmonary TB worldwide (Palomino, 2009). The method relies on the direct examination of sputum smears for the presence of acid fast bacilli (AFB) in suspected TB patients. Although smear microscopy remains one of the cornerstones for TB diagnosis, the limitations of this method are compounded by low sensitivity and specificity. The method cannot detect a lower bacterial load less than 10 000 cells /ml of the sputum specimen, hence generating false results (Shah and Rauff, 2001).

It has been shown that between 5 000 and 10 000/ ml of sputum are required to allow visualization. This is a far higher number than is required for culture isolation but the detection of a symptomatic patient with a positive pulmonary smear produces the clinician with a presumptive diagnosis and allows the patient to be placed in. Furthermore, this technique cannot differentiate among MTBC as well as distinguishing it from non-tuberculosis mycobacterium (NTM) (Palomino, 2009).

2.6.4. Culture

Culture, although not always positive in patients who are treated for TB, is considered the gold standard for TB diagnosis. Health Protection Agency 2009 showed the United Kingdom data which showed that only 56% of notified TB patients were confirmed with a positive culture (Health Protection Agency, 2009). The culture method is the gold method for TB diagnosis. The method uses the Lowestein-Jensen (LJ) medium which is agar base and Middlebrook's (egg based) medium for propagating *M. tuberculosis* complex in most TB laboratories (Sun *et al*., 2009). Culture's sensitivity is higher than that of microscopy, it is as few as 10 bacilli/ ml which has been detected (Yeager *et al*., 1967).

In addition to the advantage of increased sensitivity, culture should be performed on all specimens to provide definitive species identification, drug susceptibility testing and genotyping. The BACTEC liquid culture system has an advantage over the conventional LJ solid culture technique which is an early detection of the presence of tubercle bacilli in clinical specimens (Lee *et al*., 2011; Polomino, 2009; Yam, 2006). Diagnosis depends upon visual observation of the morphological characteristics such as shape and coloration. *M. tuberculosis* when grown on media appears as small, but colour eugenic colonies (WHO/EMC/ZOO/96.4). This method offers high sensitivity and specificity, but it is very slow, (it takes 3-6 weeks) (Palomino, 2009; Sjobring *et al*., 1990). Culturing of *M. bovis* is performed on LJ medium supplemented with sodium pyruvate, the orgasm do not grow on a culture media containing glycerol. *M. bovis* appears as small, flat and smooth colonies on solid media (WHO/EMC/ZOO/96.4). *M. pinnipedii* diagnosis relies on bacteriological culture in which the isolates dysgonic, rough flat colonies which are non photochromogenic (Cousins *et al*., 2003).

M. africanum when grown on solid culture media produces rough colonies resembling *M. tuberculosis* (Cousins *et al*., 2003). *M. canettii* exhibits smooth, round and glossy colonies with a whitish colour solid LJ or Middlebrook's media (Somoskovi *et al*., 2001). The diagnosis of *M. microti* uses traditional methods which is hampered by the extremely show growth of the pathogen. However, the colonies exhibit an S-shaped morphology which can be used for preliminary identification of the organism (van Soolingen *et al*., 1998).

2.7. Antimicrobial Susceptibility Testing and TB drugs

The prompt detection of drug resistance is central to controlling the disease (TB). A number of assays are in use for the phenotypic detection of drug resistance or susceptibility. The resistance ratio (RR) method is the most accurate at measuring the drug susceptibilities of *M. tuberculosis* (Heifets and Cangelosi, 1999). RR measures the Minimum Inhibitory Concentration (MIC- the lowest concentration of antimicrobial capable of inhibiting growth) of the strain, compared to reference strain $H_{37}Rv$ when measured in the same experiment. The MIC of tested strain is divided by the MIC of $H_{37}Rv$. If the RR is 2 or less the strain is susceptible. An RR of 8 and higher indicates resistance. The modified proportion method is less labour expensive and may utilize automated liquid culture systems (Pfyffer *et al*., 2002).

The strain tested for resistance is incubated in the presence of first line drugs. A growth control (1:1000 dilution of the strain) is incubated in the absence of drugs. Should growth be detected in any of the drug-containing tubes prior to, or at the same time as the growth control, this indicates 1% of the bacterial population is resistant to that agent. The phenotypic detection of antimicrobial susceptibilities is the gold-standard, but this takes several days following the isolation of the organism. Microscopic-observation drug-susceptibility assays (MODS) allow the growth of resistant organisms in the presence of anti-tuberculosis drugs to

be visualised. This assay may be performed directly on clinical samples and has shown some potential particularly in resource poor settings (Moore *et al*., 2006).

There are several strategies that *Mycobacterium tuberculosis* complex use to resist the action of antimicrobial agents. The mycobacterial cells have decreased permeability (Lee *et al*., 1996; Nikaido, 1994). *M. tuberculosis* has been found to be active drug efflux systems, and the genes that are associated with these functions (Cole *et al*., 1998; Kwon *et al*., 1995). Genetic studies have shown that resistance of *M. tuberculosis* to anti-microbacterial drugs is the consequence of spontaneous mutations in genes that encode either the target of the drug, or enzymes that are involved in drug activation. After the first anti-TB drugs were introduced, Streptomycin (STR), para-aminosalicylic acid (PAS), Isoniazid (INH) resistance to these drugs was observed in clinical isolates of *M. tuberculosis* (Johnson *et al*., 2009). This therefore brought a need to measure resistance accuracy and easily led by this.

In 1961 the Pasteur institute introduced the critical proportion for drug susceptibility testing in TB and this method became the standard method of use (Espinal, 2003). In the 1960s, studies on drug resistance in various countries had a much higher incidence of drug resistance than developed countries (Espinal, 2003). Rifampicin (RIF) was introduced and with the use of combination therapy by the end of 1960s. Drug resistant and drug susceptible TB in developed countries declined, and this led to a decline in funding and interest in TB control programs and there was no concrete monitoring of drug resistance carried out for about 20 years (Espinal, 2003). In the 1980s, the arrival of HIV/AIDS resulted in an increase of Drug-resistant TB (MDR-TB) (Fischl *et al*., 1992) i.e. resistant to INH and RIF. Anti-TB antibiotics by some MTBC strains is a serious threat to public health, which has impended effective control of TB worldwide (Ani *et al*., 2009; Assam-Assam *et al*., 2009). Culturing

and drug susceptibility (DST) are commonly used for detecting drug resistance (Miotto *et al*., 2008).

Drug susceptibility testing is carried out on sub-cultured bacteria after the initial positive culture is obtained for diagnosis. It usually takes 3-6 weeks to obtain the initial positive culture with an additional 3 weeks susceptibility testing (reduced to about 15 days when using the BACTEC system). Susceptibility testing is time consuming and costly and there are numerous problems associated with standardization of tests and the stability of drugs in different culture media (Moskrousovi *et al*., 2002). The slow diagnosis of drug resistance may be a major contributor to the transmission of MDR-TB (Victor *et al*., 2001). MDR-TB detection methods currently available such as INNO-LiPA Rif.TB kit for detecting *rpoB* mutations are expensive and require sophisticated equipment and highly skilled personnel (Nikolayesky *et al*., 2004; Moskrousov *et al*., 2002).

2.8. Molecular diagnosis

2.8.1. Approaches to diagnosis

The approaches to diagnosis includes GenoType MTBDR test which is able to detect mutations in the *rpoB* gene for RIF resistance, and the most frequent mutation at codon 315 of the *katG* gene for INH resistance, either in isolates or clinical samples. The assay is said to have a specificity and sensitivity for RIF resistance nearly 100% for INH-resistance the sensitivity of the test is said to range from 70%-90% depending on the prevalence of the particular mutation at the *katG* locus (Hilleman *et al*., 2007) and a high specifity of nearly 100%.

The GenoType MTBDR*plus* (Hain Life Science) assay is said to be advanced and it uses probes for identification of other mutations in the region of the *rpoB* gene for RIF resistance, and probes to detect mutations in the promoter region of *inhA* gene involved in INH resistance. This assay has improvements which are implemented by the use of line probe assays which are accurate and useful for rapid detection of drug resistance directly in clinical specimen. The assay also have disadvantages which includes that the number of genes that can be analyzed remains limited and the test fails to distinguish insertion mutation. They also retain a lower sensitivity among acid fast bacilli-negative sample and the assay line probe is expensive (Therese, 2012). There is the DNA sequencing method, which is the gold standard for sequencing DNA of PCR amplified products. It is the most widely used method; it is accurate and reliable. It has been widely used for characterizing mutations in the *rpoB* gene in rifampicin-resistant strains and to detect mutations responsible for resistance to other anti-tuberculosis drugs (Therese, 2012).

2.8.2. Nucleic Acid Amplification (Polymerase Chain Reaction-PCR)

Several molecular tools have become available over the past 15 years to aid in the rapid diagnosis of TB. Polymerase chain reaction (PCR) line emerged as a valuable alternative tool for rapid diagnosis of TB with increased sensitivity and specificity (Palomino, 2009). This molecular techniques targets to amplify *M. tuberculosis* DNA to aid diagnosis, it relies on amplification of target genes specific to the *M. tuberculosis* complex. The insertion element IS*6110* found exclusively in the DNA of MTBC is the most targeted genes. The method said to be reliable for rapid TB diagnosis because of its reports from different studies that says it has high specificity and sensitivity (Palomino, 2009). In most laboratories in developed countries commercially available PCR methods have been used for TB diagnosis. The use of PCR based has brought unprecedented reliability for rapid diagnosis of TB, commercially

available diagnostic kits are costly and also require sophisticated equipment which may be unaffordable for routine in laboratories in most low resource settings (Palomino, 2009; Sun *et al*., 2009).

2.8.3. Region of differences analysis (RD)

Region of differences analysis is a technique that differentiates amongst the different TB causing species and it relies on the variability of several genomic regions in the MTBC (Huard *et al*., 2003). Several experiments have used different DNA hybridization techniques to study the genome of *M. tuberculosis* and the studies have revealed several variable regions in the DNA of MTBC. Genomic comparative studies of *M. bovis* BCG and *M. tuberculosis* H37Rv have concluded that there was availability in genomic regions of these MTBC members (Parsons *et al*., 2002). From the findings based on the genomic comparative studies showed that RD analysis has been widely used to differentiate MTBC (Huard *et al*., 2003). It has been reported that there are 16 RD deletions in *M. bovis* BCG compared to other MTBC (Parsons *et al*., 2002).

2.9 Investigations presented in this dissertation

This dissertation is divided into 6 chapters: Chapter 1 provides the introduction and background of the study; Chapter 2 provides the review of literature of the study. The next successive chapters deal with specific issues, which this work set out to investigate. Chapter 3 deals with the identification of MTBC DNA isolates from NHLS in Port Elisabeth, South Africa. This will also include factors such as the prevalence of *Mycobacterium tuberculosis* complex (MTBC) amongst different villagers in Port Elizabeth, South Africa. The differentiation of the *Mycobacterium tuberculosis* complex is presented in Chapter 4.

There is a paucity of information on the molecular epidemiology of resistant MTBC from NHLS in Port Elizabeth, South Africa. Molecular characterization using sequencing of resistant MTBC isolates is investigated in Chapter 5. This chapter provides information on the genetic diversity of multidrug resistant MTBC isolates as well as the potential impact on the clinical management of patients. Lastly is chapter 6, a general overview of the entire study is made where the main findings are highlighted in a holistic context, with recommendations and future prospects. References are also included at the end of the dissertation.

REFERENCES

Abbadi S, Rashed H.G, Morlock G.P, Woodley C.L, Shanawy O & Cooksey R.C (2001). Characterization of IS*6110* restriction fragment length polymorphism patterns and mechanisms of antimicrobial resistance for multidrug-resistant isolates of *Mycobacterium tuberculosis* from a major reference hospital in Assiut, Egypt. Journal of Clinical Microbiology. 39:2330– 2334.

Aftab R, Amjad F, Khurshid R & Ahmed N (2008). Detection of *Mycobacterium tuberculosis* in clinical samples by smear and culture. Rawal Medical Journal. 33:134-136.

Alexander K.A, Laver P.N, Michel A.L, Williams M, van Helden P.D & Gey van Pittius N.C (2010) Novel *Mycobacterium tuberculosis* complex pathogen, *M. mungi.* Emergency Infectious Disease. 16:1296-1299.

Allix C, Philip S & Maryse F (2004). Utility of fast mycobacterial interspersed repetitive unit-variable number tandem repeat genotyping in clinical mycobacteriological analysis. Clinical Infectious Diseases. 39:783–789.

Ameni G, Aseffa A, Engers H, Young D, Hewinson G & Vordermeier M (2011). Cattle husbandry in Ethiopia is a predominant factor affecting the pathology of bovine tuberculosis

and gamma interferon responses to mycobacterial antigens. Clinical and Vaccine Immunology. 13:1030–1036.

Angela D.P, Giuseppuna C, Tong F.V, Bijo B, Fatmira S & Giuseppina T (2006). Detection of *Mycobacterium tuberculosis* complex in milk using polymerase chain reaction (PCR). Food Control. 17:776-780.

Ani A, Bruvik T, Okoh Y, Agaba P, Agbaji O & Dahle U.R (2010). Genetic diversity of *Mycobacterium tuberculosis* complex in Jos, Nigeria. BMC Infectious Diseases. 10:189. doi 10.1186/1471-2334-10-189.

Ani A.E, Idoko J, Dalyop Y.B & Pitmang S.L (2009). Drug resistance profile of *Mycobacterium tuberculosis* isolates from pulmonary tuberculosis patients in Jos, Nigeria. Tropical Medicine and Hygiene. 103:67-71.

Aranaz A, Cousins D, Mateos A & Dominguez L (2003). Elevation of *Mycobacterium tuberculosis* subsp. caprae to species rank as *Mycobacterium caprae* comb. nov., sp. nov. Journal of Systematic and Evolutionary Microbiology.53:1785-1789.

Arend S.M, Franken W.P.J, Aggerbeck H, Prins C, van Dissel J.T, Carstensen B.T. *et al.* 2008). Double-blind randomized Phase I study comparing rdESAT-6 to tuberculin as skin test reagent in the diagnosis of tuberculosis infection. Tuberculosis. 88:249-261.

Aslan G, Tezcan S, Serin M.S & Emekdas G (2008). Genotypic analysis of isoniazid and rifampin resistance in drug resistant. Clinical *Mycobacterium tuberculosis* complex isolates in Southern Turkey. Journal of Infectious Disease. 61:255-260.

Assam Assam J.P, Penlap V.B, Cho-Ngwa F, Tedom J.C, Anyangwe I & Titanji, V.P (2009). *Mycobacterium tuberculosis* complex drug resistance pattern and identification of species causing TB in the West and Centre regions of Cameroon. BMC Infectious Diseases. 11:94-100.

Barnard M, Albert H, Coetzee G, O'Brien R & Bosman M.E (2008). Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. American Journal of Respiratory and Critical Care. 177:787-792.

Bezos J, de Tuan L, Romeo B, Alvarez J, Mazzucchdli F, Melzos A. *et al.* (2010). Experimental Infection with *Mycobacterium caprae* in goats and evaluation of immunological status in tuberculosis and paratuberculosis co-infected animals. Journal of Veterinary Immunology and immunopathology. 133:269-275.

Bigi F, Garcia-Pelayo M.C, Nunez-Garcia J, Peralta A, Caimi K.C, Golby P. *et al.* (2005): Identification of genetic markers for *Mycobacterium pinnipedii* through genome analysis. FEMS Microbiology Letters 248:147–152.

Brosch R, Gordon S.V, Marmiesse M. Brodin P, Buchrieser C, Eiglmeier K. *et al*. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proceedings of National Academy of Science USA, 99:3684–3689.

Brudey K, Driscoll J.R, Rigouts L, Prodinger W.M, Gori A, Al-Hajoj S.A. *et al*. (2006). *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC* Microbiology. 6:23.

Brzostek A, Sajduda A, Sliwinski T, Augustynowicz-Kopec E, Jaworski A & Dziadek, J (2004). Molecular characterisation of streptomycinresistant *Mycobacterium tuberculosis* strains isolated in Poland. International Journal of Tuberculosis and Lung Disease. 8:1032–1035.

Cadmus S, Palmer S, Okker M, Dale J, Gover K, Smith N. *et al*. (2006). Molecular analysis of human and bovine turbecle bacilli from a local setting in Nigeria. Journal of Clinical Microbiology. 44(1):29-34.

Cadmus S.I.B, Adesokan H.K, Adedokun B.O & Stack, J.A (2010). Seroprevalence of bovine brucellosis in trade cattle slaughtered in Ibadan, Nigeria, from 2004–2006. Journal of the South African Veterinary Association. 81:50–53.

Campbell P.J, Morlock G.P, Sikes R.D, Dalton T.L, Metchock B, Starks A.M. *et al*. (2011). Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 55(5):2032–2041.

Castets M, Boisvert H, Grumbach F, Bruned M & Rist M (1968). Tuberculosis bacilli of the African type; preliminary note. Revesized Tuberculosis pneumology (paris).

Chan E.D, Laurel V, Strand M.J, Chan J.F, Huynh M.L & Iseman M.D (2004). Treatment and outcome analysis of 205 patients with multidrug-resistant tuberculosis. American Journal of Respiratory and Critical Care. 169:1103-1109.

Chen Y, Chao Y, Deng Q, Liu T, Xiang J, Chen J. *et al*. (2009). Potential challenges to the stop TB plan for humans in China; Cattle maintain *M. bovis* & *M. tuberculosis*. Journal of Tuberculosis. 89:95-100.

Cheng A.F, Yew W.W, Chan E.W, Chin M.L, Hui M.M & Chan R.C (2004). Multiplex PCR amplimer conformation analysis for rapid detection of gyrA mutations in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrobial Agents Chemotherapy. 48:596–601.

Cho E.H, Bae H.K, Kang S.K & Lee E.H (2009). Detection of isoniazid and rifampicin resistance by sequencing of *katG*, *inhA*, and *rpoB* genes in Korea. Korean Journal of Laboratory Medicine. 29:455-60.

Christianson S, Wolfe J, Orr P, Karlowsky J, Levett P.N, Horsman G.B, Thibert L. *et al*. (2010). Evaluation of 24 locus MIRU-VNTR genotyping of *Mycobacterium tuberculosis* isolates in Canada. Tuberculosis. 90:31-38.

Cockle P.J, Gordon S.V, Hewinson R.G, & Vordermeier H.M (2006). Field evaluation of a novel differential diagnostic reagent for detection of *Mycobacterium bovis* in cattle. Journal of Clinical and Vaccine Immunology. 13(10):1119-1124.

Cole S.T, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D. *et al*. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. 393:537-544.

Collins M.D (2011). Advances in molecular diagnostics for *Mycobacterium bovis*. Veterinary Microbiology. doi:10.1016/j.vetmic.2011.02.019. 67.

Cooksey R.C, Morlodi G.P, McQuen A, Glickman S.E & Crawfor J.T (1996). Characterization of streptomycin resistance mechanisms amoung *Mycobacterium*

tuberculosis isolates from patients in New York City. Antimicrobial Agents and Chemotherapy. 40(5):1186-1188.

Coros A, Deconno E. & Derbyshire K (2008). IS*6110*, a *Mycobacterium tuberculosis* complex Specific Insertion Sequence, Is also present in the genome of *Mycobacterium* of *Smegmatis* suggestion of lateral gene Transfer among Mycobacterial species. Journal of Bacteriology. 190(9):3408-3410.

Cosivi O, Meslin F.X, Daborn C.J & Grange J.M (1998). Epidemiology of Mycobacterium bovis infection in animals and humans, with particular reference to Africa. Revised Science of Technology. 14(3):733–746.

Cousins D.V, Bastida R, Cataldi A, Viviana Q, Redrobe S, Dow S. *et al.* (2003). Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. Journal of Systematic and Evolutionary Microbiology. 53:1305-1314.

Cousins D.V, Williams S.N, Reuter R, Forshow D, Chadwick B & gakes N (1993). Tuberculosis in wild seals and characterization of the seal bacillus. Australian Veterinary Journal. 70:92-97.

Da Silva P.E.A & Palomino J.C (2011). Molecular basis and mechanisms of drug resistance: strategies, sources and newmolecules. Current Medicinal Chemistry. 16:1898–1904.

Dale J.W, Brittain D, Cataldi A.A, Cousins D, Crawford J.T, Driscoll J. *et al*. (2001). Spacer oligonucleotide typing of bacteria of the *Mycobacterium tuberculosis* complex: recommendations for standardised nomenclature. International Journal of Tuberculosis and Lung Disease. 5:216-219.

de Jong B.C, Antonio M & Gagneux S (2010). *Mycobacterium africanum* - Review of an important cause of human tuberculosis in West Africa. PLoS Neglected Tropical Diseases 4(9):e744.

Deforges L, Boulouis H.J, Thibaud J.L, Boulouha L, Souigakoff W, Blot S. *et al*. (2004). First isolation of *Mycobacterium microti* (Llama-type) from a dog. Veterinary Microbiology. 103:249-253.

Demers A.M, Mostowy S, Coetzee D, Warren R, van Helden P & Behr M.A (2010). *Mycobacterium africanum* is not a major cause of human tuberculosis in Cape Town, South Africa. Tuberculosis. 90:143-144.

Dheda K, Shean K, Zumla A, Badri M, Streicher E.M, Page-Shipp L. *et al.* (2010). Early treatment outcomes and HIV status of patients with extensively drug-resistant tuberculosis in South Africa: a retrospective cohort study. Lancet, 22:1798-1807.

Dou H.Y, Lu J.J, Lin C.W, Chang J.R, Sun J.R & Su I.J (2008). Utility and evaluation of new variable–number tandem-repeat systems for genotyping mycobacterial tuberculosis isolates. Journal of Microbiological Methods. 77:127-129.

Ducati R.G, Ruffino-Netto A, Basso L.A & Santos D.S (2006). The resumption of consumption - a review on tuberculosis. Memorias Instituto Oswaldo Cruz. 101:697-714.

Eisenach J.C, Lysak S.Z & Viscomi C.M (1988). Epidural clonidine analgesia following surgery: phase I. Anesthesiology. 71:640 -646.

Engström A, Perskvist N, Werngren J, Hoffner S.E, Jureen P (2011). Comparison of isolates and in vitro selected mutants reveals that tlyA is not a sensitive genetic marker for capreomycin resistance in *Mycobacterium tuberculosis*. Journal of Antimicrobacterium chemotherapy. 66:1247-1254.

Espinal M.A (2003). The global situation of MDR-TB. Tuberculosis. (Edinb.) 83:44–51.

Evans J.T, Gibson A, Khanom S, Overton-Lewis C, Smith E.G & Hawkey P.M (2012) Introduction of 24-loci MIRU-VNTR typing in a region-wide scheme considerably reduces the number of cases requiring epidemiological investigation. Clinical Microbiology and Infect. 17: In press.

Fabre M, Hauck Y, Soler C, Koeck J.L, Van Ingen J, van Sloongen D. *et al.* (2010). Molecular characteristics of *"Mycobacterium canettii''* the smooth *Mycobacterium tuberculosis* bacilli. Journal of Infection, Genetics and Evolution. doi: 10.1016/j.meegid

Filliol I, Driscoll J.R, Van Soolingen D, Kreiswirth B.N, Kremer K, Valetudie G. *et al.* (2002). Global distribution of *Mycobacterium tuberculosis* spoligotypes. Emerging Infectious Diseases. 8:1347-1349.

Filliol I, Driscoll J.R, van Soolingen D, Kreiswirth B.N, Kremer K, Valetudie G. *et al.* (2003). Snapshot of moving and expanding clones of *Mycobacterium tuberculosis* and their global distribution assessed by spoligotyping in an international study. Journal of Clinical Microbiology. 41:1963-1970.

Fischl M.A, Uttamchandani R.B, Daikos G.L, Poblete R.B, Moreno J.N, Reyes R.R. *et al.* (1992). An outbreak of tuberculosis caused by multiple-drug-resistant tubercle bacilli among patients with HIV infection. Annals of International Medicine. 117:177–183.

Friedland G, Harries A & Coetzee D (2007). Implementation issues in tuberculosis/HIV program collaboration and integration: 3 case studies. The Journal of Infectious Diseases. 196:S114-S123.

Frothingham R & Meeker-O'Connell W.A (1998). Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology. 144:1189-1196.

Gandhi N.R, Moll A, Sturm A.W, Pawinski R, Govender T, Lalloo U. *et al*. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 368:1575-1580.

Gibson A.L, Hewinson G, Goodchild T, Watt B, Story A & Drobniewski F.A (2004). Molecular epidemiology of disease due to *Mycobacterium bovis* in humans in the United Kingdom. Journal of Clinical Microbiology. 42(1):431-434.

Ginsburg A.S, Grosset J.H & Bishai W.R (2003). Fluoroquinolones, tuberculosis, and resistance. Lancet Infectious Disease. 3:432–442.

Godreuil S, Torrea G, Terru D, Chevenet F, Diagbouga S, Supply P. *et al.* (2007). First molecular epidemiology study of *Mycobacterium tuberculosis* in Burkina Faso. Journal of Clinical Microbiology. 45:921-927.

Goh K.S, Fabre M, Huard R.C, Schmid S, Sola C & Rastogi N (2006). Study of the gyrB gene polymorphism as a tool to differentiate among *Mycobacterium tuberculosis* complex subspecies further underlines the older evolutionary age of 'Mycobacterium canettii'. Molecular and Cellular Probes. 20:182-190.

Goyal M, Saunders N.A, van Embden J.D, Young D.B & Shaw R.J (1997). Differentiation of *Mycobacterium tuberculosis* isolates by spoligotyping and IS*6110* restriction fragment length polymorphism. Journal of Clinical Microbiology. 35:647-651.

Green E, Obi L.C, Nchabeleng M, de Villiers B.E, Sein P.P, Letsoalo T. *et al.* (2008). Molecular characterisation of resistant Mycobacterium tuberculosis isolates from Dr George Mukhari Hospital, Pretoria, South Africa. South African Journal of Epidemiology Infection. 23(3):11-14.

Green E, Obi L, Nchabeleng M, de Villiers B.E, Sein P.P, Letsoalo T, Hoosen A.A. *et al.* (2010). Drug-susceptibility patterns of *Mycobacterium tuberculosis* in Mpumalanga Province, South Africa: Possible guiding design of retreatment regimen. Journal of Health Population and Nutrition. 28(1):7-13.

Groenen P.M, Bunschoten A.E, van Soolingen D & van Embden J.D (1993). Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. Molecular Microbiology. 10:1057-1065.

Hauck M, Jürgens S-R, Willenbruch K, Huneck S & Leuschner C (2009).Dissociation and metal-binding characteristics of yellow lichen substances suggest a relationship with site preferences of lichens. Annals of Botany.103:13-22.

Hall N (2007). Advanced sequencing technologies and their wider impact in microbiology. Journal of Experimental Biology. 210:1518–1525.

Health Protection Agency Annual Report and Accounts (2009). The Health Protection Agency's Annual Report and accounts for the financial year 2008/09.

Heifets L.B, & Cangelosi G.A (1999). Drug susceptibility testing of *Mycobacterium tuberculosis:* a neglected problem at the turn of the century. International Journal of Tuberculosis and Lung Disease. 3(7):564–581.

Hermans P.W, van Soolingen D, & Dale J.W *et al*. (1990). Insertion Element IS986 from *Mycobacterium tuberculosis*: a Useful Tool for Diagnosis and Epidemiology of Tuberculosis, Journal of Clinical Microbiology. 28(9):2051–2058.

Hermans P.W.M, van Soolingen D, Bik E.M, Haas P.E.W, Dale J.W & van Embden J.D (1991). Insertion element IS*987* from *Mycobacterium bovis* BCG is located in a hot-spot

integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. Infection and Immunology. 59:2695-2705.

Hermans P.W, van Soolingen D & van Embden J.D (1992). Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae*. Journal of Bacteriology. 174:4157-4165.

Hillemann D, Rusch-Gerdes S & Richter E (2007). "Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens". Journal of Clinical Microbiology. 45(8):2635-2640.

Huard R.C, de Oliveira Lazzarini L.C, Butler W.R, van Soolingen D & Ho J.L (2003). PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis c*omplex on the basis of genomic deletions. Journal of Clinical Microbiology. 41(4):1637-1650.

Huard R.C, Fabre M, de Haas P, Lazzarini L. C, van Soolingen D & Ho J.L (2006). Novel genetic polymorphisms that further delineate the phylogeny of the Jeon B.Y, Kim S.C, Je S, Kwak J, Cho J.E, Woo J.T, Seo S, Shim H.S, Park B.O, Lee S.S & Cho S.N (2010). Evaluation of enzyme-linked immunosorbent assay using milk samples as a potential

screening test of bovine tuberculosis of dairy cows in Korea. Journal of Research in Veterinary Science. 88:390-393.

Hutchison III C.A (2007). DNA sequencing: bench to bedside and beyond. Nucleic Acids Research. 35:6227–6237

Jeon B.Y, Kim S.C, Je S, Kwak J, Cho J.E, Woo J.T. *et al*. (2010). Evaluation of enzyme-linked immunosorbent assay using milk samples as a potential screening test of bovine tuberculosis of dairy cows in Korea. Journal of Research in Veterinary Science. 88:390-393.

Johansen S.K, Maus C.E, Plikaytis B.B, Douthwaite S (2006). Capreomycin binds across the ribosomal subunit interface using tly-A encoded 2'-0 methylations in 16S and 23S rRNA. Molecular cell. 23:173-182.

Johnson R, Streicher E.M, Louw G.E, Warren R.M, van Helden P.D & Victor T.C (2009). Drug resistance in *Mycobacterium tuberculosis*. Molecular Biology. 8:97-112.

Johnson K & Schultz P.G (1994). Mechanistic studies of the oxidation of isoniazid by the catalase peroxidase from Mycobacterium tuberculosis. Journal of American Chemical Society. 116:7425–7426.

Jones W.D & Greenberg J (1978). Modification of methods used in bacteriophage typing of *Mycobacterium tuberculosis* isolates. Journal of Clinical Microbiology. 7:467-469.

Jonmalung J, Prammananan T, Leechawengwongs M & Chaiprasert A (2010). Surveillance of pyrazinamide susceptibility among multidrug-resistant *Mycobacterium tuberculosis* isolates from Siriraj Hospital, Thailand. BMC Microbiology. 10:223-228.

Kallenius G, Hoffner S.E, Miorner H & Svenson S.B (1999). Novel approaches to the diagnosis of mycobacterial infections. The European Respiratory Journal. 7:1921-1924.

Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S. *et al.* (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. Journal of Clinical Microbiology. 35:907-914.

Kasai H, Ezaki T & Harayama S (2000). Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. Journal of Clinical Microbiology. 38:301-308.

Kiers A, Klarenbeek A, Mendelts B, Van Soolingen D & Koeter G (2008). Transmission of *Mycobacterium pinnipedii* to humans in a zoo with marine mammals. Journal of Tuberculosis and Lung Disease. 12(12):1469-1473.

Kim D.H, Kim H.J, Park S.K, Kong S.J, Kim Y.S, Kim T.H. *et al.* (2008). Treatment outcomes and long term survival in patients with extensively drug-resistant tuberculosis. American Journal of Respiratory and Critical Care Medicine. 178:1075-1082.

Kometsevaya I, Mironova S, Nikalayevskyy V, Balabanova Y, Michell S & Drobniewski F (2011). Evaluation of two molecular assays for rapid detection of *Mycobacterium tuberculosis* resistance of fluoroquinolones in high-tuberculosis and multidrug-resistance settings. Journal of Clinical Microbiology. 49:2832-2837.

Kong Y, Cave M.D, Zhang L, Foxman B, Marrs C.F & Yang Z.H (2005). Population-based study of deletions in five different genomic regions of *Mycobacterium tuberculosis* and possible clinical relevance of the deletions. Journal of Clinical Microbiology. 44:3940-3946.

Kremer K, Au B.K.Y, Vip P.C.W, Skuce R, Supply P & van Soolingen D (2005). Uses of variable number tandem-repeat typing of differentiate *Mycobacterium tuberculosis* Beijing family isolates from Hong Kong and comparison with IS*6110* restriction fragment length polymorphism typing and Spoligotyping. Journal of Clinical Microbilogy. 43(1):314-320.

Kremer K, van Soolingen D, van Embden J, Hughes S, Inwald J & Hewinson G (1999). *Mycobacterium microti*: More widespread than previously thought. Journal of Clinical Microbiology. 36(9):2793-2794.

Kremer K, van Soolingen D, van Embden J, Hughes S, Inwald J & Hewinson G (1998). *Mycobacterium microti*: More widespread than previously thought. Journal of Clinical Microbiology. 36(3):2793-2794.

Kwara A, Schiro R, Cowan L.S, Hyslop N.E, Wiser M.F, Roahen Harrison S. *et al.* (2003). Evaluation of the epidemiologic utility of secondary typing methods for differentiation of *Mycobacterium tuberculosis* isolates. Journal of Clinical Microbiology. 41:2683-2685.

Kwon H.H, Tomioka H & Saito H (1995). Distribution and characterization of beta-lactamases of mycobacteria and related organisms. Tuberculosis of Lung Disease. 76:141–148.

Lalvani A (2007). Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. Chest. 131(6):1898-1906.

Lee J.E, Kim H.J & Lee S.W (2011). The clinical utility of tuberculin skin test and interferon-g release assay in the diagnosis of active tuberculosis among young adults: A prospective observational study. BMC Infectious Diseases. 11:96-103.74.

Lee R.E, Brennan P.J & Besra G. S (1996). *Mycobacterium tuberculosis* cell envelope. Occurrence Top Microbiology Immunology. 215:1–27.

Leimane V, Riekstina V, Holtz T.H, Zarovska E, Skripconoka V, Thorpe L.E. *et al.* (2005). Clinical outcome of individualised treatment of multidrug-resistant tuberculosis in Latvia: a retrospective cohort study. Lancet. 365:318-326.

LoBue P.A, Enarson D.A & Thoen C.O (2010). Tuberculosis in humans and animals: an overview. Journal of Tuberculosis and Lung Disease. 14(9):1075-1078.

Louw C, Young P.R, Van Rensburg P & Divol B (2009). Regulation of endo-polygalacturonase activity in *Saccharomyces cerevisiae*. FEMS Yeast Research. 10:1111.

Lyashchenko K, Whelan A.O, Greenwald R, Pollock J.M, Andersen P, Hewinson R. *et al.* (2004). Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis*. Journal of Infection and Immunity. 72(5):2462-2467.

Mathema B, Kurepina N.E, Bifani P.J & Kreiswirth B.N (2006). Molecular epidemiology of tuberculosis: current insights. Clinical Microbiology Reviews. 19(4):658-685.

Maus C.E, Plikaytis B.B, Shinnick T.M (2005). Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin and viomycin in *Mycobacterium tuberculosis*. Antimicrobiological Agents and Chemotherapy. 49:3192-3197.

Mc Adam R.A, Hermans P.W, van Soolingen D, Zainuddin Z.F, Catty D & Dale J.W (1990). Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. Molecular Microbiology. 4:1607-1613.

Mierer A, Sander P, Schaper K.J, Scholz M & Bottger E.C (1996). Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 40(11):2452-2454.

Migliori G.B, Besozzi G, Girardi E, Kliiman K, Lange C, Toungoussova O.S. *et al.* (2007). Clinical and operational value of the extensively drug-resistant tuberculosis definition. European Respiratory Journal. 30:623-626.

Miotto P, Saleri N, Dembele M, Ouedraogo M, Badoum G, Pinsi G. *et al.* (2008). Molecular detection of rifampin and isoniazid resistance to guide chronic TB patient management in Burkina Faso. BMC Infectious Diseases. 9:142-150.

Mizuno M, Suzuki M, Matsumoto K & Murakami M (2009). Clinical practice and research activities for early psychiatric intervention at Japanese leading centres. Early Intervention in Psychiatry 3(1):5-9.

Mlambo C.K, Warren R.M, Poswa X, Victor T.C, Duse A.G & Marais E (2008). Genotypic diversity of extensively drug-resistant tuberculosis (XDR-TB) in South Africa. International Journal of Tuberculosis and Lung Disease. 12:99-104.

Moore D.A, Evans C.A, Gilman R.H, Caviedes L, Coronel J, Vivar A. *et al.* (2006). Microscopic-observation drug-susceptibility assay for the diagnosis of TB. New England Journal of Medicine. 355:1539-1550.

Moskrousovi I, Filliol I, Legrand E, Sola C, Otten T, Vyshnevskaya E. *et al*. (2002). Molecular characterization of multiple-drug-resistant *Mycobacterium tuberculosis* isolates from north-western Russia and analysis of rifampin resistance using RNA/RNA mismatch analysis as compared to the line probe assay and sequencing of the *rpoB* gene. Research in Microbiology. 153: 213-219.

Mphahlele M, Syre H, Valvatne H,Stavrum R, Mannsaker T, Muthivhi T. *et al.* (2008). Pyrazinamide resistance among South African multidrug-resistant *Mycobacterium tuberculosis i*solates. Journal of Clinical Microbiology. 46(10):3459-3464.76.

Neonakis I.K, Gitti Z, Krambovitis E & Spandidos D.A (2008). Molecular diagnostic tools in mycobacteriology. Journal of Microbiology Methods. 75:1-11.

NICE. NICE Tuberculosis Guidelines (2006). National Institute for Health and Clinical Excellence. Clinical diagnosis and management of tuberculosis, and measures for its prevention and control.

Niemann S, Koser C.U, Gagneux S, Plinke C, Homolka S, Bignell H. *et al*. (1993). Genomic diversity among drug sensitive and multidrug resistant isolates of *Mycobacterium tuberculosis* with identical DNA fingerprints. PLoS one 4:e7407.

Niemann S, Richter E & Rüsch-Gerdes S (2000). Differentiation among members of the *Mycobacterium tuberculosis* complex strains by *gyrB* DNAsequence polymorphism analysis. Journal of Clinical Microbiology. 38:3231-3234.

Niemann S, Rusch-Gerdes S, Joloba M.L,Whalen C.C, Guwatudde D, Ellner J.J. *et al*. (2002). *Mycobacterium africanum s*ubtype II is associated with two distinct genotypes and is a major cause of human tuberculosis in Kampala, Uganda. Journal of Clinical Microbiology. 40(9):3398-3405.

Nikaido H, Kim S.H & Rosenberg E.Y (1994) Physical organization of lipids in the cell wall of Mycobacterium chelonae. Molecular Microbiology. 8:1025–1030.

Nikolayevsky V, Brown T, Balabanova Y, Fedorin R.M & Drobniewski F (2004). Detection of mutations associated with isoniazid and rifampin resistance in *Mycobacterium tuberculosis*

isolates from Samara Region, Russian Federation. Journal of Clinical Microbiology. 42(10):4498-4502. 77.

O'Donnell M.R, Padayatchi N, Master I, Osburn G & Horsburgh C.R (2009). Improved early results for patients with extensively drug-resistant tuberculosis and HIV in South Africa. International Journal of Tuberculosis and Lung Disease. 13:855-861.

Onipede A.O, de Jong B & Adegbola R.A (2005). *Mycobcaterium africanum*: A review. Journal of Clinical and Experimental Microbiology. 6(2):167-198.

Padayatchi N, Stiefvater E, Naidoo K, Naidoo K, Ndung'u T & Abdool Karim Q (2009). Expanding HIV surveillance to include TB patients in resource-limited settings with a generalized epidemic. International Journal of Tuberculosis and Lung Disease. 13:1447-1449.

Palomino J.C (2009). Molecular detection, identification and drug resistance detection in *Mycobacterium tuberculosis*. Journal of Immunology and Medical Microbiology. 2:1-3.

Parsons L.M, Brosch R, Cole S.T, Somoskovi A, Loder A, Bretzel G. *et al*. (2002). Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. Journal of Clinical Microbiology. 40(7):2339-2345.

Pepper T, Joseph P, Mwenya C, McKee G.S, Haushalter A, Carter A. *et al.* (2008). "Normal chest radiography in pulmonary tuberculosis: implications for obtaining respiratory specimen cultures", International Journal of Tuberculosis and Lung Disease. 12(4):397-403.

Pfyffer G. E & F. Palicova (2002). *Mycobacterium*: General characteristics, laboratory detection, and staining procedures. In: P. R. Murray *et al.* (eds.), Manual of Clinical Microbiology, 10th ed. American Society for Microbiology Press, Washington, D.C. (in press).

Pfyffer G.E, Auckenthaler R, van Embde, J.D & van Soolingen D (1998). *Mycobacterium canettii*, the smooth variant of *M. tuberculosis*, isolated from a Swiss patient exposed in Africa. Emerging Infectious Diseases. 4:631-634.

Piton J, Petrella S, Dalarue M, Andre-Leroux G, Jarlier V, Aubry A. *et al.* (2010). Structural insights into the quinolone resistance mechanism of *Mycobacteriom tuberculosis* DNA gyrase. PLoS one 5e12245.

Prammananan T, Sander P, Brown B.A, Frischkorn K, Onyi G.O, Zhang Y. *et al.* (1998). A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in *Mycobacterium abscessus* and *Mycobacterium chelonae*. Journal of Infectious Disease. 177:1573-1581.

Prodinger W.M, Brandstatter A, Naumann L, Pacciarini M, Kubica T, Boschiroli M.L. *et al*. (2005). Characterization of *Mycobacterium caprae* isolates from Europe by mycobacterial interspersed repetitive unit genotyping. Journal of Clinical Microbiology. 43(10):4984-4992.

Ramaswamy S & Musser J.M (1998). Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuberculosis and Lung Disease. 79(1):3-29.

Rattan L, Awdhesh K &Nishat A (1998) Multidrug-Resistant *Mycobacterium tuberculosis*: Molecular perspectives. Emerging Infectious Disease. 4:195-209.

Richeldi L (2006). An update on the diagnosis of tuberculosis infection. American Journal of Respiratory and Critical Care Medicine 174(7):736-742.

Richter E, Weizenegger M, Fahr A & Rüsch-Gerdes S (2004). Usefulness of the GenoType MTBC Assay for differentiating species of the *Myobacterium tuberculosis* complex in cultures obtained from clinical specimens. Journal of Clinical Microbiology. 4303-4306.

Richter E, Weizenegger M, Rüsch-Gerdes S & Niemann S (2003). Evaluation of the GenoType MTBC Assay for differentiating of clinical *Myobacterium tuberculosis* complex in isolates. Journal of Clinical Microbiology. 41:2672-2675.

Rodriguez E, Sanchez L.P, Perez S, Herrera L, Jimenez M.S, Samper S. *et al.* (2009). Human tuberculosis due to *Mycobacterium bovis* and *M. caprae* in Spain, 2004–2007. International Journal of Tuberculosis and Lung Disease. 13(12):1536-1541.

Romero B, Aranaz A, Bezos J, Alvarez J, de Juan L, Javed M.T. *et al.* (2007). Drug susceptibility of Spanish *Mycobacterium tuberculosis* complex isolates from animals. Journal of Tuberculosis. 87:565-571.

Roring S.A, Scott D, Brittain I, Walker G, Hewinson S & Skuce R (2002). Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. Journal of clinical Microbiology. 40:2126-2133.

Rouse D.A, Devito J.A, Li Z, Byer H & Morris S.L (1996). Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis* isolates originating from the St. Petersburg area in Russia. Antimicrobial Agents and Chemotherapy. 42:2443-2445.

Rufenacht S, Stuber K.B, Bodmer T, Jaunin V.F.B, Jmaa D.C.G & Gunn-Moore D.A (2011). *Mycobacterium microti* infection in the cat: A case report, literature review and recent clinical experience. Journal of Feline Medicine & Surgery. 13:195-204.

Rüsch-Gerdes S, Domehl C, Nardi G, Gismondo M.R, Welscher M &Pfyffer G.E (1999). Multicenter evaluation of mycobacterial growth indicator tube for testing susceptibility of *Mycobacterium tuberculosis* to first-line drugs. Journal of Clinical Microbiology 37:45-48.

Salo W.L, Auferheide A.C, Buikstra J & Holcomb T.A (1994). Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. Proceedings of the National Academy of Science USA. 91:2091-2094.

Scorpio A & Y. Zhang (1996). Mutations in *pncA*, a gene encoding pyrazinamidase/ nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle *Mycobacterium tuberculosis*. Journal of Clinical Microbiology. 28:2200-2204.

Shah A & Rauf Y (2001). Newer methods for the laboratory diagnosis of tuberculosis. Journal of Laboratory Medicine. 8(4):266-267.

Singh U.B, Arora J, Suresh N, Pant H, Rana T, Sola C. *et al*. (2007). Genetic biodiversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in India. Infection, Genetics and Evolution. 7:441-448.

Sjobring U, Mecklenburg M, Andersen A.B & Miorner H (1990). Polymerase Chain Reaction for detection of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology. 28(10):2200-2204.

Skuce R.A, McCorry T.P, McCarroll J.F, Roring S.M, Scott A.N, Brittain D. *et al*. (2002). Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. Microbiology. 148:519-528.

Smith N.H, Crashaw T, Parry J & Birtles R.J (2009). *Mycobacterium microti*: More diverse than previously thought. Journal of Clinical Microbiology. 47(8):2551–2559.

Soini H, Pan X, Amin A, Graviss E.A, Siddiqui A & Musser J.M (2001). Characterization of *Mycobacterium tuberculosis* isolates from patients in Houston, Texas, by spoligotyping. Journal of Clinical Microbiology. 38:669-676.

Sola C, Filliol I, Gutierrez M.C, Mokrousov I, Vincent V & Rastogi N (2001). Spoligotype database of *Mycobacterium tuberculosis*: biogeographic distribution of shared types and epidemiologic and phylogenetic perspectives. Emerging Infectious Diseases. 7:390-396.

Sola C, Filliol I, Legrand E, Lesjean S, Locht C, Supply P. *et al.* (2003). Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infection on Genetic Evolution. 3:125-133.

Somoskovi A, Parsons L.M & Salfinger M (2001). The molecular basis of resistance to isoniazid, rifampin and pyrazinamide in *Mycobacterium tuberculosis*. Respiratory Research. 2(3):164-168.

Sreevatsan S, Stockbauer K.E, Pan X, Kreiswirth B.N, Moghazeh S.L, Jacobs W.R. *et al.* (1997). Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of embB mutations. Antimicrobial Agents and Chemotherapy. 41:1677-1681.

Stead W.W, Eisenach K.D, Cave M.D, Beggs M.L, Templeton G.L, Thoen C.O. *et al.* (1995). When did Mycobacterium tuberculosis infection first occurrence in the new world an important question with public health omplications. American Journal of Respiratory and Critical Care Medicine Home. 151(4):1267-8.

Stover C.K, Pham X.Q, Erwin A.L, Mizoguchi S.D, Warrener P, Hickey M.J. *et al.* (2000). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature. 406:959–964.

Sun J.R, Lee S.Y, Perng C.L & Lu J.J (2009). Detecting *Mycobacterium tuberculosis* in Bactec MGIT 960 cultures by in-house IS*6110*-based PCR assay in routine clinical practice. Journal of Formosan Medical Association. 108(2):119-125.

Sun Y.J, Luo J.T, Wong S.Y & Lee A.S.G (2010). Analysis of *rpsL* and *rrs* mutations in Beijing and non-Beijing streptomycin-resistant *Mycobacterium tuberculosis* isolates from Singapore. Clinical Microbiology Infection. 16(3):287-289.

Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E. *et al.* (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology. 44:4498-4510.

Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D & Locht C (2001). Automated high throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. Journal of Clinical Microbiology. 39:3563-3571.

Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B & Locht C (2000). Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Molecular Microbiology. 36:762-771.

Supply P & Multilocus (2005). Variable Number Tandem Repeat Genotyping of *Mycobacterium tuberculosis* Technical Guide. Institut de Biologie/Institut Pasteur de Lille.

Suzuki Y, Ono Y & Hirabayashi Y (1998). Rapid and specific reactive oxygen species generation via NADPH oxidase activation during Fas-mediated apoptosis. FEBS Letters. 425:209-212.

Taniguchi H, Chang B, Abe C, Nikaido Y, Mizuguchi Y & Yoshida S.I (1997). Molecular analysis of kanamycin and viomycin resistance in *Mycobacterium smegmatis* by use of the conjugation system. Journal of Bacteriology. 179:4795-4801.

Therese K.L, Gayathri R & Madhavan H.N (2012). Molecular Biological Techniques for Detection of Multidrug Resistant Tuberculosis (MDR) and Extremely Drug Resistant Tuberculosis (XDR) in Clinical Isolates of Mycobacterium tuberculosis, Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis, Dr. Pere-Joan Cardona (Ed.), ISBN:978-953-307-938-7.

Torun T, Tahaoglu K, Ozmen I, Sevim T, Atac G, Kir A. *et al.* (2007). The role of surgery and fluoroquinolones in the treatment of multidrug-resistant tuberculosis. International Journal of Tuberculosis and Lung Disease. 11:979-985.

Tudo G, Rey E, Borrell S, Alcaide F, Codina G, Coll P. *et al.* (2010). Characterization of mutations in streptomycin-resistant *Mycobacterium tuberculosis* clinical isolates in the area of Barcelona. Journal of Antimicrobial Chemotherapy. doi:10.1093/jac/dkq322.

Valvatine H, Syrel H, Kross M, Staurum R, Phyu S & Grewal H.M.S (2009). Isoniazid and rifampin resistance-associated mutations in *Mycobacterium tuberculosis* isolated from Kazakhstan. Journal of Clinical Microbiology. 15(2):1413-8670

van Embbden J, Cave M.D, Crawford J.T, Dale J.W, Eisenach K.D, Gicquel B. *et al.* (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: Recomendations for a standardized methodology. Journal of Clinical Microbiology. 31(2):406-409.

van Embden J.D, Cave M.D, Crawford J.T. *et al*. (2000). Strain Identification of *Mycobacterium tuberculosis* by DNA Fingerprinting: Recommendations for a Standardized Methodology. Journal of Clinical Microbiology. 31(2):406–409.

van Soolingen D, Hoogenboezem T, de Haas P.E.W, Hermans P.W.M, Koedam M.A, Teppema K.S. *et al.* (1997). A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: Characterization of an exceptional isolate from Africa. International Journal of Systematic Bacteriology. 47(4):1236-1245.

van Soolingen D, Qian D.L, de Haas P.E, Douglas J.T, Traore H, Portaels F. *et al.* (1994). Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. Journal of Clinical Microbiology. 33:323-328.

Van Soolingen D, vander Zanden A.G, de Haas P.E, Noordhoek G.T, Kiers A, Foudraine N.A. *et al.* (1998). Diagnosis of *Mycobacterium micriti* infections amoung humans by using novel genetic marker. Journal of Clinical Microbiology. 36(7): 1840-1845.

Vester B & Douthwaite S (2001). Macrolide resistance conferred by base substitutions in 23S rRNA. Antimicrob. Agents Chemotherapy. 45:1–12.

Victor T.C, van Rie A, Jordaan A.M, Richardson M, Der Spuy G.D, Beyers N. *et al.* (2001). Sequence polymorphism in the *rrs* gene of *Mycobacterium tuberculosis* is deeply rooted within an evolutionary clade and is not associated with streptomycin resistance. Journal of Clinical Microbiology. 39:4184–4186.

Vitol I, Driscoll J, Kreiswirth B, Kurepina N & Bennett K.P (2006). Identifying *Mycobacterium tuberculosis* complex strain families using spoligotypes. Infection, Genetics and Evolution. 6:491–504.

Warren R.M, Streicher E.M, Sampon S.L, Vander G.D, Richardson M, Nguyen D. *et al.* (2002). Microevolution of the direct repeat region of *Mycobacterium tuberculosis*: implications for interpretation of Spoligotyping data. Journal of Clinical Microbiology. 40:4457-4467.

Warren R.M, Victor T.C, Streicher E.M, Richardson M, Beyers N, Gey van Pittius N.C. *et al.* (2009). Patients with active tuberculosis often have different strains in the same sputum specimen. American Journal of Respiratory and Critical Care. 169:610-614.

Waters W.R, Whelan A.O, Lyashchenko K.P, Greenwald R, Palmer M.V, Harris B.N. *et al.* (2010). Immune responses in cattle inoculated with *Mycobacterium bovis*, *Mycobacterium tuberculosis* or *Mycobacterium kansasii*. Journal of Clinical and Vaccine Immunology. 17(2):247-252.

WHO/EMC/ZOO/94.6. Guidelines for speciation within the *Mycobacterium tuberculosis* complex. Pages 8-14.

World Health Organisation (2008). Global Tuberculosis Control: Surveillance, Planning, Financing. Geneva, Switzerland.

World Health Organisation (2008). Anti-tuberculosis drug resistance in the world. Fourth global report. The WHO/IUATLD global project on anti-tuberculosis drug resistance surveillance, 2002 -2007, WHO Press, Geneva Switzerland.

World Health Organization, 2012. Global Tuberculosis Report. http://www.who.int/tb/publications/global_report/en/. (Accessed 03 August 2013).

World Health Organization (2001). Guidelines for drug susceptibility testing for second-line anti-tuberculosis drugs for DOTS-plus. 45:1–12.

Winivesky J.P, Henschke C, Balentine J, Wilner C, Delorie A & McGinn T (2000). Prospective validation of a prediction rule to assess the need for respiratory isolation of patients with suspected tuberculosis. Archieves of International Medicine. 165:453-457.

Xu C, Kreiswirth B.N, Sreevatsan S, Musser J.M & Drlica K (1996). Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*. Journal of Infectious Disease. 174:1127-1130.

Yam W.C (2006). Recent advances in rapid laboratory diagnosis of tuberculosis. The Hong Kong Medical Diagnosis. 11(1):6-7.

Yeager H, Jr Lacy J, Smith L.R & LeMaistre C.A (1967). Quantitative studies of mycobacterial populations in sputum and saliva. Am Revised Respiratory Disease. 95:998-1004.

Yew W.W, Chan C.K, Chau C.H, Tam C.M, Leung C.C, Wong P.C. *et al*. (2000). Outcomes of patients with multidrug-resistant pulmonary tuberculosis treated with ofloxacin/levofloxacin-containing regimens. Chest. 117:744-751.

Zanolari P, Robert N, Lyashchenko K.P, Pfyffer G.E, Greenwald R, Esfandiari J (2009). Tuberculosis caused by *Mycobacterium microti* in South American camelids. Journal of Veterinary Internal Medicine. 23:1266-1272.

Zhang Y & Telenti A (2000). Genetics of drug resistance in Mycobacterium tuberculosis. In: Molecular Genetics of Mycobacteria. Edited by Hatful GF, Jacobs WR Jr. Washington DC: ASM Press: 235–254.

Zhang Y (1992). Strain Variation in the *katG* region of *Mycobacterium tuberculosis* assay. Journal of Medical Microbiology. 59:285-294.

Zimhony O, Cox J.S, Welch J.T, Vilcheze C & Jacobs W.R Jr (2000). Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of Mycobacterium tuberculosis. Nature Medicine. 6:1043–1047.

Zimhony O, Vilcheze C & Jacobs W.R.Jr (2004). Characterization of Mycobacterium smegmatis expressing the *Mycobacterium tuberculosis* fatty acid synthase I (fas1) gene. Journal of Bacteriology. 186:4051–4055.

CHAPTER 3

Detection of *Mycobacterium tuberculosis* complex (MTBC) DNA using Seeplex® MTB Nested ACE detection assay

3.0. Abstract

The *Mycobacterium tuberculosis* complex (MTBC) causes tuberculosis (TB) in both animals and humans. South Africa has a heavy burden of TB worsened by the concurrent epidemic of HIV. We investigated the prevalence of MTBC among TB patients in Port Elizabeth. Three thousand eight hundred and ten (3810) sputum specimens were processed and DNA was isolated from sputum specimens collected from the National Health Laboratory Service (NHLS) in Port Elizabeth. DNA was amplified using the Seeplex® MTB Nested ACE detection assay. One hundred and ninety (5%) DNA samples tested positive for MTBC using the Seeplex® MTB Nested ACE assay. These results raise an alarm for the prevalence of MTBC in Port Elizabeth and further investigations and actions should be taken such as encouraging people to go to clinics for check-ups.

3.1. Introduction

The *Mycobacterium tuberculosis* Complex (MTBC) consists of nine bacterial species that cause tuberculosis (TB) in mammals, including human beings (Brosch *et al*., 2002). MTBC results in substantial economic losses in cattle herds and humans as it is usually found in the more economically active humans (Riemann and Abbas, 1983). Tuberculosis is a major public health concern a third's of the world's population is infected with MTBC (Brosch *et al*., 2002). According to a World Bank report in 2012, there were 981 per 100 000 people infected with tuberculosis in 2010 (WHO, 2013). The World Health Organization reported that in 2012, in the world 8.6 million people fell ill with TB and 1.3 million died from it (WHO, 2013). In the same year 2012, an estimated 530 000 children became ill with TB and 74 000 HIV-negative children died of TB (WHO, 2013).

In terms of TB incidence South Africa ranks second among the 22 high burden TB countries and in terms of overall TB burden (WHO, 2007). South Africa is a country with high incidence of TB, there were 550 cases per 100 000 population in 2003, 718 case per 100 000 population in 2004 (Day and Gray 2005; WHO, 2008) and 600 cases per 1000 000 population in 2005 (WHO, 2007). South Africa is a middle income country and is well provided with 143 laboratories performing sputum smears and 18 culture laboratories also capable of performing drug sensitivity testing (WHO, 2007). The country had one of the worst recorded epidemics in the world in 2008 caused by the rising rates of HIV and the emergence of multidrug resistant TB (WHO, 2013). The epidemic of TB was blamed on historical neglect, health service fragmentation and poor patient management due to the fastest growing HIV epidemics ever recorded (WHO, 2007; Weyer *et al*., 1999).

South Africa is divided into nine Provinces with Gauteng Province being the economical attention of many foreigners (Census, 2011). Among the nine Provinces, the most affected Provinces are KwaZulu-Natal, the Western Cape, Eastern Cape and Gauteng, which have 80% of TB cases in South Africa. Out of these 80% only 54% are cured and 13% of patients stop taking treatment (WHO, 2007; Wayer *et al*., 1999). In 1997, in the Eastern Cape Province there was a dramatic increase in reported cases of pulmonary TB, from 10 358 in 1995 to 28 800 in 1997 (Mahlalela *et al.*, 1998). The incidence was about 500 per 10 000 in 1997 (Mahlalela *et al*., 1998). The provincial cure rate for all TB cases in 1997 was 38.3% with treatment interruption rate of 21.9% (Meidary and Puchert 1999). The Eastern Cape is one of the poorest Provinces in South Africa and because of its poverty, illiteracy, TB and HIV; it is heading for disaster (Kaona, 2008). This Province has the second worst burden of poverty in South Africa with approximately 47% households living below the poverty line. There is also a 55% rate of unemployment (Bradshaw *et al.*, 2000).

Several methods have been used to identify MTBC, including culture and biochemical tests such as acid-fast smears and sputum cultures. The diagnosis of TB includes history, physical examination and radiological findings in lung apices. Acid-fast smears and cultures of sputum are also required (Nawaz *et al.*, 2012). The different smear microscopy is an expensive and rapid test for diagnosis of TB (Kesarwani *et al*., 2004), but though this technique does not permit differentiation between species of *M. tuberculosis* complex (Nawaz *et al*., 2012). Culture is the gold standard and its disadvantage is that it requires a lot of time (it takes 3-4 weeks) while clinical urgency constrain immediate back up (Palomino, 2009). There are mediums used by this method such as the agar based Lowestein-Jensen (LJ)

medium and an egg-based Middlebrook,s medium (Sun *et al*., 2009). There are also liquid cultures such as BACTEC which detects the turbecle bacilli early compared to LJ medium in clinical specimen (palomino, 2009). Culturing and drug susceptibility are used to detect drug resistance (Miotto *et al*., 2008).

The reliable method for rapid diagnosis is the PCR amplification of *Mycobacterium tuberculosis* DNA, which can detect TB within a day (Mc Adam *et al*., 2007). There has been an introduction of a new test for TB called GeneXpert. This test takes approximately 2 hours to detect if a person has TB and it is more sensitive than any other TB tests (Müller, 2011). Although the test is good it has a disadvantage of being expensive at a market price approximately $60 000 (Müller, 2011). This study aimed to use multiplex PCR targeting two genes (*mpb64* and IS*6110*) for the detection of MTBC in different individuals in Port Elizabeth in the Eastern Cape Province.

3.2. Material and Methods

3.2.1. Study area and sample collection

Port Elizabeth is one of the largest cities in South Africa, situated in the Eastern Cape Province, 770 km east of Cape Town. It has a population of over 1.3 million (Census, 2011). Three thousand eight hundred and ten specimens in the Eastern Cape Province were collected from different hospitals and clinics from patients that showed clinical signs of TB and transported to the National Health Laboratory Service (NHLS) in Port Elizabeth, South Africa. At the NHLS, isolation, identification and susceptibility profiles were done and DNA was isolated from MTBC isolates. Demographic profiles including (age 0-20; 21-40; 41-60 and 60 years and above) of the patients were also collected from NHLS database.

3.2.2. Bacteriological procedure.

Three consecutive morning sputum samples (5 – 10 ml) were collected in McCartney bottles and processed on the same day. Petroff's method was used to decontaminate and increase the bacillary concentration (Balows *et al*., 1991). NaOH (4%) was used to kill any other contaminants in this procedure. Two Lowenstein_Jensen (LJ) slants were inoculated and incubated at 37 ºC for 6–8 weeks. A smear was prepared from each of the processed samples on a grease-free slide and stained by carbol fuschin using the Ziehl_Neelsen technique. Slides were checked for AFB under a microscope

3.2.3. Drug susceptibility.

The agar-dilution proportion method was used to perform drug-sensitivity testing using 7H10 Middlebrook medium (Difco) (Balows *et al*., 1991). Lyophilized drugs were reconstituted aseptically in water. The stock was diluted in such a manner that a 5µl aliquot contained the requisite amount of each drug. The drug concentrations used in this study were isoniazid (1 µg ml^{-1}) Rifampicin (5 µg ml^{-1}), Streptomycin (10 µg ml^{-1}), Ethambutol (10 µg ml^{-1}), Ethionamide (5 µg ml^{-1}), ofloxacin (2 µg ml^{-1}), Amikacin (6 µg ml^{-1}) and capreomycin (40 µg ml^{-1}).

3.2.4. Preparation of inoculum for drug sensitivity.

Several spade-fulls (2 – 5 mg) of growth were scraped from LJ slants, transferred to a sterile screw-cap tube containing six to eight glass beads and 3 ml normal saline (0.85 %) and mixed well on a vortex mixer. Turbidity was matched against McFarland standard no. 1. Inoculum (100µl) was added to each plate, containing 5 ml 7H10 Middlebrook medium with drug in each quadrant. *M. tuberculosis* strain H37Rv was used as control in all sets of experiments. The inoculated plates were incubated at 37 ºC in an atmosphere of 10 % CO_2. Results were recorded after 3 weeks. Each drug-sensitivity test was carried out at least three times and the average was recorded.

3.2.5. Identification of MTBC species using Seeplex® MTB Nested ACE detection assay

The Seeplex® MTB Nested ACE detection assay (Seegene Inc, Korea) was carried according to the manufacturer's instructions using a thermal cycler (Bio-Rad, South Africa). The assay is a multiplex PCR involving the first PCR (1 cycle at 94℃ for 15 minutes; 40 cycles at 94℃ for 30 seconds, 60℃ for 30 seconds, 72℃ for 30 seconds; 1 cycle at 72℃ for 5 minutes) and a nested PCR (1 cycle at 94℃ for 15 minutes; 30 cycles at 94℃ for 30 seconds, 62℃ for 30 seconds, 72℃ for 30 seconds; 1 cycle at 72℃ for 5 minutes). The amplicons were separated on 2% agarose gel electrophoresis, at 100 V for 90 minutes using TBE buffer pH 8.3. The gel was thereafter visualized under Alliance 4.7 transilluminator (UVITEC Limited, Cambridge, UK).

3.3. Results and Discussion

One hundred and ninety (190) DNA samples out of the three thousand eight hundred and ten (3810) specimens were used in the study. This is 5% of the specimens that were positive for MTBC.

3.3.1. Amplification of Seeplex DNA samples

The Seeplex® MTB Nested ACE detection assay target multi DNA regions (*mpb*64 and IS*6110*). The internal control (520 bp) is used to identify processed samples containing substances that may interfere with PCR amplification. The *M. tuberculosis* band corresponds to 190 bp and this is the band that confirms that this is *M. tuberculosis* complex. Our results indicate that all samples tested positive for MTBC (Fig.3.1).

The main strength of the Seeplex® MTB Nested ACE detection assay is that it uses multi-target PCR (IS*6110* and *mpb*64) for the specific detection of MTBC only; this prevents false positive results that are caused by other mycobacteria. However, the assay has a weakness of not differentiating amongst the different members of the MTBC. We noted that MTBC was detected among the black and the coloured race. No whites were detected with MTBC.

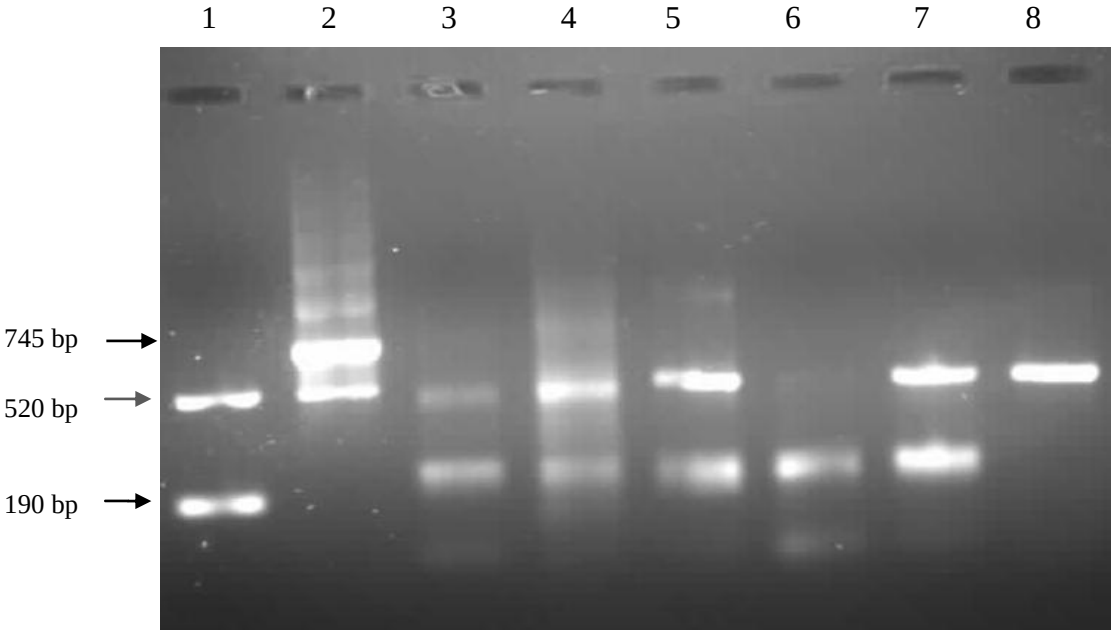

Fig.3.1: Agarose gel electrophoresis of amplicons generated using the Seeplex® MTB Nested ACE detection assay. Lane 1: DNA Marker Seegen, USA (shows how positive samples should be); lane 2: Positive control; lane 3-7 DNA samples; lane 8: Negative control. The DNA Marker shows two bands which are the internal control band (520 bp) and the *M. tuberculosis* band at (190 bp). This shows how positive samples should be. The positive control shows two bands as well, the internal control (520 bp) and an upper band corresponding to 745 bp (instead of 190 bp) which is designed by the manufacturer to eliminate false positive resulting from cross contamination. The negative control shows only the internal control (520 bp), negative samples shows only this band. All 190 samples that were collected from the NHLS showed to be positive for MTBC.

The 190 bp band shows that MTBC was detected. The sample in lane 6 showed a band at 190 bp and a very faint one at 520 bp, therefore we then re-ran a gel to be sure of the band and we properly mixed the DNA before loading to a 2% agarose gel and two visible bands were obtained at 190 bp and 520 bp. The drawback of the Seeplex is that it is incapable of differentiating between the members of the MTBC.

Table 3.1: Patient's demographic profile

Age (years)	**Males**	**Females**	**Race**		
	N (%)	**N (%)**	Blacks N (%)	Coloured N (%)	Whites N (%)
0-14	2 (1.05)	7 (3.68)	6 (3.160)	3 (1.58)	0
15-24	8 (4.21)	24 (12.63)	28 (14.74)	4 (2.11)	0
25-44	52 (27.4)	65 (34.21)	100 (52.6)	17 (8.95)	0
45-64	19 (10)	9 (4.74)	22 (1.58)	6 (3.16)	0
65+	1 (0.53)	3 (1.58)	4 (2.11)	0	0
Total	82 (43.16)	108(56.84)	160 (84.2)	30 (15.79)	0

N (%), number and percentage

From the demographic data (Table 3.1) we observed that most of the patients were from the black race with 84.2% (160/190) and the rest were of the coloured race 15.8% (30/190). We then compared our findings with the population of the Nelson Mandela Bay Metropolitan Municipality (Port Elizabeth) which is 1 152 115 (Census, 2011). According to Census 2011, 60.1% are black African, 23.6% coloured, 14.4% white and 1.1% Indian/Asian. Of the population, 552 994 (48%) are male and 599 121 (52%) are female. Young people (0–14 years) constitute 25.5% of the population, youth (15–35 years) 37.1%, adults (36–64 years) 31.4% and the elderly (65+ years) 6% (Statistics South Africa, 2011). In our study we had 190 patients that we detected with MTBC from, 160 (84.2%) were black African, 30 (15.8%) were coloured people and there were no white people where MTBC was detected from (Table 3.1).

When looking at the statistics by Census 2011 black Africans are more than any other race in this Municipality. This results show high prevalence of MTBC amongst the black race which could be due to the fact that these samples were collected from public clinics, where most black people frequently visit because of the low cost. Some coloured people attend public clinics and some attend private clinics and that could be one of the reasons there was 15.8% coloured race that had MTBC. However it could also be because of sampling bias where only 190 DNA isolates were chosen irrespective of gender or race. Not having any whites detected with MTBC could be due to the fact that most white people attend private clinics and this data was collected from public clinics. The coloured and black people work together most of the time and some attend the same schools and stay in the same location which increases the risk of transmission of MTBC between the two races.

A fascinating observation from our results (Table 3.1) was made in this study whereby it was observed that there are more females (56.8%) in this study that were detected to have MTBC in comparison to males (43.16%) which contradicts what most studies have reported on (WHO, 2013; Onifade *et al*., 2010). It has been reported that in most of the world, more men than women are infected by MTBC (WHO, 2013). A report by Census (2011) gave the population of the people in Nelson Mandela Bay Metropolitan Municipality 552 994 (48%) are male and 599 121 (52%) are female which shows that there are more females than males in this region. However, our results concerning females being more infected by MTBC compared to males might be due to the fact that females care for the sick, both children and their husbands or brothers. It can also be as a result of selection bias where more samples for females were chosen than males.

Nevertheless most women die due to TB (WHO, 2013) and this affect women mainly in their economically and reproductively active years (WHO, 2013). This was shown in our study (Table 3.1) where 47% of women aged 15-44 years are the most infected. Our results are also in agreement with Murray *et al*. (1990) who found out that in women aged 15–44 years in developing countries TB is the most common cause of morbidity and mortality combined, and it kills more women than any other infectious disease including malaria and AIDS (Murray *et al*., 1990). It was noted that the group from 15-44 years is a sexually and economically active age groups which could be another reason of having more females detected with MTBC. This does not mean that TB is contracted through sexual intercourse bit it is transmitted by having a close contact with someone infected or an animal. This result (Table 3.1) indicates that TB can infect any race exposed to MTBC despite their genetic makeup and age group.

Table 3.2: Anti-TB resistance to both first line and second line drugs stratified for *M. tuberculosis* genotypes

	Anti-TB drug resistance profiles (FL)					Anti-TB drug resistance profiles (SL)					
Parameter Age	**INH N (%)**	**RIF N (%)**	**STM N (%)**	**EMB N (%)**	**MDR N (%)**	**ETHIO N (%)**	**OFL N (%)**	**CAP N (%)**	**AMIK N (%)**	**Pre-XDR N (%)**	**XDR N (%)**
≤ 20	23 (12.1)	22 (11.6)	12 (6.3)	2 (1.1)	22 (11.6)	2 (1.1)	1 (0.53)	2 (1.1)	4 (2.1)	2 (1.1)	23 (12.1)
21-40	114 (60)	114 (60)	73 (38.4)	20 (10.5)	114 (60)	30 (15.8)	37 (19.5)	14 (7.4)	65 (34.2)	25 (13.2)	114 (60)
41-60	46 (24.2)	46 (24.2)	21 (11.1)	3 (1.58)	46 (24.2)	5 (2.63)	3 (1.6)	4 (2.1)	18 (9.5)	12 (6.3)	46 (24.2)
≥61	5 (2.6)	5 (2.6)	2 (1.1)	0 (0)	5 (2.6)	2 (1.1)	2 (1.1)	0 (0)	3 (1.6)	1 (0.53)	5 (2.6)
Total Gender	**188 (98.9)**	**187 (98.4)**	**108 (56.8)**	**25(12.7)**	**187 (98.4)**	**39 (20.5)**	**43 (22.6)**	**20 (10.5)**	**90 (47.4)**	**40 (21.1)**	**188 (98.9)**
MALE	85 (43)	84 (42.6)	47 (23.5)	8 (4)	84 (42.6)	17 (8.6)	14 (7.1)	5 (2.5)	36 (18.2)	17 (8.6)	85 (43)
FEMALE	105 (55.3)	105 (55.3)	60 (31.6)	10 (5.3)	105 (55.3)	15 (7.9)	22 (11.6)	8 (4.21)	47 (24.7)	16 (8.42)	105 (55.3)
190 (100%)	**190 (100%)**	**189 (99.5)**	**107 (56.3)**	**18 (9.5)**	**189 (99.5)**	**32 (16.8)**	**36 (18.9)**	**13 (6.8)**	**83 (43.7)**	**33 (17.4)**	**189 (99.5)**

FL=First line, SL=Second line, MDR the other 2 samples did not have age; therefore they were not counted in the age group. INH =isoniazid; RIF =rifampicin; STM =streptomycin; EMB =ethambutol; MDR =multi-resistant drug; ETHIO =ethionimide; OFL =ofloxacin; CAP =capreomycin; AMIK = amykacin; pre-XDR =pre-extensively drug resistant; XDR =extensively drug resistant.

From the resistant profiles (Table 3.2) we noted that multidrug-resistant TB was identified in 190 (100%) patients infected with MTB resistant to isoniazid and 189 (99.5%) patients infected with MTBresistant to rifampicin). Drug resistant TB develops from inadequate treatment of pulmonary TB (Otu *et al*., 2013). Resistance to anti-TB drugs may also be due to poor drug selection by medical doctors (Sharma and Mohan, 2006). Suggestion of several biological mechanisms linking drug-resistant TB and HIV has been made (Dye *et al*., 2002). Drug malabsorption in HIV-infected patients, especially rifampicin and ethambutol can lead to drug resistance leading to treatment failure (Otu *et al*., 2013). Malbsorption is caused by the damage to the intestinal villi caused by HIV, *Cryptosporidium*, one of the commoner and more serious opportunistic gut infections (Amade *et al*., 2002; Arapradi, 2000; Sharostone *et al*., 1999). Possible mechanisms responsible for malabsorption HIV/AIDS include the impact of HIV on villi specitre enzyme deficiencies in intestinal mucosa, the opportunistic infections and altered intestinal transit have all been considered but these are mainly conjectural and effective treatments remain to be developed (WHO, 2005).

Data that supports this hypothetic statement has not yet been observed in humans (Patel *et al*., 1995). This is supported by three studies that were done in South Africa which also did not find association between HIV-infection and MDR-TB. In a retrospective study conducted in Durban, 2.4% of 42 HIV co-infected and 11.5% of 253 HIV negative patients had MDR-TB (Anastasis *et al*., 1997). In a study in Cape Town MDR-TB was 2.6% in 155 HIV negative in comparison of 32% in 93 HIV co-infected patients (Post and Wood, 1997). In gold miners, MDR-TB rate was 5.3% among 207 HIV co-infected and 6.5% of 218 HIV negative miners (Murray *et al*., 2000).

One hundred and ninety (100%) of the patients were found to be resistant to at least one or more anti-TB drugs. Resistnce to only one drug was found in all 190 patients who were resistant to isoniazid 190 (100%); rifampicin 189 (99.5%); streptomycin 107 (56.3%); ethambutol 18 (9.5%) for first line drugs. The drug with highest resistant profile was isoniazid with 100% and with the lowest resistant profile ethambutol with 9.5%. Almost all the samples (99.5%) that were resistant to isozianid were also resistant to rifampicin. The findings of the study supports what other authors hypothesised which states that rifampicin can be used as a surrogate marker for MDR, this is due to the fact that 99.5% of rifampicin resistant *Mycobacterium tuberculosis* strains are equal to isoniazid (Mokrousov *et al*., 2002; El-Hajji *et al*., 2001; Somoskovi *et al*., 2001). Only one (0.53%) sample was susceptible to rifampicin. This is a first report of high drug resistant MTBC Port Elizabeth; these results are higher than those that were reported by Green *et al*. (2010) who obtained 58.4% MDR-TB in Mpumalanga Province of South Africa. This raises a serious concern which calls for immediate measures which can decrease the spread of resistant strains.

On the second line drugs ethionimide, was identified in 32 (16.8%) isolates; ofloxacin in 36 (18.9.6%); capreomycin in 13 (6.8%) and amykacin in 83 (43.7%). Pre-extensively drug tuberculosis was detected in 33 (17.4%) patients and extensively drug resistant TB was detected in 157 patients. Thirty three isolates (17.4%) were identified as pre-XDR and fifty seven isolates were identified as XDR-TB. Comparing our results with the study by Campbell *et al*. (2011), Campbell reported ofloxacin (69: 21.9%) and amykacin (1:0.3%). Of the tested isolates 55 (16%) were susceptible to all the study antibiotics and 10 (3%) were determined to be XDR- *M. tuberculosis* (Campbell *et al.*, 2011). Our results show a high drug resistance of second line drugs in Port Elizabeth.

3.4. Conclusions

The results obtained from this study show a high prevalence of MTBC among Port Elizabeth villagers. Of noteworthy is the fact that women at their reproductive years are mostly infected and this could lead to a vicious cycle, hence women are exposed to a lot of people. They can infect their children at home the children will pass it to others at school and they can also transmit it to their husbands. There is a high risk of MBTC transmission in Port Elizabeth and something needs to be done agently to prevent the spread of TB.

REFERENCES

Amadi B, Mwiya M, Musuku J, Watuka A, Sianongo S, Ayoub A. *et al*. (2002). Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: a randomised controlled trial. Lancet. 360(9343):1375-80.

Anastasis D, Pillai G, Rambiritch V & Abdool Karim S.S (1997). A retrospective study of human immunodeficiency virus infection and drug-resistant tuberculosis in Durban, South Africa. International Journal of Tuberculosis and Lung Disease. 1(3):220–224.

Arpadi S.M (2000). Growth failure in children with HIV infection. Journal of Acquired Immune Deficiency Syndrome. 25(Suppl 1):S37-S42.

Balows A, Hausler W.J, Jr Herrmann K.L, Isenberg H.D & Shadomy H.J (editors) (1991). Manual of Clinical Microbiology, 5th edn. Washington, DC: American Society for Microbiology.

Bradshaw D, Nannan N, Laubscher R, Groenewald P, Joubert J, Nojilana B. *et al.* (2000). Mortality estimates for Eastern Cape Province. South African National Burden of Disease Study. 3-10.

Brosch R, Gordon S.V, Marmiesse M, Brodin P, Buchrieser C, Eiglmeir K. *et al*. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proceedings of the National Academy of Science of the United States of America. 99:3684–3689.

Campbell P.J, Morlock G.P, Sikes R.D, Dalton T.L, Netchock B, Starks A.M. *et al.* (2011). Molecular detection of mutations associated with first- and second line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrobial Agent and Chemotherapy. 55(5):2032-2041.

Census 2011 Statistical release – P0301.4 / Statistics South Africa. Pretoria: Statistics South Africa, 2012.

Day C & Gray A (2005). Health and related indicators. Chapter 17. In: Ijumba P, Barron P, editors. South African Health Review 2005. Durban: Health Systems Trust. 248-367.

Dye C, Williams B.G, Espinal M.A & Raviglione M.C (2002). Erasing the world's slow stain: strategies to beat multidrug-resistant tuberculosis. Science. 295(5562):2042–2046.

EL-Hajji H, Marras S.A.E, Tyagi S, Kramer F.R & Alland D (2001). Detection of rifampin resistance in *Mycobacterium tuberculosis* in a single tube with molecular beacons. Journal of Clinical Microbiology. 39(11):4131-4137.

Green E, Obi L, Nchabeleng M, de Villiers B.E, Sein P.P, Letsoalo T. *et al.* (2010). Drug-susceptibility patterns of *Mycobacterium tuberculosis* in Mpumalanga Province, South

Africa: Possible guiding design of retreatment regimen. Journal of Health Population and Nutrition. 28(1):7-13.

Kaona F.A, Tuba M & Siziya S (2008). An assessment of factors contributing to treatment adherence and knowledge of TB transmission among patients on TB treatment. BMC Public Health. 41:2004. (Accessed 18/03/2008).

Kesarwani R.C, Anjana P, Ashutosh M & Kumar S. A (2004). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Indian Journal of Surgery. 66:84-88.

Mahlalela X, Rohde J & Bennett J (1998). The status of primary health care services in the Eastern Cape Province. Report on the 1st equity primary health care survey of clinics and district hospitals of the Eastern Cape Province, S A.1997. MSH/EQUITY Project.

Mc Adam R.A, Hermans P.W, van Soolingen D, Zainuddin Z.F, Catty D & Dale J.W (2007). Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. Molecular Microbiology. 4:1607-1613.

Meidany F & Puchert R (1999). Eastern Cape Epidemiological notes. Department of Health, Province of the Eastern Cape. South Africa. 7(3).

Miotto P, Saleri N, Dembele M, Ouedraogo M, Badoum G, Pinsi G. *et al.* (2008). Molecular detection of rifampin and isoniazid resistance to guide chronic TB patient management in Burkina Faso. BMC Infectious Diseases. 9:142-150.

Moskrousovi I, Filliol I, Legrand E, Sola C, Otten T, Vyshnevskaya E. *et al.* (2002). Molecular characterization of multiple-drug-resistant *Mycobacterium tuberculosis* isolates from north-western Russia and analysis of rifampin resistance using RNA/RNA mismatch analysis as compared to the line probe assay and sequencing of the *rpoB* gene. Research in Microbiology. 153: 213-219.

Müller A (2011). Gene Xpert. http://www.tbonline.info/posts/2011/10/3/gene-xpert/. (Accessed 20 October 2013).

Murray C.J.L, Styblo K & Rouillon A (1990). Tuberculosis in developing countries: burden, intervention and cost. Bulleting of the International Union against Tuberculosis and Lung Disease. 65:6-24.

Murray J, Sonnenberg P, Shearer S & Godfrey-Faussett P (2000). Drug-resistant pulmonary tuberculosis in a cohort of Southern African goldminers with a high prevalence of HIV infection. South African Medical Journal. 90(4):381–386.

Nawaz A, Chaudhry Z.I, Shahid M, Gul S, Khan F.A & Hussain M (2012). Detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis*in sputum and blood samples of human. The Journal of Animal and Plant Sciences. 22(2 Suppl.):117-120 ISSN: 1018-708.1

Onifade D.A, Bayer A.M, Mantoya R, Haro M, Alva J, Franco J. *et al*. (2010). Gender-related factors influencing tuberculosis control in Shanty towns: qualitative study. BMC public health. 10:381.

Otu A, Umoh V, Habib A, Soter Ameh S, Lawson L & Ansa V (2013). Drug Resistance among Pulmonary Tuberculosis Patients in Calabar, Nigeria. Pulmonary Medicine. (2013):235190, 6.

Palomino J.C (2009). Molecular detection, identification and drug resistance detection in *Mycobacterium tuberculosis*. Journal of Immunology and Medical Microbiology. 2:1-3.

Patel K.B, Belmonte R & Crowe H. M (1995). Drug malabsorption and resistant tuberculosis in HIV-infected patients. The New England Journal of Medicine. 332(5):336–337.

Post F.A & Wood R (1997). HIV infection is not associated with an increased rate of drug-resistant tuberculosis. South African Medical Journal. 87(7):903.

Riemann H.P & Abbas B (1983). Diagnosis and control of bovine paratuberculosis (Johne's disease). Advances in Veterinary Science and Comparative Medicine. 27:481-503.

Sharma S.K & Mohan A (2006). Multidrug-resistant tuberculosis: a menace that threatens to destabilize tuberculosis control. Chest. 130(1):261-272.

Sharpstone D, Neild P, Crane R, Taylor C, Hodgson C, Sherwood R. *et al*. (1999). Small intestinal transit, absorption, and permeability in patients with AIDS with and without diarrhoea. Gut. 45(1):70-6.

Somoskovi A, Parsons L.M & Salfinger M (2001). The molecular basis of resistance to isoniazid, rifampin and pyrazinamide in *Mycobacterium tuberculosis*. Respiratory Research. 2(3):164-168.

Statistics South Africa (2011). http://beta2.statssa.gov.za/?page_id=1021&id=buffalo-city-municipality. (Accessed 28 August 2013).

Sun J.R, Lee S.Y, Perng C.L & Lu J.J (2009). Detecting *Mycobacterium tuberculosis* in Bactec MGIT 960 cultures by in-house IS*6110*-based PCR assay in routine clinical practice. Journal of Formosan Medical Association. 108(2):119-125.

Weyer K, Fourie P.B & Nardell E.A (1999). A Noxious Synergy: Tuberculosis and HIV in South Africa. The Global Impact of Drug – Resistant Tuberculosis. Harvard Medical School and Open Society Institute, Boston, USA.

World Health Organisation (2013) http://www.who.int/tb/challenges/gender/. (Accessed 11 November 2013).

World Health Organization (2007). Strategic and technical advisory group for tuberculosis (STAG-TB) report on conclusions and recommendations. Seventh meeting Geneva.

World Health Organization (2008). Global tuberculosis control: surveillance, planning, financing: WHO report 2008. Geneva: World Health Organization, page 294. (WHO/HTM/TB/2008.393).

World Health Organization (2005). Macronutrients and HIV/AIDS: a review of current evidence Consultation on Nutrition and HIV/AIDS in Africa. http://www.who.int/nutrition/topics/PN1_Macronutrients_Durban.pdf (Accessed 10 March 2014).

CHAPTER 4

Differentiation of *Mycobacterium tuberculosis* complex (MTBC) using Region of differences (RDs)

4.0. Abstract

Tuberculosis (TB) still continues to be the leading cause of death in developing countries. The spread of tuberculosis in the community can be controlled by accurate early diagnosis and proper treatment. The diagnosis tests that have been used for some time have limitations such as low sensitivity and being time consuming. The introduction of diagnostic methods that are more sensitive and specific can increase the efficiency of strategies to control tuberculosis. This study aims to differentiate the *Mycobacterium tuberculosis* complex (MTBC) members using a multiplex polymerase chain reaction (PCR) method based on genomic regions of differences such as RD1, RD1^{mic}, RD2^{seal}, RD4, RD9 and RD12 for *M. tuberculosis, M. bovis, M. bovis* BCG, *M. microti, M. africanum, M. canetti, M. pinnipediii* and *M. caprae*. The size of the respective multiplex PCR amplification products corresponded to the presence of the different members of MTBC. From the 184 DNA isolates 45 (24.5%) isolates were identified to be *M. tuberculosis*, 94 isolates (51.1%) to be *M. bovis* BCG and 3 isolates (1.6%) to be *M. cannetti*. From the results that were obtained it can be concluded that a multiplex PCR can be used to differentiate between the MTBC members that cause disease in both animals and humans.

4.1. Introduction

It has been discovered that most bacterial species consist of a wide spectrum of distinct clones and conal complexes (Spratt, 2004; Maiden, 2000) and they differ from one another by 1% or more at synonymous nucleotide sites (Feil and Spratt 2001; Palus *et al*., 1997). Tuberculosis (TB) continues to be the leading cause of modility and mortality in developing countries (Parkash *et al*., 2009). Laboratory diagnosis of human TB relies on culture, acid-fast staining and microscopy mainly (Warren *et al*., 2006). These traditional diagnostic methods have limitations such as sensitivity, specificity and they are time consuming (Parkash *et al*., 2009). Due to the mentioned limitations, a new tool was sought and Warren *et al*. (2006) introduced the Region of Differences differentiating *Mycobacterium tuberculosis* complex (MTBC) isolates into different species.

The agents responsible for TB are among the most successful human pathogen and they are members of the MTBC. The MTBC members include *M. tuberculosis, M. bovis, M. bovis* BCG, *M. microti, M. africanum, M. cannetti, M. pinnipediii, M. caprae* and *M. mungi* species. Differentiation of MTBC is important for the accurate molecular identification of species of the MTBC, accurate diagnosis of mycobacterial disease, public health surveillance and appropriate cause management (Warren *et al*., 2006). In a genomic comparative study of the members of the MTBC it was revealed that region of differences (RDs) reveals sequential losses of genetic material in the cause of evolution from a common descendent strain (Brosch *et al*., 2002; Machairas *et al*., 1996). The presence or absence of several RDs detection has been used to differentiate between the members of the MTBC in patient isolates using PCR-based approaches (Warren *et al*., 2006; Parsons *et al*., 2002). The study aims to differentiate

MTBC members known to cause disease in both animals and humans using a single multiplex PCR diagnostic method.

4.2. Materials and Methods

4.2.1. Multiplex PCR amplification

One hundred and eighty four (184/190) DNA isolates from TB patients were used. The DNA isolates were those from the 190 MTBC detected isolates and we ended working with 184 because we ran out of DNA, and we could not go back and get the samples from the affected individuals due to the fact that some patients died, while some have been released to go to their homes. These 184 DNA samples were amplified using the multiplex PCR which consisted of four primer sets per reaction. The 4 primer sets used were as described by Warren *et al*. (2006) and they were as followsRD1, RD4, RD9 and RD12.

A single multiplex PCR diagnostic method was used to differentiate the MTBC members detected in chapter 3 known to cause to disease in humans and animals. Each PCR reaction contained 1 µl DNA template, 5 µl Q-buffer, 2.5 µl of 10X buffer, 2 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTPs, 0.5 µl of each primer (50 pmol/µl) (Table 4.1), 0.125 µl HotStarTaq DNA polymerase (Fermentas, Thermo Scientific, US) and was made up to 25 µl with sterile nuclease free H_2O. To differentiate among the different TB causing species DNA was amplified using the primers in table 4.1. Amplification was initiated by incubation at 95^0C for 15 minutes, followed by 45 cycles at 94^0C for 1 minute, 62^0C for 1 minute, and 72^0C for 1 minute. After the last cycle, the samples were incubated at 72^0C for 10 minutes. PCR amplification products were electrophoretically fractionated in 3.0% agarose in 1X TAE pH 8·0 at 6V/cm for 4 hours and visualised by staining with ethidium bromide. The results were interpreted as explained by Warren *et al*. (2006) and table 4.1.

Table 4.1: PCR primer sequences and corresponding amplification product sizes indicating the presence or absence of genomic regions of difference in different MTC members

Primers sequence	**RD**	***M. canettii***	***M. tuberculosis***	***M. Africanum***	***M. microti***	***M. pinnipedii***	***M. caprae***	***M. bovis***	***M. bovis* BCG**	**PCR conditions**
AAGCGGTTGCCGCCGACCGACC* CTGGCTATATTCCTGGGCCCGG* GAGGCGATCTGGCGGTTTGGGG*	1 1 1	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 absent (169 bp)	Initial denaturation at 95^{0}C for 15 min; 3-step cycling for 40X, 1min at 95^{0}C, 1 min at 62^{0}C, 1min at 72^{0}C; 10min at 72^{0}C.
ATGTGCGAGCTGAGCGATG TGTACTATGCTGACCCATGCG AAAGGAGCACCATCGTCCAC	4 4 4	RD4 present (172 bp)	RD4 present (172 bp)	RD4 present (172 bp)	RD4 present (172 bp)	RD4 present (172 bp)	RD4 present (172 bp)	RD4 absent (172 bp)	RD4 absent (172 bp)	Initial denaturation at 95^{0}C for 15 min; 3-step cycling for 40X, 1min at 95^{0}C, 1 min at 62^{0}C, 1min at 72^{0}C; 10min at 72^{0}C.
CAAGTTGCCGTTTCGAGCC CAATGTTTGTTGCGCTGC* GCTACCCTCGACCAAGTGTT*	9 9 9	RD9 present (235 bp)	RD9 present (235 bp	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	Initial denaturation at 95^{0}C for 15 min; 3-step cycling for 40X, 1min at 95^{0}C, 1 min at 62^{0}C, 1min at 72^{0}C; 10min at 72^{0}C.
GGGAGCCCAGCATTTACCTC GTGTTGCGGGAATTACTCGG AGCAGGAGCGGTTGGATATTC	12 12 12	RD12 absent†	RD12 present (369 bp)	RD12 present (369 bp)	RD12 present (369 bp)	RD12 present (369 bp)	RD12 absent (306 bp)	RD12 absent (306 bp)	RD12 absent (306 bp)	Initial denaturation at 95^{0}C for 15 min; 3-step cycling for 40X, 1min at 95^{0}C, 1 min at 62^{0}C, 1min at 72^{0}C; 10min at 72^{0}C.
CGGTTCGTCGCTGTTCAAAC CGCGTATCGGAGACGTATTTG CAATCAGCCAAGACGAGGTTG	1mic 1mic 1mic			RD1mic present (195 bp)	RD1mic absent (127 bp)	RD1mic present (195 bp)				Initial denaturation at 95^{0}C for 15 min; 3-step cycling for 40X, 1min at 95^{0}C, 1 min at 62^{0}C, 1min at 72^{0}C; 10min at 72^{0}C.
TCAGCGGTCTCATAGCATTGC CGGGTTGGGAATGTCAGAAAC GCGGCAAGGTACGTCAGAAC	2seal 2seal			RD2seal absent†	RD2seal present (293 bp)	RD2seal absent (168bp)				Initial denaturation at 95^{0}C for 15 min; 3-step cycling for 40X, 1min at 95^{0}C, 1 min at 62^{0}C, 1min at 72^{0}C; 10min at 72^{0}C.

* Primer sequences according to Parsons *et al*. (2002)

PCR (Polymerase Chain Reaction); MTBC _ *Mycobacterium tuberculosis* complex; RD _ regions of difference; BCG _ Bacille Calmette-Guérin (Warren *et al*., 2006).

The size of amplicons was estimated by comparison with the 100 bp DNA ladder (Thermo Scientific). Images were visualized under Alliance 4.7 transilluminator (UVITEC Limited, Cambridge, UK). DNA template from *M. tuberculosis* strain H37Rv was used as a positive control and sterile nuclease free H_2O was used as a negative control.

4.3. Results and Discussion

Using the four primer sets RD1, RD4, RD9 and RD12 in one reaction was possible to differentiate between *M. tuberculosis, M. bovis, M. bovis* BCG, *M. caprae* and *M. canettii*. For the differentiation of *M. africanum, M. microti* and *M. pinnipedii* the 1^{mic} and the 2^{seal} primer sets were used for the amplification. Region of differences (RDs) are generated primary by IS*6110* insertion, homologous recombination and excision of intermediary DNA that occurs in an undirectional manner (Evans, 2011).

The results from this study (Fig 4.1, 4.2 and 4.3) shows the products generated after PCR amplification of DNA from nine MTBC members using the four primer sets. Out of 184 DNA isolates, 45 (24.5%) were identified to be *M. tuberculosis* (Fig. 4.1).MTBC can be detected in humans because humans are the primary hosts. The implication of *M. tuberculosis* is that it has developed some mechanisms that interfere with the host immune system (Doherly and Andersen, 2005; Flynn *et al*., 2005). *M. tuberculosis* can modulate various aspects of both the innate and adaptive immune response (North and Jins, 2004; van Crevel *et al*., 2002). Not much information has been published in this area.

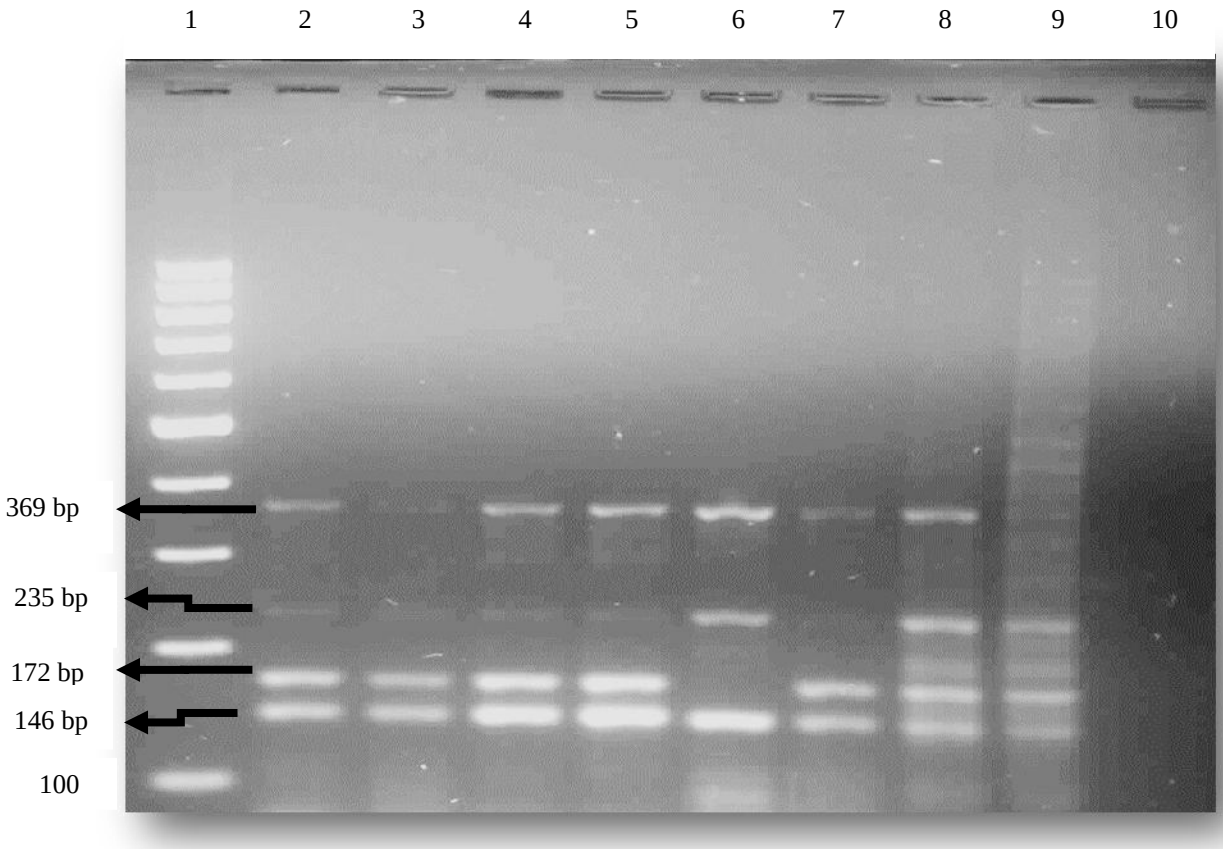

Fig.4.1: Electronic fractionation of representative PCR products in 3% agarose generated using Multiplex PCR amplification with four sets of primers RD1, RD4, RD9 and RD12 for *Mycobacterium tuberculosis*. Lane 1: 100 bp DNA Marker; lane 2: *Mycobacterium tuberculosis* positive control; lane 3-9 DNA samples which shows *Mycobacterium tuberculosis* species; lane 10: Negative control.

From the results that were obtained we noted that 4 isolates (2.2%) were *M. bovis* and 94 isolates (51.1%) were *M. bovis* BCG (Bacillus Calmette-Guérin). The host of *M. bovis* is cattle, but a variety of domestic animals, wild animals' as well as humans can be infected with this species (Gibson *et al*., 2004). The disease (*M. bovis*) has a public health implication due to the fact that it can be transmitted to humans. The disease can be transmitted to humans through the consumption of unpasteurized milk from infected cattle (Angela *et al*., 2006).

This means that it is important to accurately detect and screen for MTBC in milk before it can be consumed by humans. This statement was corroborated by Angela *et al*. (2006). *M. bovis* is one of the most recognised members of MTBC that transmits TB from animals to humans (Angela *et al*., 2006). This is also supported by a study of Vitale *et al*. (1997) which reported on high prevalence (87%) of MTBC in milk from cattle in Italy. Domestic and wild animals as well as humans can be infected by *M. bovis* (Gibson *et al.*, 2004). The danger of untreated *M. bovis* is that it may progress to gastrointestinal or pulmonary disease (Heymann, 2008).

In our study we noted that *M. bovis* BCG (Fig. 4.2 and Fig. 4.3) was the most identified strain from the MTBC and this raises a concern because BCG is an attenuated derivative of virulent strain of *Mycobacterium bovis* (Talbot *et al*., 1996). BCG has been used as a vaccine against *M. tuberculosis*, as a cancer immunetherapy and as a recombinant vehicle for multivalent vaccines against other infectious diseases (Brosman, 1992; Lugosi, 1992). BCG can cause disease in humans more especially those with immunodeficiency (Lamm *et al*., 1992; Lugosi, 1992) and it is not recommended for use in infants known to be infected with HIV, due to the risk of disseminated BCG disease (WHO, 2011). This shows the clinical importance of the ability to rapidly and specifically identify BCG. The ability to differentiate BCG from other members of *M. tuberculosis* complex is important because BCG can cause disease in humans although it is less virulent from its parent strain (Lotte *et al*., 1988). The accurate identification of BCG will provide important epidemiological and treatment information. The other importance is the ability to identify BCG all over South Africa in areas where it is used as a vaccine (Edward and Kernodle, 1996). *M. bovis* BCG's failings have important implication for current efforts to develop new and better vaccines against tuberculosis.

Infection with the turbecle bacillus itself provides only incomplete protection; the bacilli remain dormant in many infected individuals and can reactivate to cause diseases due to previous infection (Flne, 1995). This raise a concern on the use of BCG in children who are immune compromised, such as children with HIV; these children could end up having an infection caused by BCG vaccine itself.

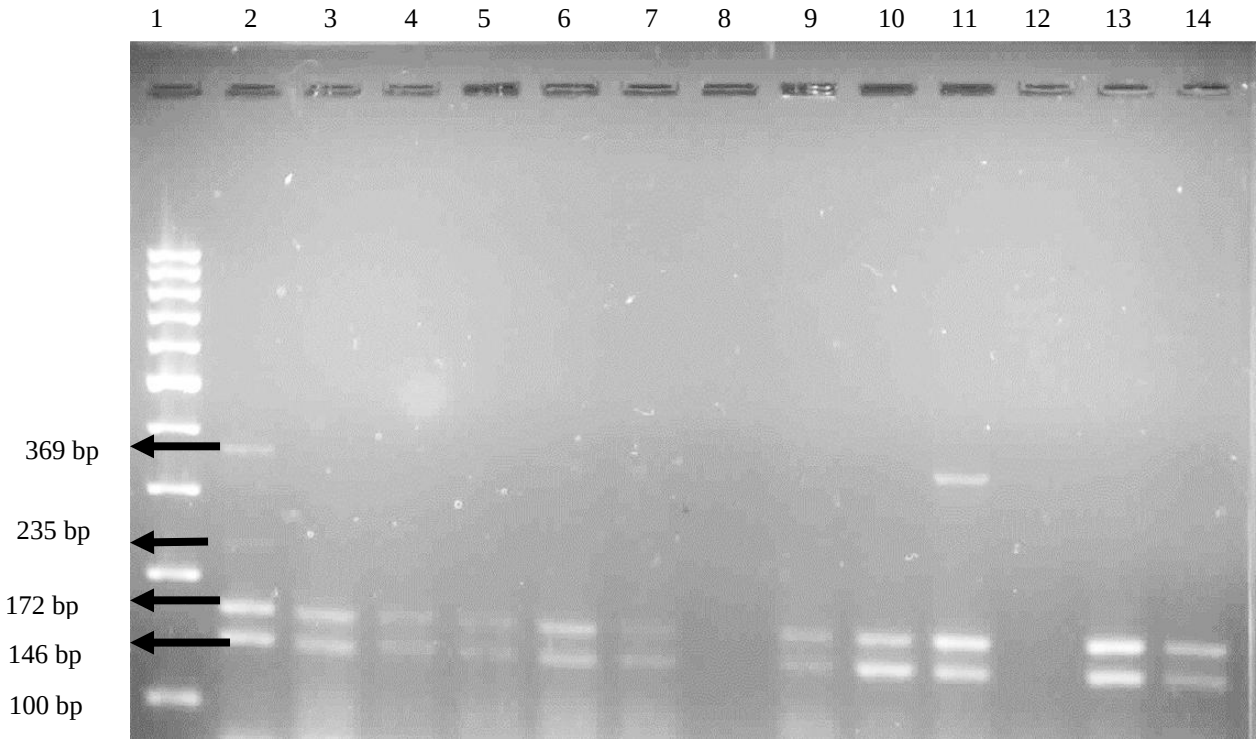

Fig. 4.2: Electronic fractionation representative of PCR products in 3% agarose generated using Multiplex PCR amplification with four sets of primers RD1, RD4, RD9 and RD12 for *Mycobacterium tuberculosis*. Lane 1: DNA Marker (100 bp ladder); lane 2: *M. tuberculosis* positive control which shows *Mycobacterium tuberculosis* species (146 bp, 172 bp, 235 bp and 369 bp); lane 3-7: *M. caprae* species (146 bp and 172 bp); lane 8: *M. bovis* BCG isolates (no amplification); lane 9-10: *M. caprae* species; lane 11; *M. africanum* species(146 bp, 172 bp and 369 bp); lane 12: *M. bovis* BCG isolate and lane13-14: *M. caprae* species.

M. caprae was found in 34 isolates (18.4%) (Fig. 4.2 and Fig. 4.3), the species or the main causative agent of animal tuberculosis has been isolated from several domestic and wild animal species (Aranaz *et al*., 2003). This pathogen has been recognized mainly in Central Europe where it was isolated from cattle (Duet *et al*., 2008) and pigs (Pavlik *et al.,* 2002). *M. caprae* isolates are usually characterized from goats and other domestic and wild animals. Fascinating results were observed in this study hence (18.4%) of 184 patients were found to be infected by this species.

This contradicts with what has been reported from previous studies which reported that these pathogen has never been isolated outside the European continent but from a European patient in Australia (Sintchenko *et al*., 2006) and in a cow in Algeria (Sahraoui *et al*., 2009). From our results we can suggest a possibility of transmission of this pathogen from livestock to humans (Prodinger *et al*., 2002). Our study results agree with reports that were reported by Aranaz *et al*. (2003) and Rodriguez *et al*. (2009) which report that this species has been isolated from humans in European countries.

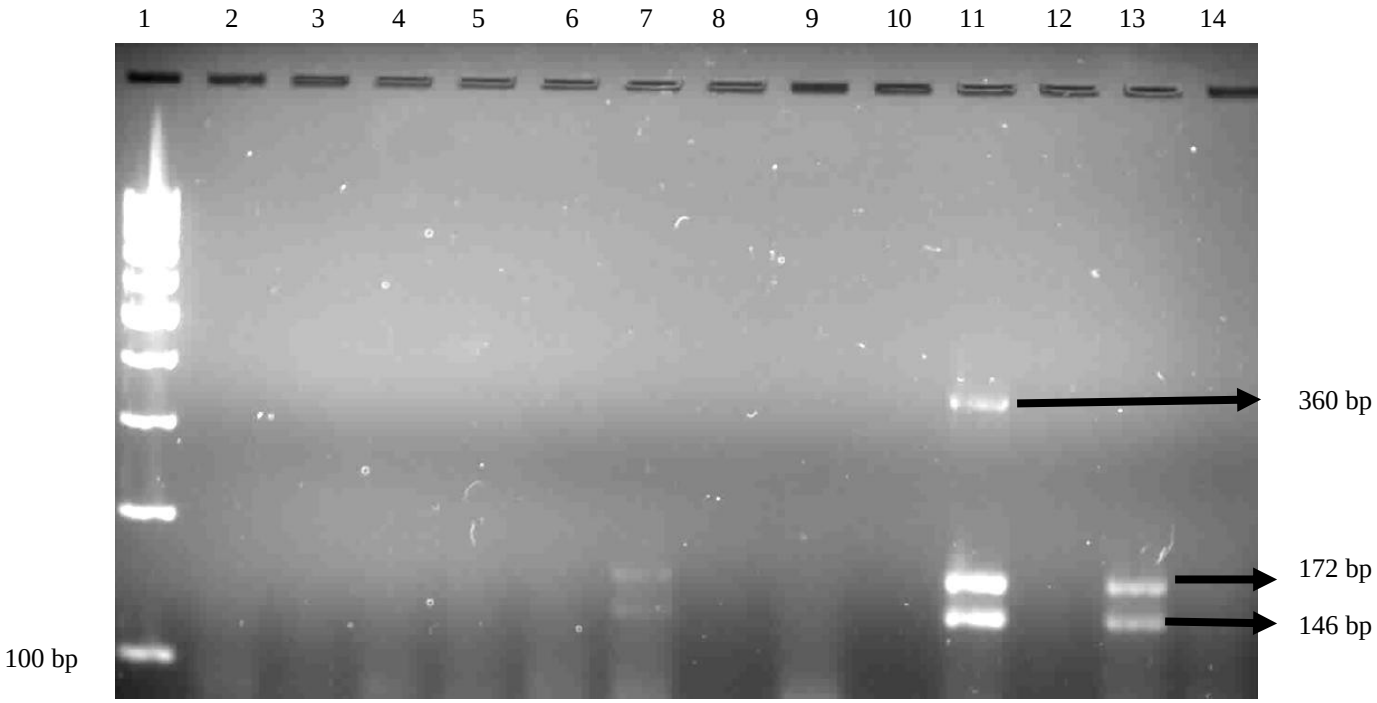

Fig. 4.3: Electronic fractionation representative of PCR products in 3% agarose generated using Multiplex PCR amplification with four sets of primers RD1, RD4, RD9 and RD12 for *Mycobacterium tuberculosis*. Lane1: DNA Marker (100 bp ladder); lane 2-6: *M. bovis* BCG isolates (no amplification); lane 7: *M. caprae* species; lane 8-10 (146 bp and 172 bp): *M. bovis* BCG isolates lane 11: DNA sample which shows *M. africanum* species (146 bp, 172 bp and 360 bp); lane 12: *M. bovis* BCG isolate; lane 13: *M. caprae* species; lane14: Negative control.

The current observation totals of 4 isolates were identified to be *M. africanum* (Fig 4.2 and Fig 4.3). *M. africanum* is a heterogeneous group causing human tuberculosis in Africa. *M. africanum* exhibits biochemical characteristics that are somewhat intermediate between those of *M. bovis* as well as *M. tuberculosis* (Cousins *et al.*, 2003). *M. africanum* (subtype I) has traits that resembles those of *M. bovis* and is widespread in West Africa. Conversely, *M.*

africanum (subtype II) has biochemical properties that bear a resemblance to *M. tuberculosis* and is highly prevalent in East Africa (Mostowy *et al*., 2004). The variability in its biochemical characteristics impedes the ability to diagnose infection due to *M. africanum* (de Jong *et al*., 2010).

M. canettii (data not shown because the gel picture was not clear) was also found to be part of the species that infected the patients, 3 isolates (1.6%) were detected. *M. canettii* is a rare, smooth, variant of *M. tuberculosis* usually isolated in Africa from humans. The species has been reported worldwide and the majority of reported cases are from patients who have either travelled or lived in Africa (Fabre *et al*., 2010). The multiplex PCR method used in this study gave results within a short period of time. Multiplex PCR amplification is faster and simpler compared to conventional mycobacteriological methods which takes more than eight weeks to achieve the same results.

4.4. Conclusions

Our results indicated that *M. caprae* infection poses a serious health risk not only in domestic animals such as goats as reported in other studies but it also pose a serious health risk also in humans. We discovered that all the RDs were deleted from all BCG sub-strains and present in all other strains evaluated. We also discovered that *M. bovis* BCG was highly prevalent in organisms causing TB in Port Elisabeth, Eastern Cape Province. Multiplex PCR can be used in the identification of the different *M. tuberculosis* complex species.

REFERENCES

Angela D.P, Giuseppina C, Tony F.V, Bijo B, Fatmira S & Giuseppina T (2006). Detection of *Mycobacterium tuberculosis* complex in milk using polymerase chain reaction (PCR). Food Control. 17:776-780.

Aranaz A, Cousins D, Mateos A & Dominguez L (2003). The elevation of *Mycobacterium tuberculosis* subsp. caprae Aranaz et al. 1999 to species rank as *Mycobacterium caprae* comb Nov., sp. Nov. International Journal of Systematic Evolution of Microbiology. 53:1785-1789.

Brosch R, Gordon S.V, Marmiesse M, Brodin P, Buchreser C, Eiglmeir K. *et al*. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proceeding National Academy of Science USA. 99:3684-3689.

Brosman S.A (1992). Bacillus Calmette-Guerin immunotherapy. Urologic Clinics of North America. 19:557-564.

Cousins D.V, Bastida R, Cataldi A, Viviana Q, Redrobe S, Dow S. *et al.* (2003). Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. Journal of Systematic and Evolutionary Microbiology. 53:1305-1314.

de Jong B.C, Antonio M & Gagneux S (2010). *Mycobacterium africanum* - Review of an important cause of human tuberculosis in West Africa. PLoS Neglected Tropical Diseases 4(9):e744.

Doherly T.M & Andersen P (2005). Vaccines for tuberculosis: novel concepts and recent progress. Clinical of Microbiology Review. 18:687-702.

Duet E.L, Domingos M, Amado A & Botelho A (2008). Spoligotyping diversity of *Mycobacterium caprae* animal isolates. Veterinary Microbiology. 130:415-421.

Edwards K.M & Kernodle D.S (1996). Possible hazards of routine bacillus Calmette Guerin immunization in human immunodeficiency virusinfected children. Pediatric Infectious Disease Journal. 15:836-838.

Evans J.T (2011). Molecular Epidemiology of Tuberculosis in the Midlands. School of Immunity and Infection. Page 45.

Fabre M, Hauck Y, Soler C, Koeck J.L, van Ingen J, van Soolingen D. *et al.* (2010). Molecular characteristics of *Mycobacterium canettii*, the smooth *Mycobacterium tuberculosis* bacilli. Journal of Infection, Genetics and Evolution.

Feil E.J & Spratt B.G (2001). Recombination and the population structures of bacterial pathogens. Annual Review of Microbiology. 55:561–590.

Flne P.E.M (1995). Variation ion protection by BCG: implications of and for heterogous immunity. The lancet. 346:1330-1345.

Flynn J.L & Chan J (2005). What's good for the host is good for the bug. Trends in Microbiology. 13:98-102.

Gibson G.R, Probert H.M, Van loo J, Rastall, R.A & Roberfroid M.B (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition Research Reviews 17:259-275.

Heymann G, Jacobsz S.W, Vorster T.E.B & Storry R.B (2008). Comparison of ground stiffness from seismic surface wave and large scale load tests. Geotechnical and Geophysical Site Characterization (ISC'3). Huang & Mayne (eds), Taylor & Francis, London.843-848.

Lamm D.L, van der Meijden A.P.N, Morales A, Brosman S.A, Catalona W.J, Herr H.W. *et al*. (1992). Incidence and treatment of complications of bacillus Calmette-Guerin intravesical therapy in superficial bladder cancer. Journal of Urology. 147:596-600.

Lotte A, Wasz-Hockert O & Poisson P (1988). Second IUATLD study on complications induced by intradermal BCG-vaccination. Bull. International Union Against Tuberculosis and Lung Disease. 63:47-59.

Lugosi L (1992) Theoretical and methodological aspects of BCG vaccine from the discovery of Calmette and Guerin to molecular biology. A review. Tubercle and Lung Disease. 73:252-261.

Machairas G.G, Sabo P.J, Hickey M.J, Singh D.C & Stover C.K (1996). Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. Journal of Bacteriology. 178:1274-1282.

Maiden M.C (2000). High-throughput sequencing in the population analysis of bacterial pathogens of humans. International Journal of Medical Microbiology. 290: 183-190.

Mostowy S, Onipede A, Gagneux S, Niemann S, Kremer K, Desmond E.P. *et al.* (2004). Genomic analysis distinguishes *Mycobacterium africanum.* Journal of Clinical Microbiology. 42(8):3594-3599.

North R.J & Jung Y.J (2004). Immunity to tuberculosis. Annual Review of Immunology. 22:599-623.

Palus T, Nakamura L.K & Cohan FM (1997) Discovery and classification of ecological diversity in the bacterial world: The role of DNA sequence data. International Journal of Systematic and Evolutionary Bacteriology. 47:1145-1156.

Parkash O, Singh B.P & Pai M (2009). Regions of Differences Encoded Antigens as Targets for Immunodiagnosis of Tuberculosis in Humans. Scandinavian Journal of Immunology. 1365-3083.

Parsons L.M, Brosch R, Cole S.T, Somoskövi A, Loder A, Bretzel G. *et al*. (2002). Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. Journal of Clinical Microbiology. 40:2339-2345.

Pavlik I, Dvorska L, Bartos M, Barmova I, Meliciiarek I, Jesenska A. *et al*. (2002). Molecular epidemiology of *bovine tuberculosis* Czech Republic and Slovakia in the period 1965-2001 studied by Spoligotyping. Veterinary medicine (Praha). 47: 181-194.

Prodinger W.M, Eigentler A, Allerberger F, Schonbauer M & Glawisching W (2002). Infection of red deer, cattle and human with *Mycobacterium bovis* subsp *capare* in Western Australia. Journal of Clinical Microbiology. 40: 2270-2272.

Rodriguez E, Sanchez L.P, Perez S, Herrera L, Jimenez M.S, Samper S. *et al*. (2009). Human tuberculosis due to *Mycobacterium bovis* and *M. caprae* in Spain, 2004–2007. International Journal of Tuberculosis and Lung Disease. 13(12):1536-1541.

Sahraoui N, Muller B, Guetarni D, Boulahhba F, Yala D, Ouzrout R. *et al*. (2009). Molecular characterization of *Mycobacterium bovis* strains isolated from cattle slaughtered at two abattoirs in Algeria. BCM Veterinary Res. 5:4. DOI 10.1186/1746-6148-5-4.

Spratt B.G (2004). Exploring the concept of clonality in bacteria. Methods of Molecular Biology. 266:323-352.

Stintchenko V, Jelfs P, Dally M, Crighton T & Gilbert G.L (2006). A case of urinary tuberculosis due to *Mycobbacterium bovis* subspecies *caprae*, pathology. 38:376-378.

Talbot E.A.S, Perkins M.D, Silva S.F.M & Frothingham R (1996). Disseminated BCG disease after vaccination: case report and review of the literature. Clinical Infectious Disease., in press.

van Crevel R, Ottenhoff T.H & van der Meer J.W (2002). Innate immunity to *Mycobacterium tuberculosis*. Clinical Microbiology Reviews. 15:294-309.

Vitale F, Capra G, Maxia L, Reale S, Vesco G & Caracappa S (1997). Detection of *Mycobacterium tuberculosis* complex in cattle by PCR using milk, lymphnode aspirates and nasal swabs. Journal of Clinical Microbiology. 36(4):1050-1055.

Warren R.M, Gey van Pittius N.C, Barnard M, Hesseling A, Engelke E, de Kock M, Gutierrez M.C. *et al*. (2006). Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. International Journal of Tuberculosis and Lung Disease. 10:818-822.

World Health Organization (2011). Global Tuberculosis Control. Geneva: World Health Organization.

CHAPTER 5

Detection of mutations coding for resistance to first and second-line drugs in *Mycobatcerium tuberculosis* isolates from Port Elizabeth, South Africa.

5.0 Abstract

South Africa is ranked third amongst countries that are faced with the burden of tuberculosis (TB) worldwide. *Mycobacterium tuberculosis* complex (MTBC) is primarily transmitted by inhalation of aerosolised droplets containing the organism. Multi-drug resistance (MDR) TB is defined as tuberculosis disease caused by a strain of *M. tuberculosis* that is resistant to at least isoniazid and rifampin. Extensively resistant drug resistant (XDR) is defined as TB resistance to at least isoniazid and rifampin plus a fluoroquinolone and one of the three injectable second-line drugs such as kanamycin, amikacin and capreomycin. One hundred and ninety DNA samples in this study detected to be MTBC using the Seeplex® MTB Nested Ace Assay were used. The study aimed to determine the incidence of MTBC in DNA samples and amplification and sequencing of the DNA amplicons known to confer resistance to TB drugs. Target genes known to confer resistance to first and second-line drugs were amplified and the amplicons sequenced using a Big Dye Terminator DNA sequencing kit v3.1 (Applied Biosystems, UK). Mutation resulting in MDR (100%), 40% pre-XDR and 98.9% of XDR was observed. Results show a high prevalence of extensive drug resistant TB in Port Elizabeth, Eastern Cape Province and that resistance of MTBC to drugs seems to be increasing. This is a serious health concern which calls for a need to strategise on the identification of extensive drug resistant TB patients from multi-drug resistant TB patients and ensure monitoring of their treatment to ensure total cure.

5. 1. Introduction

Tuberculosis (TB) still remains a public health problem worldwide with a global mortality of 1.2 to 1.5 million in 2010 (WHO, 2011). There has been an increase in TB incidence from 2009 t0 2013. In 2009, 10 million children were orphans as a result of one parent dying of TB (WHO, 2013). In 20011 there was an estimate of 8.7 million incidents of TB cases (WHO, 2013). In 2012, an estimated 8.6 million people developed TB and 1.3 million died from it (WHO, 2013), this include 320 000 deaths among HIV positive people. The cases of people infected with tuberculosis and the number of deaths from it fell in 2012, but there is a progression on uncontrolled contagious lung disease which is under the threat of drug resistance growth (WHO, 2013). Most people take TB as a disease of the past decade caused by strain that cannot be treated with existing drugs; this disease has turned to be one of the world's most pressing health problems (WHO, 2013). Anti-TB drug resistance is a major public health problem that threatens the progress made in TB control world wild. Drug resistance arises due to improper use of antibiotics in chemotherapy of drug susceptible (WHO, 2013).

Multidrug-resistance TB (MDR-TB) is resistance to the two most commonly used drugs (isoniazid and rifampin) in the common four drug regimen (TB Alliance, 2013). In 2010, the World Health Organisation (WHO) estimated that there were globally 290 000 cases of MDR-TB among cases of pulmonary TB that were reported (WHO, 2012). There have been 1.8 increases in MDR-TB cases in South Africa. There are several factors that contribute to the development of MDR-TB, such as poor adherence of patients to first line anti-TB drugs, dosage and duration oftreatment, inappropriate treatment regiments and non-compliance to

national guidelines and TB protocol by TB clinicians (Olusoji *et al.*, 2013). In appropriate use of second line anti-TB drugs leads to amplification of resistance and development of XDR-TB (WHO, 2012). Extensively drug resistance TB (XDR-TB) is the TB resistant to any fluoroquinolone and atleast one of the injectable drugs (capreomycin, kanamycin and amikacin) in addition to isoniazid and rifampin. Due to the above mentioned fact, this makes XDR-TB treatment extremely complicated in resource limited settings. In an XDR-TB outbreak in KwaZulu Natal, South Africa year 2006, 52 out of 53 people who contracted the disease died within a month (WHO, 2013).

It is estimated that 70% of XDR-TB patients die within a month of diagnosis (WHO, 2013). There is also Pre-extensively resistant drug resistance (Pre-XDR) which is defined as a disease caused by a TB strain resistant to isoniazid and rifampin and either a fluroquinolone or a second-line injectable drug but not both of them (WHO, 2008). The most recent drug-resistance surveillance data issued by WHO estimates that an average of roughly 5 percent of MDR-TB cases are XDR-TB. Most laboratories are still ill-equipped which makes the estimation detection and diagnosis of drug resistant TB to be extremely difficult and it brings a thought such as that most of the XDR-TB cases go undetected (WHO, 2013).

The emergency of drug resistant TB is a major concern to TB control in South Africa (Klopper *et al.*, 2013). In 2002 a medical survey revealed that 1.8% of all new TB patient and 6.7% of TB patients had MDR-TB who had undergone previous treatment (WHO, 2008). There is an annual case load estimated to be 13 000 MDR-TB cases, which places South Africa fourth among countries where MDR-TB is highly prevalent (WHO, 2008). Studies by Cox and McDermid (2010) and WHO (2010) suggested that there may be an underestimation

of the number and also that the proportion of MDR-TB case maybe substantially higher than the World Health Organisation estimate (WHO, 2010). Only 4 143 of the 9070 patients which is (46%) received a diagnosis of MDR-TB in 2009 received treatment and this can be due to the fact that resource constraints creating a situation in which control was bound to fail (NHLS, 2009). In South Africa most cases of MDR-TB and XDR-TB have been detected in four Provinces and the Eastern Cape Province is one of the four Provinces (WHO, 2008). Statistics from the Eastern Cape showed the largest increase in number of MDR-TB cases rising from 836 cases in 2006 to 1 858 cases in 2009 (WHO, 2008). The reason for the dramatic increase in MDR-TB cases is still to be investigated.

Molecular epidemiologic data from the neighbouring Province which is the Western Cape have demonstrated that MDR-TB is spread by primary transmission (Johnson *et al*., 2010; Johnson *et al*., 2006; van Rie *et al*., 2001) and it accounts for nearly 80% of reported MDR-TB cases (Cox and McDermid, 2010). So far there is only one molecular study that has been reported in the Eastern Cape Province (Strauss *et al*., 2008) and it showed that 50% of rifampin resistant TB which also includes MDR-TB isolates belonged to the Beijing genotype and Beijing strains were over represented. An epidemiologic study conducted in the Eastern Cape by Kvasnovsky *et al*. (2011) estimated that 75.6% of XDR-TB cases with complete data were a result of ongoing transmission. Treatment outcomes were inhospitable; 58% of case patients died within a year and culture conversion was observed in 8.4% of cases patients after 143 days of treatment (Kvasnovsky *et al*., 2011). This raises a concern that these patients had an untreated form of TB. The current study aimed to investigate the incidence of MDR and XDR-TB patients in Port Elizabeth.

5.2. Materials and Methods

5.2.1. Detection of resistance and mutation

Molecular detection of resistant genes was done by amplifying the DNA with first line drug primers that are shown in table 5.2.1. The molecular detection of resistant genes to second line drugs was done by amplifying the DNA with second line drugs primers shown in table 5.2.2. The samples were amplified by PCR using the synthetic oligonucleotide primers shown in the tables below (table 5.2.1 and 5.2.2). PCR was carried out in 25µl tube containing 12.5 µl of master mix with (Tris pH 8.0, $MgCl_2$, dNTP, 1UTaq polymerase, 8.5µl water (DDW molecular grade), 1 µl of each primer and 2 µl of DNA template (Sekiguchi *et al*., 2007). *Mycobacterium* H37RV strain was used as a positive control and sterile water used as a negative control.

5.2.2. DNA sequencing

To check for resistance and possible mutations, twelve amplicons per gene of the amplified products were sequenced. DNA sequencing was performed using a Big Dye Terminator DNA sequencing kit v3.1 (Applied Biosystems, UK). Direct sequencing was done with 2 µL of chromosomal DNA, 0.25 µL of primer (10 pmol per µL), 2 µL of Big Dye buffer and 2 µL of Big Dye. Cycle parameters included a denaturation at 96 ℃ for 10 sec, annealing at 50℃ for 20 sec, and extension at 60℃ for 4 min over 30 cycles, followed by Agencourt CleanSeq clean up. Sequences were determined by electrophoresis with the ABI 3130xl DNA sequencer (Applied Biosystems, UK). Editing of the sequences was performed using Bioedit Alignment Editor. Cleaned sequences were sent to BLAST using the nBLAST in NCBI (http://www.ncbi.nlm.nih.gov/); resistant genes were categorized to resistance types by their

resistance profiles and sequence similarity (Tamura *et al*., 2011). Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software Version 5.2; sequences were aligned using ClustalW (Tamura *et al.*, 2011).

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The significance of branching of the phylogenetic tree was evaluated by the bootstrap analysis of 500 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2011).

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Table 5.2.1: Primers that were used in the amplification of DNAfrom samples of patients who were using first line drugs

Drug	Gene	Prime	Primer sequence (5'-3' direction)	Size of PCR product	PCR conditions	Reference
Isoniazid (INH)	katG	RTB511 RTB 311	TGGCACGCTGCCGGCACCTA CGAAGCCGAACCCGAACGTC	242	Initial denaturing at 93^0C for 5 min; 3-step cycling for 30X, 1min at 95^0C, 1 min at 64^0C, 2 min at 72^0C; 10min at 72^0C.	van Rie, (2001)
Ethambutol (EMB)	embCAB	embB3-F embB3-R	TTCGCCCGAGCAAAGATG TCGCGGGACAGGTAGGTG	368	Initial denaturing at 95^0C for 15 min; 3-step cycling for 40X, 1min at 95^0C, 1 min at 65.5^0C, 1min at 72^0C; 10min at 72^0C.	Jadaun *et al*. (2009)
Rifampin	rpoB	RDRS RDRA	GTCGGTCATGTTCGCGATCG TCGGCCAGGTAGTCGCTGAT	238	Initial denaturing at94 ℃ for 5 min; 3-step cycling for 40X, 1 min at 94℃, 1 min at 57°C, 1 min at 72°C; 10 min at 72°C.	Prabhu *et al*. (2009)
Pyrazinamide	pncA	pncA P1 pncA P2	GAGCGGATGACCACCCAGGAC TACACCGACAGCGAGCCGA	305	Initial denaturing at 95^0C for 15 min; 3-step cycling for 40X, 1min at 95^0C, 1 min at 68^0C, 1min at 72^0C; 10min at 72^0C.	Anilkumar *et al*. (2011)

Table 5.2.2: Primers that were used in the amplification of DNA from samples of patients who were using second line drugs

Drug	Gene	Prime	Primer sequence (5'-3'direction)	Size of PCR product	Cycling conditions	Reference
Kanamycin, Amikacin and Capreomycin	*rrs*	RRS2-F RRS2-R	TGCCGGGGTCAACTCGGAGG GAACCCCTCACGGCCTACGC	439	Initial denaturing at 94^0 C for 4 min; 35 cycles of den at 94^0 C for 1 min, ann at58^0 C for 1 min and ext at 72^0 C for 2 min and 30S; final ext step at 72^0C for 10 minutes.	Perdigaõ *et al*. (2012)
kanamycin	*eis*	eisF1 eisR1	GCCATGGGACCGGTACTTGC GTAGATGCCGCCCTCGCTAG	601	Initial denaturing at 94^0 C for 4 min; 35 cycles of den at 94^0 C for 1 min, ann at 56^0 C for 1 min and ext at 72^0 C for 2 min and 30S; final ext step at 72^0C for 10 minutes.	Perdigaõ *et al*. (2012)
Fluoroquinoles (monofloxacin, ofloxacin, levofloxacin, gatifloxacin and aprofloxacin)	*gyrA*	gyrA-F gyrA-R	GCCATGGGACCGGTACTTGC GTAGATGCCGCCCTCGCTAG	581	Initial denaturing at 94^0 C for 4 min; 35 cycles of den at 94^0 C for 1 min, annealing at 55^0 C for 1 min and extension at 72^0 C for 2 min and 30S; final extension step at 72^0C for 10 minutes.	Perdigaõ *et al*. (2012)

5.3. Results and Discussion

After amplification with the primers shown in table 5.2.1, 157 isolates showed positive bands to rifampicin (*rpoB* gene) and 104 isolates showed positive bands to isoniazid (*katG* gene). The samples that did not show any bands when ran on 2% agarose gel were properly mixed and they were ran on a 2% gel again. We observed that all 190 amplicons showed bands which confer amplification of DNA in samples of patients on all the first line drug regimens.

For the second line regimen after amplification with primers shown in table 5.2.2, 39 isolates showed positive bands to kanamycin (*eis* gene); 89 isolates showed positive bands to injectable drugs (*rrs* gene) and 51 showed positive bands to fluoroquinolones (*gyrA* gene). After running the amplicons on 2% agarose gel all 190 DNA isolates showed the desired bands. The agarose gel pictures (Fig 5.3.1-5.3.5) for each of the gene after amplifying with the specific primers given in tables 5.2.1 and 5.2.2.

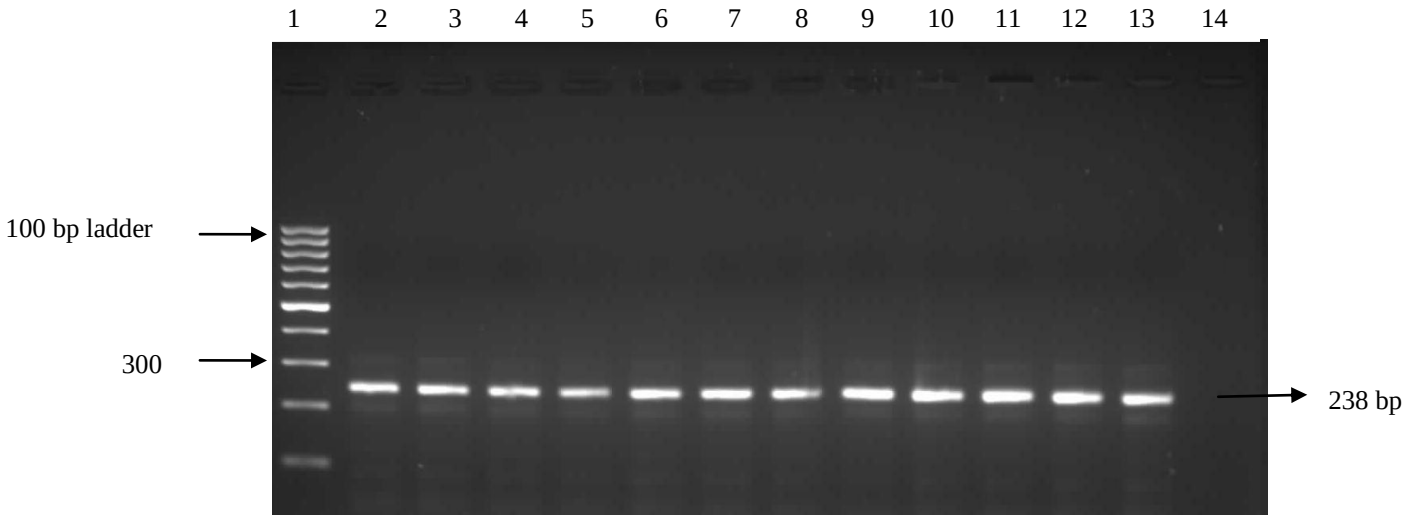


Fig.5.3.1: Agarose gel electrophoresis of amplicons generated using the *rpoB* gene. Lane 1: 100 bp ladder; lane 2: positive control; lane 3- 13: DNA isolates; lane 14: negative control.

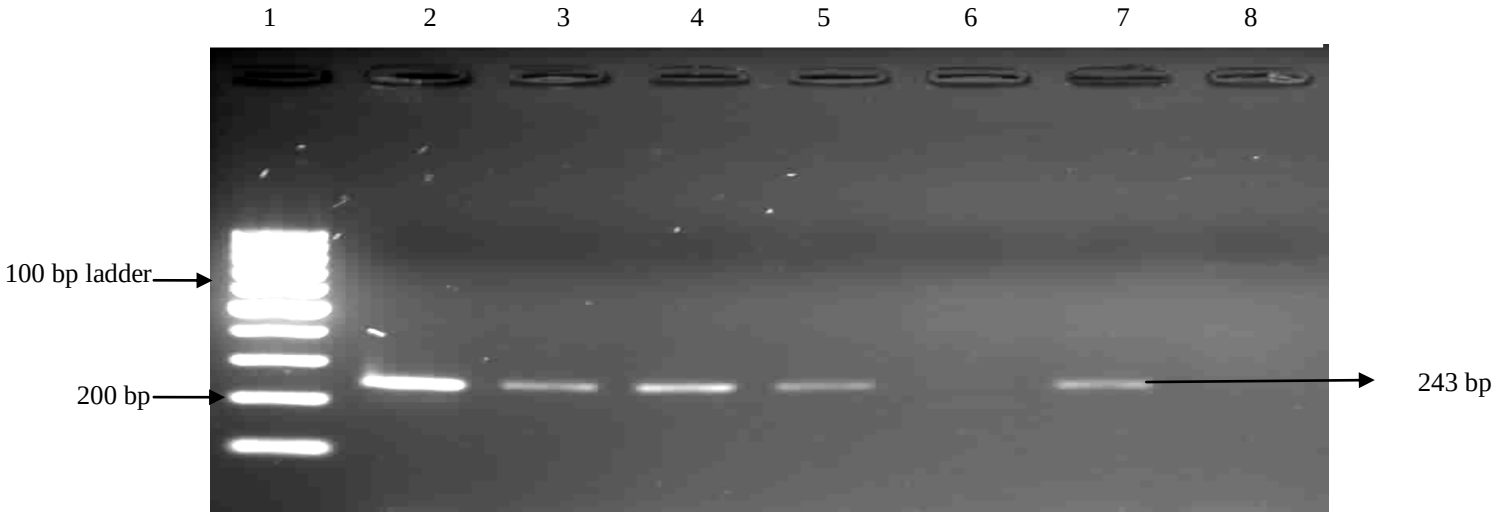


Fig.5.3.2: Agarose gel electrophoresis of amplicons generated using the *Kat G* gene. Lane 1: 100 bp ladder; lane 2: positive control; lane 3-5 and 7: DNA isolates; lane 8: negative control

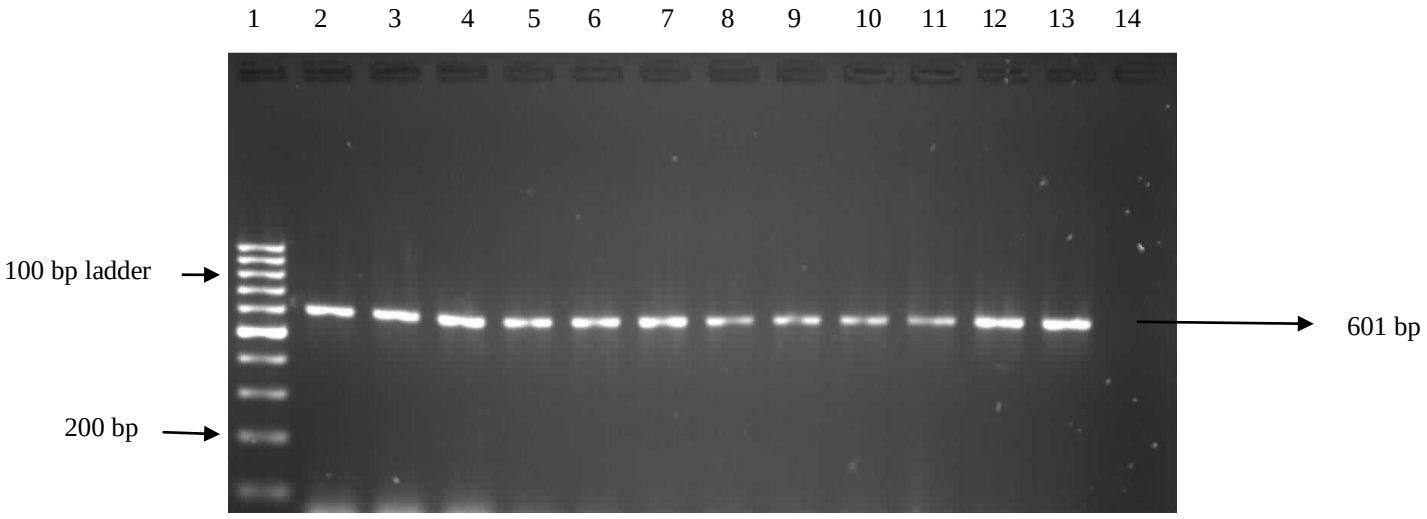


Fig.5.3.3: Agarose gel electrophoresis of amplicons generated using the *eis* gene. Lane1: 100 bp ladder; lane 2: positive control; lane 3-13: DNA samples; lane 14: Negative control.

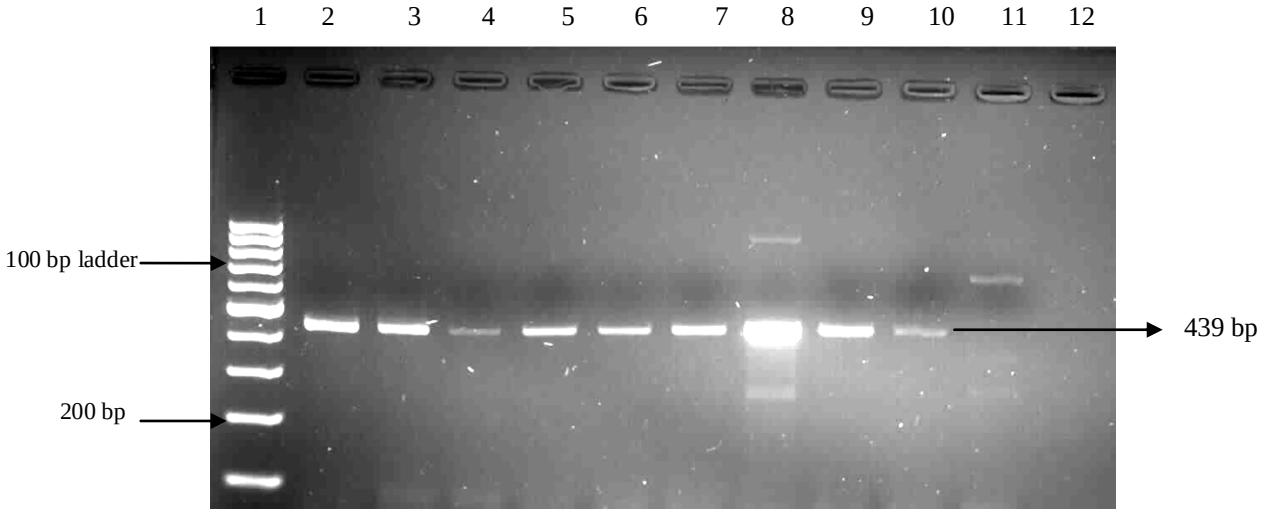


Fig.5.3.4: Agarose gel electrophoresis of amplicons generated using the *rrs* gene showing a 439bp amplicon. Lane 1: 100 bp ladder; lane 2: positive control; lane 3- 10 DNA isolates; lane 12: negative control. Lane 8 there are 3 bands which might mean there was contamination and lane 11 the band was at a higher position than the desired band size which could also mean there was contamination.

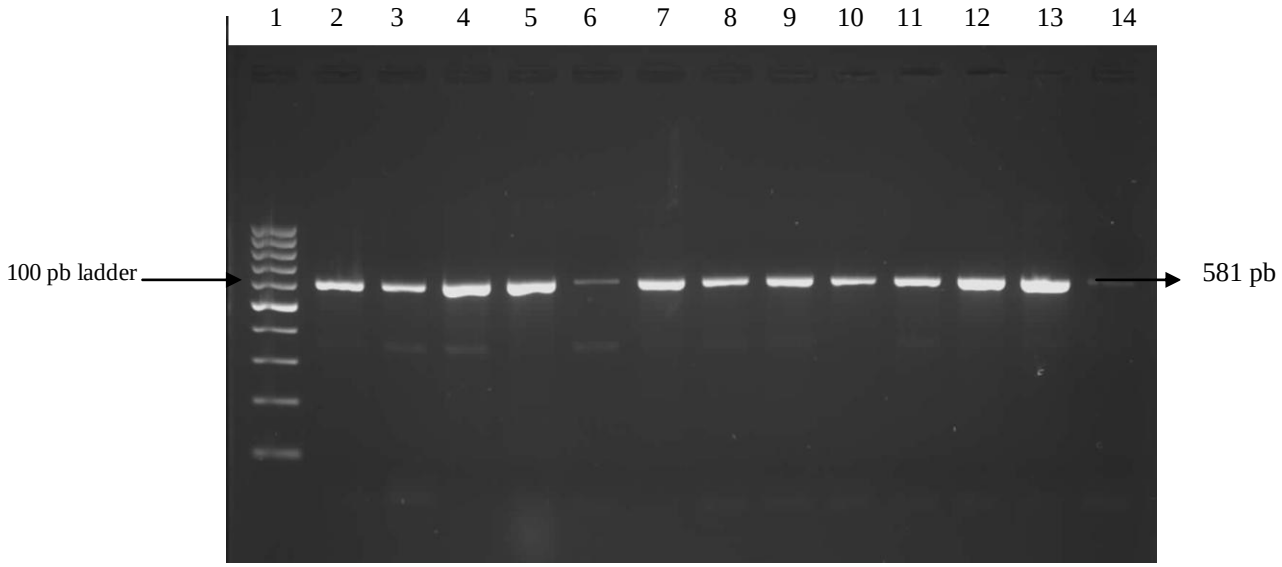


Fig.5.3.5: Agarose gel electrophoresis of amplicons generated using the *gyrA* gene. Lane1: 100 bp ladder; lane 2: positive control; Lane 3-14 DNA samples.

Drug resistance in MTBC develops due to random genetic mutations in genes conferring resistance (Zhang *et al*., 2000). The genes that confer resistance to RIF, INH, EMB, SM and PZA include the *rpoB, kat G*, *embA, rrs, rpsL* and *pncA* (Ahmed *et al*., 2000). In our study we observed INH-resistant strains in 100% (190/190) with missense mutations at codon 315 which were found in almost two- thirds of the isolates analyzed. The *kat G* gene encodes the catalase peroxidise enzyme (Green *et al*., 2008) and is present in variable regions of the MTBC genome and contains a repetitive DNA sequence. A study by Van Doorn *et al*. (2001) and Bokonyte *et al*. (2003) showed high INH-resistant strains that had mutations on codon 315 (S to T) with St Petersburg of Russia (92%), Lithuania (85.7%) and Netherlands (89%). Other isolates of our study includes 35.7% (5/14) (S t0 N), this mutation was also seen in isolates from Spain (Herera-León *et al*., 2005), 7.1% (1/14) (T to S0 and 7.1% (1/14) (T to N) which does not have much information reported on. The high percentage of mutation at

codon 315 and different substitutions demonstrates the importance of this codon in the development of INH resistance among strains from Port Elizabeth. A mutation at codon 293 seems to be rare and not much information on it has been given. In our samples two mutation 14.3% (2/14) were found at this position. We suggest that codon 293 is involved in the resistance mechanism in this isolates.

A mutation 7.1% (1/14) (R to L) was also seen in the samples. Mutations from this position have been reported before by Haas *et al*. (1997) from African strains. The very same mutations were reported by Musser *et al*. (1996) who suggested an ancestral *katG* (R) genotype for 127 isolates of *M. microti, M. bovis* and *M. africanum* (Haas *et al*., 1997). Before the report of Walter *et al*. (1997), the R mutation at position 463 was described for only *M. tuberculosis* (Walter *et al*., 1997). In the kat G gene region, seven different mutations were observed, 35.7% (5/14), 14.3% (2/14) and 7.1% (1/14) five mutations (Table.5.2.3). Two isolates did not have any mutations.

Table 5.3.1: Frequency of mutations in *katG* gene codons 293, 315 and 463 in 14 INH-resistant strains of *M. tuberculosis* complex

	***katG* gene mutation positions**						
	$N_{293} \rightarrow G$	$S_{293} \rightarrow G$	$S_{315} \rightarrow T$	$S_{315} \rightarrow N$	$T_{315} \rightarrow N$	$T_{315} \rightarrow S$	$R_{463} \rightarrow L$
No. of strains (%)	1 (7.1%)	1 (7.1%)	2 (14.3%)	5 (35.7%)	1 (7.1%)	1 (7.1%)	1 (7.1%)

N= Asparagine; G= Glycine; S= Serine; T= Threonine; R= Arginine; L= Leucine.

We also observed RIF-resistant strains in 99.5% (189/190) of the isolates with different mutations on the *rpoB* gene of 14 different strains were analysed. The results obtained from this study were interesting; hence we observed other mutations that have not been reported previously ($Y_{42} \longrightarrow D$). In our study the prevalent mutation among the RIF-resistant isolates was at codon 42 (21.4%) and not much information has been published concerning this mutations. This mutation was followed by 14.3% of mutations on codon 52 (G $\longrightarrow$ A). Codons 87 (H $\longrightarrow$G); 92 (L$\longrightarrow$ S); 441 (L$\longrightarrow$S); 450 (L$\longrightarrow$P) and 457 (showed 7.1% (1/14). Codon 531 is known to be a hot spot for *rpoB* gene mutations (Kapur *et al*., 1994).

This mutation is reported in most studies, one of the studies was reported in Taiwan where there were 54.9% mutations in codon 531 (Kapur *et al*., 1994). These mutations (at codon 531) were also observed in other countries such as Germany 71% (Tracevska *et al*., 2002), Italy 59% (Jou *et al*., 2005), Greece 56% (Mtsiota *et al*., 1998), Japan 43% (Ohno *et al*., 1996) and Mozambique 21% (Cougant *et al*., 1999). There were seven different mutations on this gene that were observed. The mutations are highlighted in (Table 5.2.4) below;

Table 5.3.2: Frequency of mutations in *rpoB* gene codons 42, 52, 87, 92, 441, 450 and 457 in 14 RIF-resistant strains of *M. tuberculosis* complex

	***rpoB* gene mutation positions**						
	$Y_{42} \rightarrow D$	$G_{52} \rightarrow A$	$H_{87} \rightarrow G$	$L_{92} \rightarrow S$	$V_{441} \rightarrow G$	$L_{450} \rightarrow S$	$L_{457} \rightarrow P$
No. of strains (%)	3 (21.4%)	2 (14.3%)	1 (7.1%)	1 (7.1%)	1 (7.1%)	1 (7.1%)	1 (7.1%)

Y= Tyrosine; D=Aspartic; G= Glycine; A= Alanine; H= Histidine; L= Leucine; S= Serine; V= Valine; P= Proline.

The *rrs* gene it consist of injectable anti-TB drugs such as amikacin (AMK), kanamycin (KAN) and capreomycin (CAP) (WHO, 2011). Proper use of injectable drugs is critical to the effective treatment of MDR-TB and in prevention of XDR-TB (WHO, 2011). Mycobacterium culture and susceptible testing in media either solid or liquid relies on conventional diagnosis of MTBC strains (Pfyffer *et al*., 1999). This method is not reliable for the detection of injectable drugs resistance (Pfyffer *et al*., 1999). AMK and KAN bind to the 16S rRNA in the 30S ribosomal subunit and inhibit protein synthesis (Magnet *et al*., 2005) and CAP interferes with translation and inhibits phenylalanine synthesis in mycobacterial ribosome (Trnka and Smith, 1970). Mutations in the MTBC that prevents the binding of the injectable drugs to the targeted pathogen gene have been associated with resistance to the three injectable drugs (Feurerriegel *et al*., 2009; Honore *et al*., 1995).

In our study among the 12 isolates sequenced, four types of mutations were observed in the rrs region; S2170A, R2201G, K2202E and a deletion in position 2207. The most observed mutation within the region was an S to A 100% (12/12) substitution at position 2170 followed by 58.3% silent mutation (7/12) R to R, 66.7% (8/12) of K to E and a deletion 41.7% (5/12) at position 2207. These mutations are shown in table 5.2.5; we note that the mutations shown in the table are more than the number of isolates that were sent for sequencing. This is because one isolate had more than one mutation in it which therefore increased the number of mutations that are seen in table 5.2.5. Mutations associated with injectable drug resistance are under studied in comparison with mutations associated with first-line drugs (Johnson *et al*., 2006). Most studies have reported on C1143G and T1521C in the *rrs* gene. In our study, these mutations were not found from our isolates and have not been described as conferring resistance (Jugheli *et al*., 2009). Studies by Maus *et al*. (2005) and Krüüner *et al*. (2003) has reported on mutations in the 500 *rrs* region A514C and C417T,

even these mutations were not found in our study. Not much of reports have been made from this gene on studies by other researchers.

Table 5.3.3: Frequency of mutations in *rrs* gene showing nucleotide change in 12 *rrs*-resistant strains of *M. tuberculosis* complex

	***rrs* gene mutation positions**			
	$S_{2169,70} \longrightarrow A$	$R_{2201} \longrightarrow R$	$K_{2202} \longrightarrow E$	$Deletion_{2207}$
No. of strains (%)	12 (100%)	7 (58.3%)	8 (66.7 %)	5 (41.7 %)

S= Serine; A= Alanine; R= Arginine; K= Lysine; E= Glutamic acid.

The last gene that we sequenced was the *gyrA* gene. The DNA gyrase encoded by *gyrA* and *gyrB* in *M. tuberculosis* is the main target of fluoroquinolones (Xu *et al.*, 1996). Mutations in two short regions known as quinolone resistance-determining regions have been associated with fluoroquinolone resistance in *M. tuberculosis* (Xu *et al*., 1996). Not much of information has been reported on *gyrA* mutations. Some reports show that mutations on Ala-90 and Asp-94 of *gyrA* have been the most commonly found (Alangaden *et al*., 1995; Kam *et al*., 2006; Pitaksajjakul *et al*., 2005). Bacterial DNA gyrase, found in all bacteria is a proven target for antibacterial chemotherapy (Ferrero *et al*., 1994). Fluoroquinololes a class of systematic antibacterial agents inhibit bacterial DNA gyrase and topoisomerase IV in most bacterial species and cause bacterial cell death (Miyamoto *et al.*, 1990).

In the study the results we got when the sequences were sent to the nBLAST (www.ncbi.nlm.nih.gov/) we got hypothetical proteins. Hypothetical proteins are proteins predicted from nucleic acid sequences and have not shown to exist by experimental protein chemical evidence (Lubec *et al*., 2005). The hypothetical proteins are of utmost importance to complete genomic and proteomic information. In this study we got hypothetical proteins where no gene name could be detected in public available databases. The sequences of the three genes (*katG, rpoB* and *rrs*) were used to draw the phylogenic trees showing the relativeness of the different drug resistant isolates.

Fig.5.3.6: A phylogenic tree showing the relativeness of isolates resistant to the Isoniazid (*KatG* gene). K82-*KatG* gene, K97-*katG*, K70-*katG*, K31-*katG*, K8-*katG*, F98-*katG*, K99-*katG*, F101-*katG*, K5-*katG*, K17-*katG* and K37-*katG* were the isolates that were used for drawing the phylogenic tree. *M. tuberculosis KatG* accession number X68081 was used as a reference strain which is a resistant strain to the antibiotic investigated.

From the diagram above we can see that the trees are from the same taxa but the strains differ from the reference strain. The reference strain is closely related to the taxa of the gene and the other strains differ from it. The seven strains in one group have a 100% similarity to each other and also the four strains in one group they have a 100% similarity to each other and they differ from the reference strain. This is shown by their distance from the taxa it is longer than that of the reference strain. The difference of the strains from the reference strain might be due to the mutations that has occurred to the resistant strains.This is seconded by what was mentioned ealier in this chapter about this gene, the mutations that are observed from the different strains.

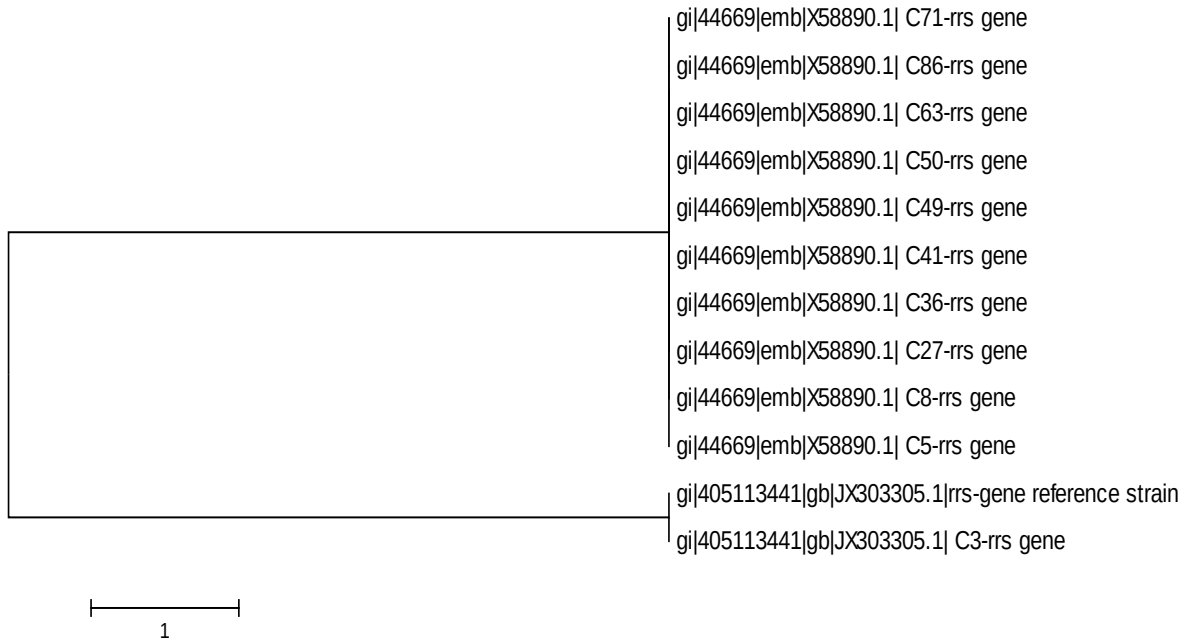


Fig.5.3.6: A phylogenic tree showing the relativeness of isolates resistant to the injectable drugs (*rrs* gene). C71-rrs gene, C86-rrs, C63-rrs, C50-rrs, C49-rrs, C41-rrs, C36-rrs, C27-rrs, C8-rrs, C5-rrs and C3-rrs are the isolates that were used for drawing the phylogenic tree. *M. tuberculosis rrs* accession number X303305 was used as a reference strain which is a resistant strain to the antibiotic investigated.

The above tree shows the relativeness of the different strains for the *rrs* gene. The reference strain and the other strains are from the taxa of the gene with the other strains and the difference is not much hence the distance from the taxa to the strain is equal. Ten strains in one group have a 100% similarity to each other and also the two strains in the other group they have a 100% similarity to each other and the second strain in the second group was not different from the reference strain. This could be due to the fact that there was silent mutation that was found from the strains. With the other group with ten strains, the difference of the

strains from the reference strain might be due to the similar mutations that have occurred to the resistant strains.

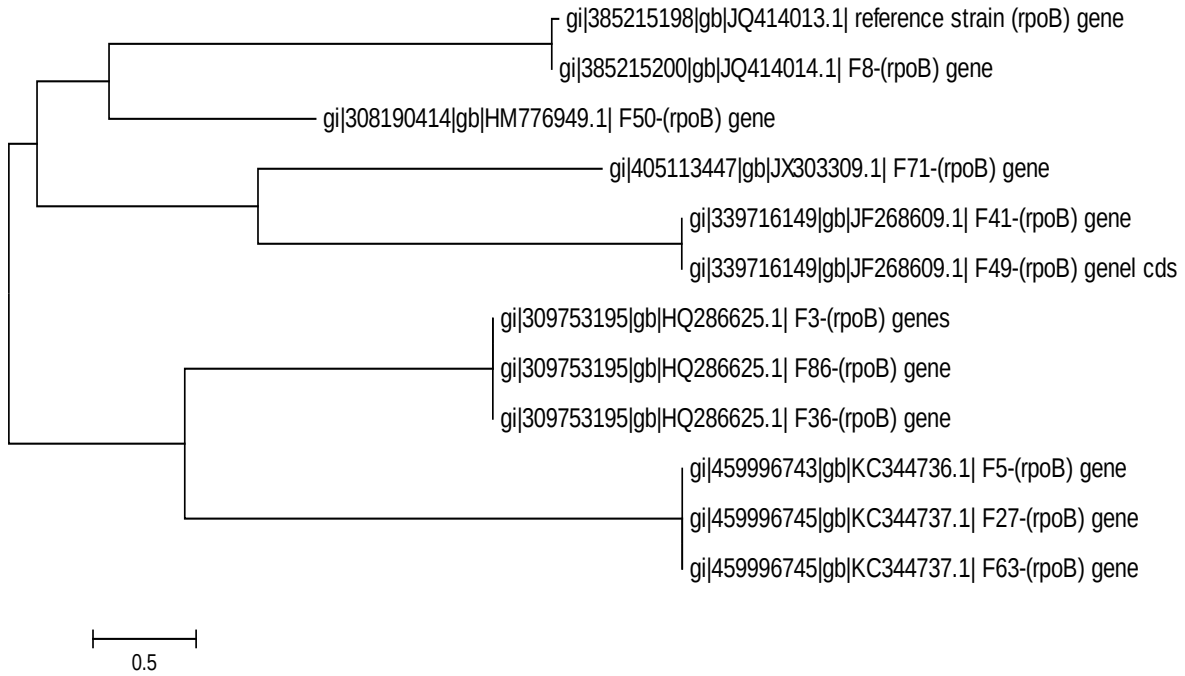


Fig.5.3.8: A phylogenic tree showing the relativeness of isolates resistant to the injectable drugs (*rpoB* gene). F27-*rpoB* gene, F63-*rpoB* gene, F5-*rpoB* gene, F50-*rpoB* gene, F36-*rpoB* gene, F-3 *rpoB* gene, F-*86*-rpoB, F8- *rpoB*, F71-rpoB, F41-rpoB and F49-rpoB are the isolates that were used for drawing the phylogenic tree. *M. tuberculosis rrs* accession number JQ414013.1 was used as a reference strain which is a resistant strain to the antibiotic investigated.

The above tree shows the relativeness of the different strains for the *rpoB* gene. The reference strain and the other strains are from the taxa of the gene with the other resistant strains. The difference between the strains is seen in this tree. The distance from the taxa to the reference strain is shorter than the other four groups this simple means the reference strain is more similar to the taxa. Six groups of the resistant strains have a 100% similarity to each other

and also there is one strain (strain F8 *rpoB*-gene) that have a 100% similarity to the reference strain. There is also a strain (F50 *rpoB*-gene) that is seen to be a little shorter than the reference strain. This means it is closely related to the taxa. The reason of such a tree with six different could be due to the fact that there were different mutations that were observed within the strains although there were common mutations. The difference of the strains from the reference strain might be due to the mutations that have occurred to the resistant strains.

5.4. Conclusions

This study provides valuable data on the different kinds of mutations occurring at various target loci in resistant MTBC strains isolated from samples obtained from the National Laboratory Service in Port Elizabeth. The results obtained reveal a high prevalence of MDR amongst the Port Elizabeth villagers. Most of MDR burdens fall in developing countries in which routine surveillance does not usually include sequence analysis and not much is known about the circulating resistant strains together with the mutation positions. This is specially seen in Port Elizabeth where not much of the information has been reported on the mutations found in *Mycobacterium tuberculosis* resistant genes. Not much information has been published on mutations found in the *gyrA* gene. In future, application of rapid methods to break chains of ongoing transmission of drug resistant TB will increasily become importens as indicated by mathematical modelling that the MDR-TB burden cannot be contained in the absence of specific efforts to limit transmission and this may include rapid detection methods of drug resistance such as geneXpert.

REFERENCES

Ahmed S, Araj G.F, Akbar P.K, Fares E, Chugh T.D & Mustafa A.S (2000). Characterization of *rpo*B mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates from the Middle East. Journal Diagnostic Microbiology and Infectious Diseases. 38:227-232.

Alangaden G.J, Manavathu E.K, Vakulenko S.B, Zvonok N.M &. Lerner S.A (1995). Characterization of fluoroquinolone-resistant mutant strains of *Mycobacterium tuberculosis* selected in the laboratory and isolated from patients. Antimicrobial. Agents and Chemotherapy. 39:1700-1703.

Anilkumar A.K, Madhavilatha G.K, Laiza K.P, Radhakrishnan I & Kumar A.R (2011). Sathish MundayoorStandardization and evaluation of a tetraplex polymerase chain reaction to detect and differentiate Mycobacterium tuberculosis complex and nontuberculous Mycobacteria—a retrospective study on pulmonary. TB patients Diagnostic Microbiology and Infectious Disease. 72:239-247.

Bokonyte D, Baranauskaie A, Gcenaite J, Sosnovkaja A & Stakenas P (2003). Molecular characterization of isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates in Lithuania. Antimicrobial Agents and Chemotherapy. 47(6):2009-2011.

Cougant D.A, Sandven P, Eng J, Jeque J.T & Tønjum T (1999). Detection of rifampicin resistance among isolates of *Mycobacterium tuberculosis* from Mozambique. Microbial Drug resistant. 4:321-326.

Cox H.S, McDermid C, Azevedo V, Muller O, Coetzee D, Simpson J. *et al.* (2010). Epidemic levels of drug resistant tuberculosis (MDR and XDR-TB) in a high HIV prevalence setting in Khayelitsha, South Africa. PLoS ONE. 5:e13901.http://dx.doi.org/10.1371/journal.pone.0013901

Ferrero L, Cameron B, Manse B, Lagneaux D, Crouzet J & Blanche F (1994). *Mycobacterium tuberculosis* DNA Gyrase as a Target for Drug Discovery. Molecular Microbiology. 13:641.

Feuerriegel S, Cox H.S, Zarkua N, Karimovich H.A & Braker K (2009). Sequence analyses of just four genes to detect extensively drug-resistant Mycobacterium tuberculosis strains in multidrug-resistant tuberculosis patients undergoing treatment. Antimicrobial Agents and Chemotherapy. 53:3353-3356. doi: 10.1128/AAC.00050-09.

Global Alliance for TB Drug Development (2013). http://www.tballiance.org/why/mdr-xdr.php

Green E, Obi L.C, Nchabeleng M, de Villiers B.E, Sein P.P, Letsoalo T. *et al.* (2008). Molecular characterisation of resistant Mycobacterium tuberculosis isolates from Dr George Mukhari Hospital, Pretoria, South Africa. South African Journal of Epidemiology Infection. 23(3):11-14.

Haas W.H, Schilke K, Brand J, Amthor B, Weyer K, Fourie B. *et al.* (1997). Molecular Analysis of *katG* Gene Mutations in Strains of *Mycobacterium tuberculosis* Complex from Africa. Antimicrobial Agents and Chemotherapy. 1601–1603. 0066-4804/97/$04.0010.

Herrera-Leon L, Molina T, Saiz P, Saez-Nieto J.A & Jimenez M.S (2005). New multiplex PCR for rapid detection of isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates. Journal of Antimicrobial Agents and Chemotherapy. 49(1): 144-147.

Honore N, Marchal G & Cole S.T (1995). Novel mutation in 16S rRNA associated with streptomycin dependence in Mycobacterium tuberculosis. Antimicrobial Agents and Chemotherapy. 39:769-770. doi: 10.1128/AAC.39.3.769.

Jadaun G.P.S, Das R, Upadhyay P, Chauhan D.S, Sharma V.D & Katoch V.M (2009). Role of embCAB gene mutations in ethambutol resistance in *Mycobacterium tuberculosis* isolates from India. International Journal of Antimicrobial Agents. 33:483-486.

Johnson R, Warren R, Strauss O.J, Jordaan A.M, Falmer A.A, Beyers N. *et al*. (2006). An outbreak of drug-resistant tuberculosis caused by a Beijing strain in the Western Cape, South Africa. International Journal of Tuberculosis and Lung Disease. 10:1412-1414.

Johnson R, Warren R.M, van der Spuy G.D, Gey Van Pittius N.C, Theron D, Streicher E.M. *et al*. (2010). Drug-resistant tuberculosis epidemic in the Western Cape driven by a virulent Beijing genotype strain. International Journal of Tuberculosis and Lung Disease. 14:119-121.

Jou R, Chen H.Y, Chiang C.Y, Yu M.C & Su I.J (2005). Genetic diversity of multidrug-resistant *Mycobacterium tuberculosis* isolates and identification of 11 novel *rpo*B alleles in Taiwan. Journal of Clinical Microbiology. 43(3):1390-1394.

Jugheli L, Bzekalava N, de Rijk P, Fissette K, Portaels F & Rigouts L (2009). High level of cross resistance between kanamycin, amikacin and capreomycin among *Mycobacterium tuberculosis* isolates from Georgia and a close relation with mutations in the *rrs* gene. Antimicrobial Agents and Chemotherapy. 53(12):5064-5068.

Kam K.M, Yip C.W, Cheung T.L Tang H.S, Leung O.C & Chan M.Y (2006). Stepwise decrease in moxifloxacin susceptibility amongst clinical isolates of multidrug-resistant *Mycobacterium tuberculosis:* correlation with ofloxacin susceptibility. Microbial Drug Resistant. 12:7-11.

Kapur V, Li L-L & Iordanescu S (1994). Characterization by automated DNA sequencing of mutations in the gene (*rpo*B) encoding the RNA polymerase b subunit in rifampicin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. Journal of Clinical Microbiology. 32:1095-1098.

Klopper M, Warren R.M, Hayes C, Pattius N.C.G, Streicher E.M, Müller B. *et al.* (2013). Emergency and spread of extensively and totally drug-resistant tuberculosis, South Africa. Centers for Disease Control and Prevention. 19(3):1-7.

Krüüner A, Jureen P, Levina K, Ghebremichael S & Hoffner S (2003). Discordant resistance to kanamycin and amikacin in drug-resistant *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 47:2971–2973.

Kvasnovsky C.L, Cegielski J.P, Erasmus R, Siwisa N.O, Thomas K & der Walt M.L (2011). Extensively drug-resistant TB in Eastern Cape, South Africa: high mortality in HIV-negative and

HIV-positive patients. Journal of Acquired Immune Deficiency Syndromw. 57:146-152. http://dx.doi.org/10.1097/QAI.0b013e31821190a3

Lubec G, Afehi-Sadat L, Yang J & Pradero J.P (2005). Searching for hypothetical proteins: Theory practice based upon original data and literature. Progress in Neurobiology. 77:90-127.

Magnet S & Blanchard J.S (2005) Molecular insights into aminoglycoside action and resistance. Chem Rev 105: 477–498. doi: 10.1021/cr0301088.

Matsiota-Bernard P, Vrioni G & Marinis E (1998). Characterization of *rpo*B mutations in rifampicin-resistant clinical *Mycobacterium tuberculosis* isolates from Greece. Journal of Clinical Microbiology. 36:20-23.

Maus C.E, Plikaytis B.B & Shinnick T.M (2005). Molecular analysis of crossresistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 49:3192–3197.

Miyamoto T, Matsumoto J, Chiba K, Egawa H, Shibamori K, Minamida A. *et al.* (1990). *Mycobacterium tuberculosis* DNA Gyrase as a Target for Drug Discovery. Journal of Medicine and Chemotherapy. 33:1645.

Musse J.M, Kapur V, Williams D.L, Kruswirth B.N, van Soolingen D & van Embeden J.D.A (1996). Characterization of catalase-peroxidase gene (*Kat G*) and *innA* locus in isoniazid-resistant and susceptible strains of *Mycobacterium tuberculosis* by automated sequencing:

restricted array of mutations associated by drug resistance. Journal of Infectious Disease. 173:196-202.

National Health Laboratory Services (2012). National Institute for Communicable Diseases annual report 2009 [cited 2012 Jan 26]. http://www.nicd.ac.za/assets/files/Annual_report_2009.pdf

Ohno H, Koga H & Kohno S (1996). Relationship between rifampicin MICs for and *rpo*B mutations of *Mycobacterium tuberculosis* strains isolated in Japan. Antimicrobial Agents and Chemotherapy. 40:1053-1056.

Olusoji D, Eltayeb O, Olanrewaju O & Olapade G.D (2013). Pre-extensive drug resistant tuberculosis (pre-XDR-TB) among MDR-TB patents in Nigeria. Global Advanced Research Journal of Microbiology. 2(2):022-025.

Pfyffer G.E, Bonato D.A, Ebrahimzadeh A, Gross W & Hotaling J (1999). Multicenter laboratory validation of susceptibility testing of Mycobacterium tuberculosis against classical second-line and newer antimicrobial drugs by using the radiometric BACTEC 460 technique and the proportion method with solid media. Journal of Clinical Microbiology 37:3179-3186.

Pitaksajjakul P, Wongwit W, Punprasit W, Eampokalap B, Peacock S & Ramasoota P (2005). Mutations in the gyrA and gyrB genes of fluoroquinolone-resistant *Mycobacterium tuberculosis* from TB patients in Thailand. The Southeast Asian Journal of Tropical Medicine Public Health 36(Suppl. 4):228-237.

Prabhu G, Shimazu H, Cerri G, Brochier T, Spinks R.L, Maier M.A. *et al*. (2009). Modulation of primary motor cortex outputs from ventral premotor cortex during visually guided grasp in the macaque monkey. The Journal of Physiology. 587:1057–1069.

Saitou N & Nei M (1987). The Neighbor-jouning methed- a new method for reconstructing phylogenic trees. Molecular Biology and Evolution. 4:406-425.

Sekiguchi J, Miyoshi-Akiyama T, Augustynowicz-Kopeć E, Zwolska Z, Kirikae F, Toyota E. *et al*. (2007). Detection of multidrug resistance in *Mycobacterium tuberculosis*. Journal of Clinical Microbiology. 45 (1):179-192.

Strauss O.J, Warren R.M, Jordaan A, Streicher E.M, Hanekom M, Falmer A.A. *et al*. (2008). Spread of a low-fitness drug-resistant *Mycobacterium tuberculosis* strain in a setting of high human immunodeficiency virus prevalence. Journal of Clinical Microbiology. 46:1514–6. http://dx.doi.org/10.1128/JCM.01938-07

Tamura K, Peterson D, Peterson N, Strecher G, Nei M & Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsiomony methods. Molecular Journal and Evolution. 28(10):2731-2739.

Tracevska T, Jansone I, Broka L & Baumanis V (2002). Mutations in the *rpo*B and *Kat*G genes leading to drug resistance in *Mycobacterium tuberculosis* in Latvia. Journal of Clinical Microbiology. 40(10): 3789-3792.

Trnka L & Smith D.W (1970) Proteosynthetic activity of isolated ribosomes of Mycobacteria and its alteration by rifampicin and related tuberculostatic drugs. Antibiotibiotics and Chemotherapy 16:369-379.

Van Doorn H.R, Kuijper E.J & Van der Ende A. (2001). The susceptibility of *Mycobacterium tuberculosis* to isoniazid and the Arg to Leu mutation at codon 463 of *kat*G are not associated. Journal of Clinical Microbiology. 39:1591-1594.

Van Rie A, Warren R, Richardson M, Gie R.P, Enarson D.A, Beyers N. *et al*. (2001). Classification of drug-resistant tuberculosis in an epidemic area. Lancet. 356:22-25. http://dx.doi.org/10.1016/S0140-6736(00)02429-6

Walter J, Capell A, Grumberg J, Pesold B, Schindzielorz A, Prior R. *et al.* ((1997). The Alzeimer's disease-associated presenilins are differentially phosphorylated proteins located predominately within the endoplasmic reticulum. Molecular Medicine. 2:273-691.

World health Organization (2012). Global Tuberculosis Control 2012, Geneva, 2012, 38 www.who.int/tb/publications/global_report/

World Health Organization (2011). Global Tuberculosis Control. Geneva: World Health Organization.

World Health Organization (2008). WHO-IUTALD Global Project on Anti-Tuberculosis Drug Resistance Surveillance. Anti-tuberculosis drug resistance in the world (report no. 4) [cited 2008

Apr 30]. http://www.who.int/tb/publications/2008/drs_report4_26feb08.pdf. (Accessed 12 August 2013).

World Health Organization (2010). Multidrug and extensively drug-resistant TB (M/XDR-TB) 2010. Global report on surveillance and response [cited 2010 Jun 26]. http://whqlibdoc.who.int/publications/2010/9789241599191_eng.pdf. (Accessed 12 July 2013).

World Health Organization (2013). http://www.foxnews.com/health/2013/10/23/who-tuberculosis-numbers-fall-but-drug-resistant-strains-on-rise/. (Accessed 11 November 2013).

Xu C, Kruswith B.N, Sreevatsan S, Musser J.M & Dilika K (1996). Journal of infectious disease. 174:1147.

Zhang Y & Telenti A (2000). Genetics of drug resistance in *Mycobacterium tuberculosis*. In: Hatfull GF, Jacobs WR. Jr. (eds). *Molecular genetics of mycobacteria*. Washington DC: ASM Press; 2000: 235-251.

CHAPTER 6

GENERAL DISCUSSION

There is a high burden of TB and MDR-TB in South Africa which is the fourth among 27 high burden MDR-TB countries worldwide and very little is known about the population structure and transmission patterns of the MDR-TB strains in most of the country's Provinces (Mlambo *et al*., 2008). The work on this study reports on the use of a molecular method, the Seeplex® MTB Nested ACE assay which is a multiplex PCR system for the detection of *M. tuberculosis* in clinical specimens such as sputum from MTBC infected patients residing in Port Elizabeth. The assay uses PCR and it amplifies the DNA. In the present study MTBC was detected in 190/190 (100%) samples. Amplicons of the positive samples corresponded to the 190 bp size which indicates the presence of MTBC DNA.

TB poses risk to public health and the use of the Seeplex® MTB Nested ACE kit may be employed as an alternative rapid diagnostic tool for screening human sputum DNA for the presence of MTBC. The method is fast and yields results quicker (approximately four hours) compared to other methods such as the culture method that takes about 42 days. Although there is a disadvantage with this method of it being expensive this makes it difficult for routine diagnosis of TB in resource limited settings. Our results show that the most infected race was the black race 160/190 (84.2%) followed by the coloured race 30/190 (15.8%) and there were no samples for the white population in this study.

The samples collected were of blacks and coloured in Port Elizabeth. We also noted that the most age group that was infected by TB was 25-44 years and this is the most active age group in communities. There was lack of information on the TB history of all the patients in this study which raises concern on the public health implications on humans interacting with this people and domestic animals such as cattle that can further transmit the organism to humans through the consumption of unpasteurized milk. The most infected patients were females while most studies report that more males are infected by MTBC in comparison with females (WHO, 2013; Onifade *et al*., 2010; WHO, 2012).

The prevalence of MTBC in Port Elizabeth villagers shows the public health problem, this is because Port Elizabeth provides job opportunities to people especially those from the Eastern Cape Province and it also attracts some illegal immigrants from some of the African countries. This therefore increases the chance of spreading the macobacterial strains amongst the villagers.

In the study we noted that the spread of tuberculosis in the community can be controlled by accurate early diagnosis and proper treatment. The diagnosis tests that have been used for some time have limitations such as low sensitivity and they take a long period of time to give results (Warren *et al*., 2006). These traditional diagnostic methods have limitations such as sensitivity, specificity and also the techniques consume a lot of time (Parkash *et al*., 2009). Due to the above mentioned limitations, a new tool was sought and they came with the Region of Differences (Warren *et al*., 2006) differentiating *Mycobacterium tuberculosis* complex (MTBC) isolates into different species. The introductions of diagnostic methods that are more sensitive and specific increase the efficiency of strategies to control tuberculosis including resistance strains of TB.

Drug resistance is a serious problem that is increasing due to improper use of chemotherapy of drug susceptible (WHO, 2013). In this study we used sequencing for the identification of mutations on the resistant strains because sequencing is said to give more results than the other methods such as the Genotype® MTBDR*plus* assay. The results indicate resistance to RIF which was observed in 189/190 (89.5%) to our interest we noted that the samples that were resistant to RIF were also resistant to INH 189/189 (100%). These findings supports the hypothesis by other authors that RIF can be used as a surrogate marker for MDR hence approximately 90% of RIF resistant *M. tuberculosis* strains are equally resistant to INH (Somoskovi *et al*., 2001; Mokrousov *et al*., 2002).

The result obtained from the study is contradictory to what several other studies reported on saying that there is a periodic improvement in MDR-TB treatment in South Africa and they are stating a change in the system used to manage MDR and XDR-TB patients (Padayatchi *et al*., 2009; Heller *et al*., 2010; Burst *et al*., 2010). In this study there is a high rate of additional (resistance to INH and RIF together with other first-line anti-TB drugs) observed in the study, we recommend that regular drug surveillance be done in South Africa. From our findings it can be indicated that among the MDR-TB isolates, more than 90% are resistant to all the first line drugs. MDR-TB is a precursor to XDR-TB and it poses challenge to the TB control programmes in the country. These findings are worrying since the MDR-TB strains seem to be established and it may be spreading as efficient as drug susceptible strains. South Africa is estimated to have a co-infection rate of TB/HIV of 70% (Abdool *et al*., 2009) and this could result in further increase in mortality.

The identification of *Mycobacterium tuberculosis* complex (MTBC) members is very important for surveillance purposes. In this study a multiplex polymerase chain reaction (PCR) method was used which amplify based on genomic regions of differences such as RD1, RD1^{mic}, RD2^{seal}, RD4, RD9 and RD12 for M. *tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti, M. africanum, M. canetti, M. pinnipediii* and *M. caprae*. The obtained results showed the size of the respective multiplex PCR amplification products corresponding to the presence of the different members of MTBC. One hundred and eighty four DNA samples were used in the study due to insufficient DNA. From the 184 DNA isolates 45 (24.5%) isolates were identified to be *M. tuberculosis,* 94 isolates (51.1%) to be *M. bovis* BCG and 3 isolates (1.6%) to be *M. canettii*. The results show that a multiplex PCR diagnostic method can be used to identify the MTBC species that cause disease in both animals and humans.

The species present amongst the patients included 4 isolates (2.2%) for M. *bovis* and 94 isolates (51.1%) for *M. bovis* BCG (Bacillus Calmette-Guérin). *M. bovis* BCG is said to cause localized abscesses, regional lymphadenopathy, and disseminated disease in immunocompromised patients (Bernatowska *et al*., 2007). The host of *M. bovis* is cattle, but a variety of domestic animals, wild animals' as well as humans can be infected with this species (Gibson *et al*., 2004). *M. bovis* is one of the most recognised members of MTBC that transmits TB from animals to humans (Angela *et al*., 2006). We observed that *M. bovis* BCG was the most identified strain from the MTBC in this study and this raises a concern because BCG is an attenuated derivative of virulent strain of *Mycobacterium bovis* (Talbot *et al*., 1996). BCG has been used as a vaccine against *M. tuberculosis,* as a cancer immune therapy and as a recombinant vehicle for multivalent vaccines against other infectious diseases (Brosman, 1992; Lugosi, 1992). We noted that BCG can also cause disease in humans more especially those with immunodeficiency (Lamm *et al*., 1992; Lugosi, 1992). This shows the

clinical importance of the ability to rapidly and specifically identify BCG. The ability to differentiate BCG from other members of *M. tuberculosis* complex is important because BCG can cause disease in humans although it is less virulent from its parent strain (Lotte *et al*., 1988). *M. bovis* BCG's failings have important implication for current efforts to develop new and better vaccines against tuberculosis. BCG vaccination cannot provide complete protection against pulmonary tuberculosis. There is a concern now that the use of the BCG in children who are immune comprised, such as children with HIV, could result in them having an infection caused by BCG vaccine itself (Flne, 2005). This is due to the fact that BCG vaccine contains a live but much weakened form of bacteria called the *Mycobacterium bovis*.

M. caprae was found in 34 isolates (18.4%). This is the main causative agent of animal tuberculosis and it has been isolated from several domestic and wild animal species (Aranaz *et al*., 2003). This pathogen has been recognized mainly in Central Europe where it was isolated from cattle (Duet *et al*., 2008) and pigs (Pavlik *et al.*, 2002). This raises concern on the public health implication on humans interacting with these domestic and wild animals as well as consumption of unpasteurized milk from the cattle. This brings us to conclude that *M. caprae* infection poses a serious health risk not only in domestic animals such as goats but also in humans. We have noted from this work that multiplex PCR can be used in the identification of the different *M. tuberculosis* complex species. The limitations we faced in the study is that we ran out of DNA while conducting the study and this resulted in us ending up using 184 DNA samples instead of the 190 that we initially started with. We could not go back to isolate DNA from the affected individuals due to the fact that some patients died, while some have been released to go to their homes. The National Health Laboratory Service (NHLS) did not grant us access to use their lab to start from culturing the organism.

6.1 Future Work

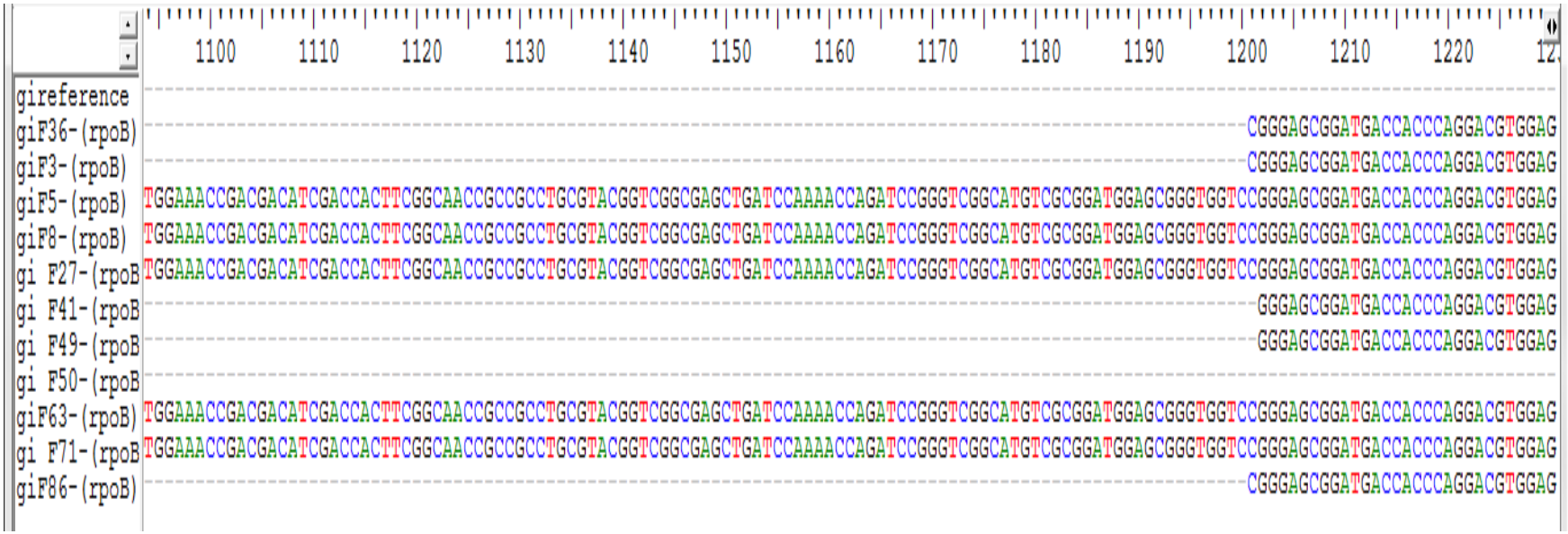
This study has provided the information on MDR and XDR-TB strains that will contribute in the understanding of the mechanisms conferring drug resistance in TB strains in the region. The serious threat of XDR-TB outbreaks in our region calls for studies into the mechanism of resistance in *M. tuberculosis* strains with extensive resistance to anti-TB drugs. We anticipate performing Spoligotyping and MIRU-VNTR and RFLP to analyse and compare our results with other laboratories and submit such information to the international Spoligotyping and MIRU-VNTR typing database that will improve on global knowledge. We also sought to further investigate the BCG identified by region of differences.

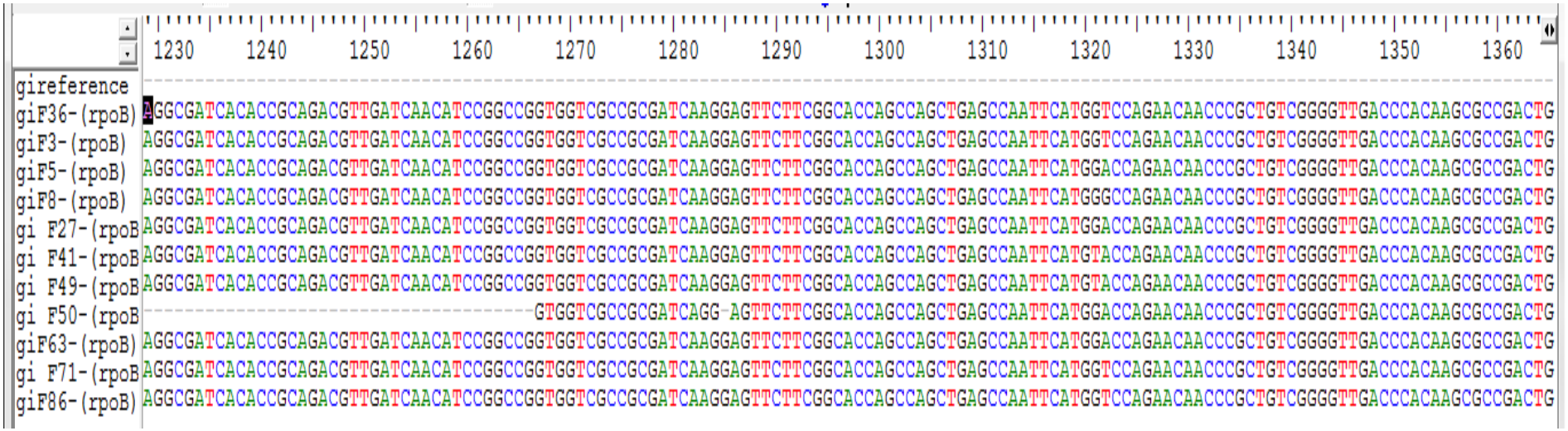
6.2 Recommendation and Conclusions

Results of this research show that resistance of MTBC to drugs seems to be increasing. This is serious health concern which calls for a need to strategise on the identification of extensive drug resistant TB patients from multi-drug resistant TB patients and ensure monitoring of their treatment to ensure total cure. The study provides valuable data on the different kinds of mutations occurring at various loci in resistant MTBC isolated in Port Elizabeth, South Africa. From this study it can be recommended that the government intensify funding for studies in development of new drugs against resistant TB and imfirmary the drug resistant TB patients.

APPENDICES

Appendix 1: *rpoB* gene allignment

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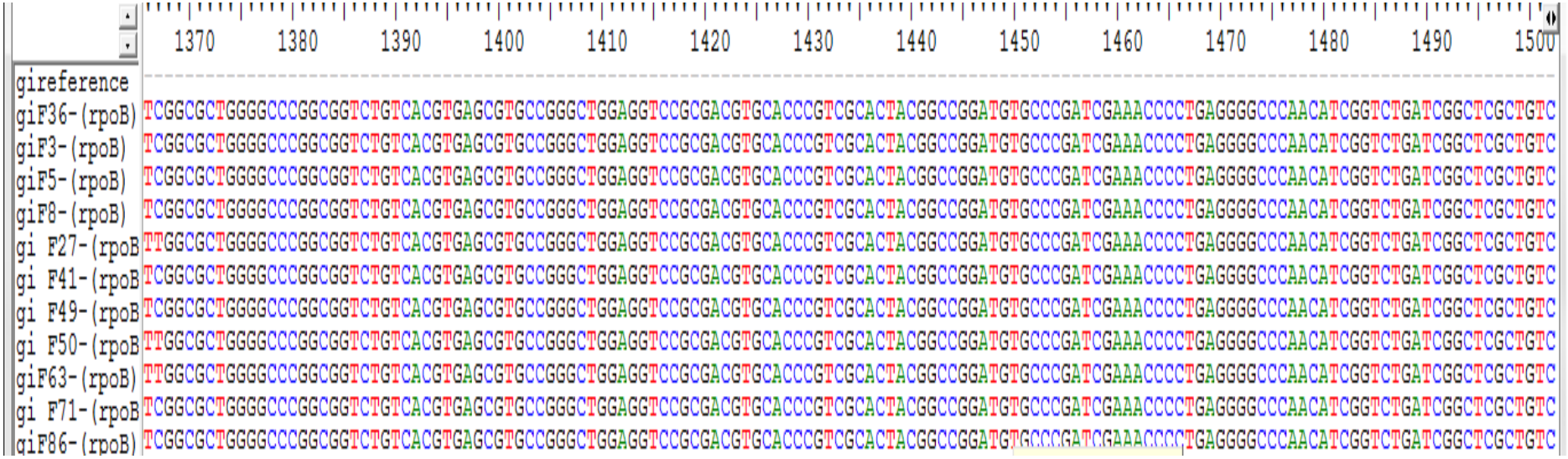
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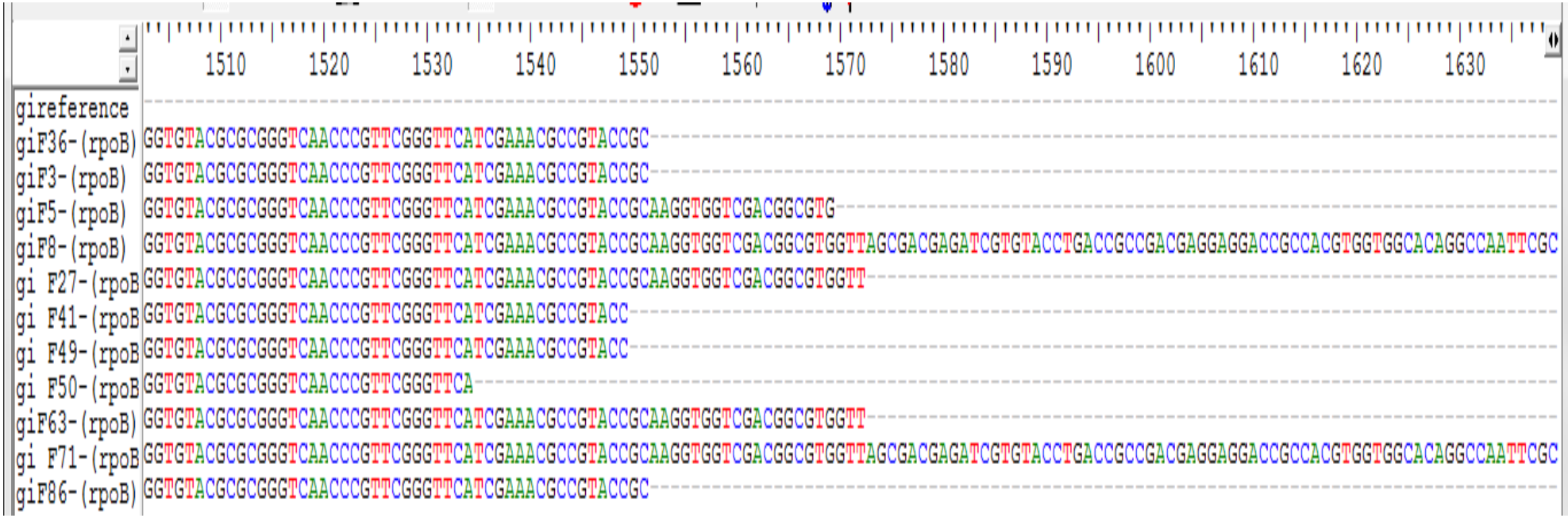
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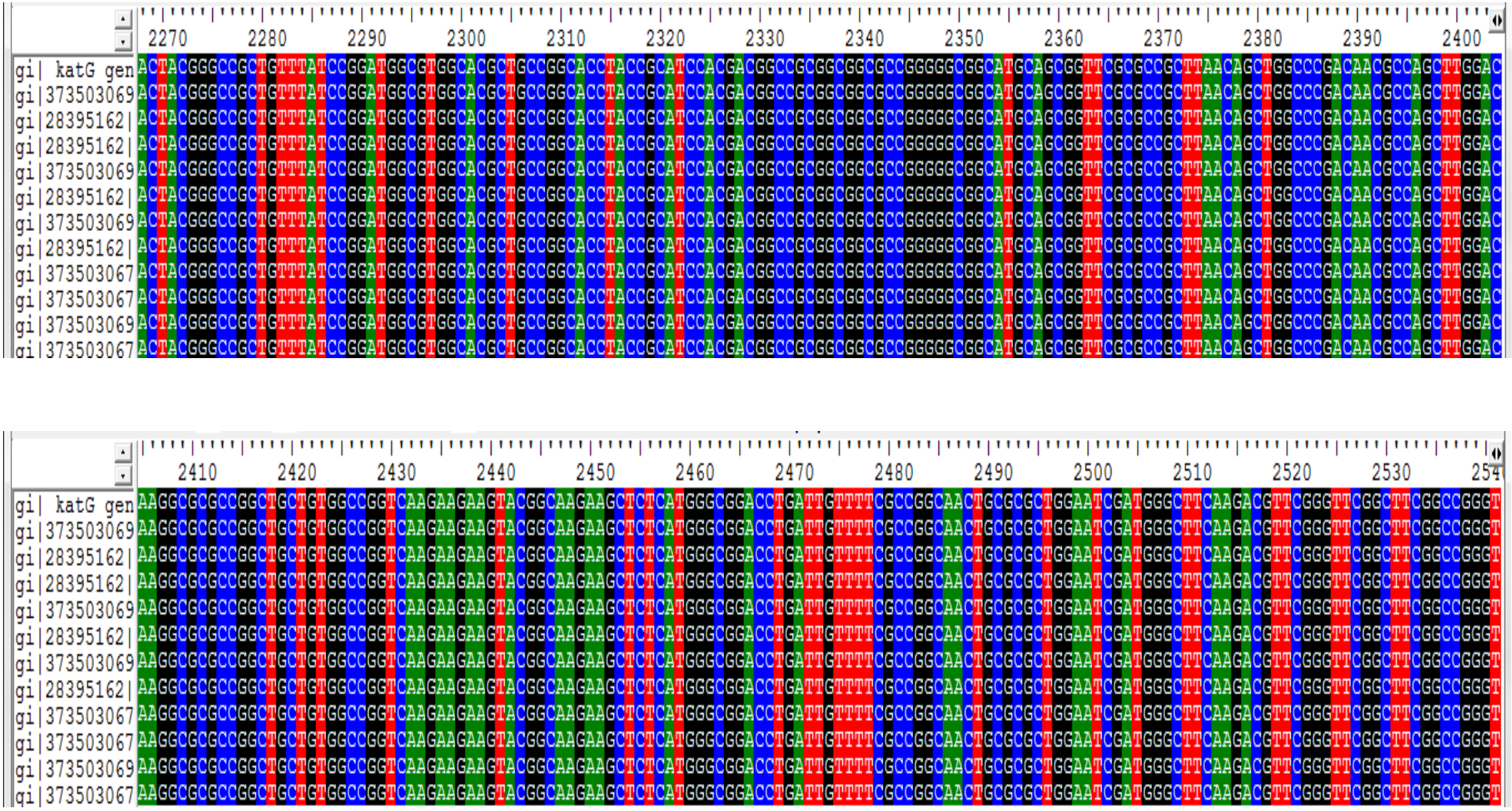
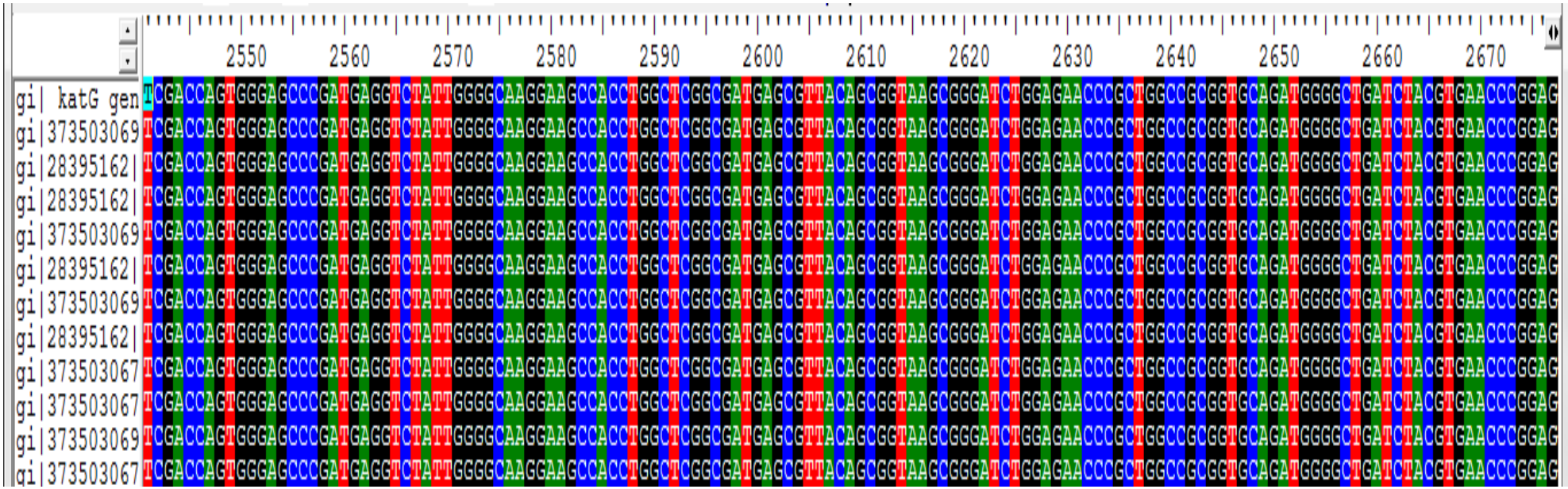
Appendix 2: *Kat G* gene alignment

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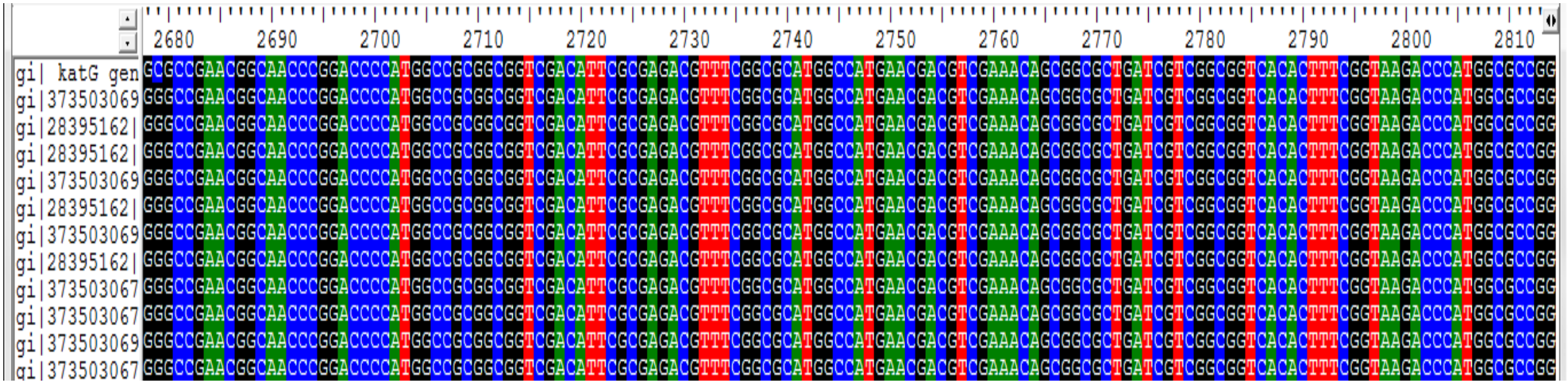
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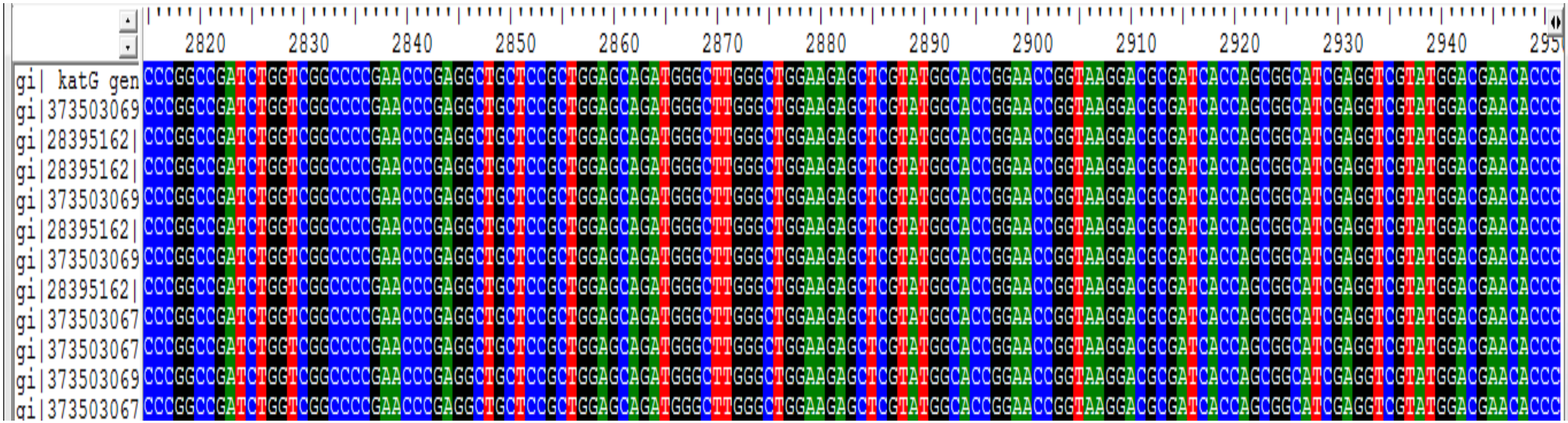
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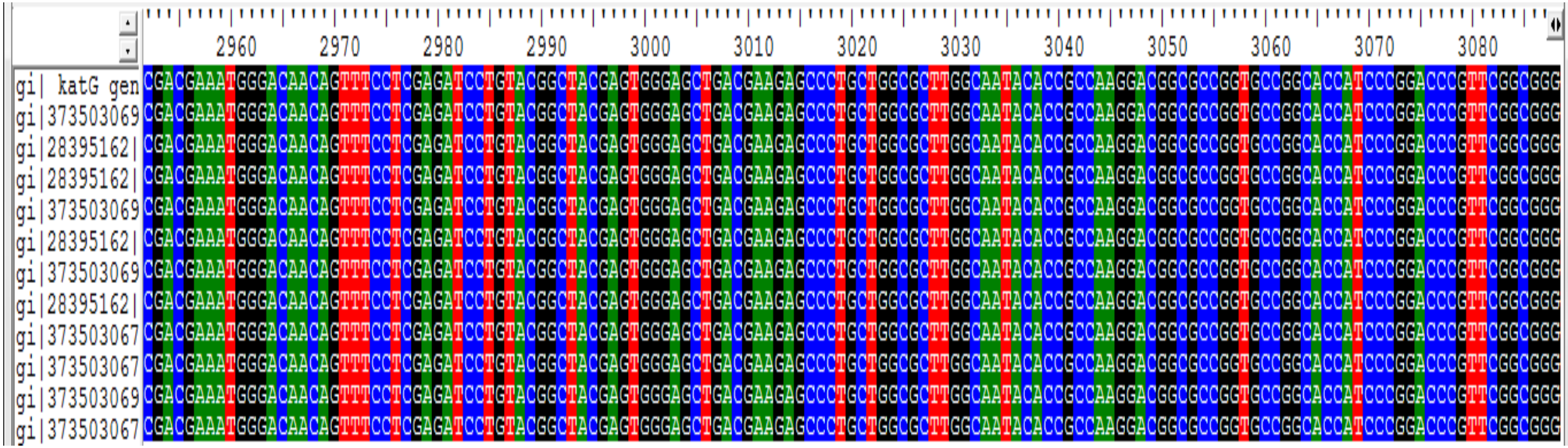
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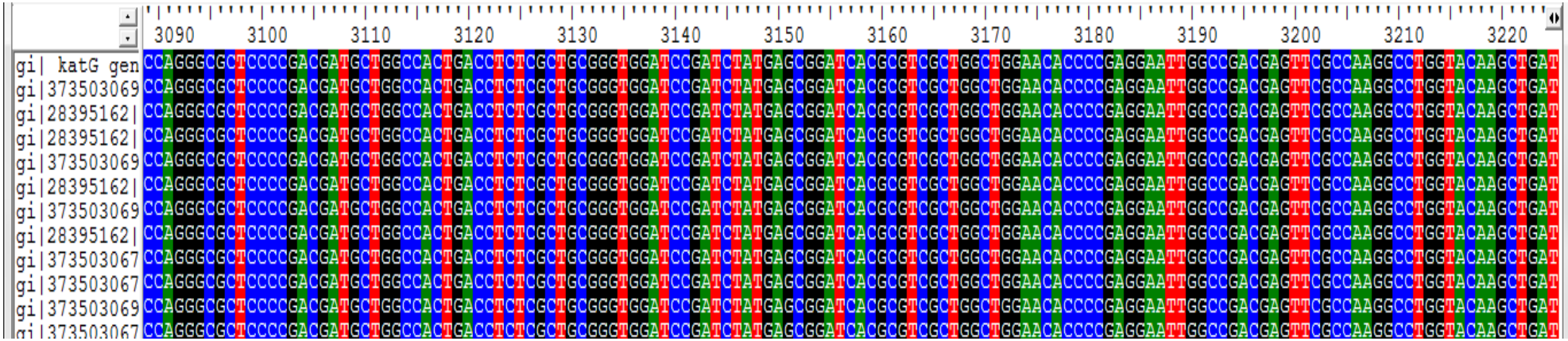
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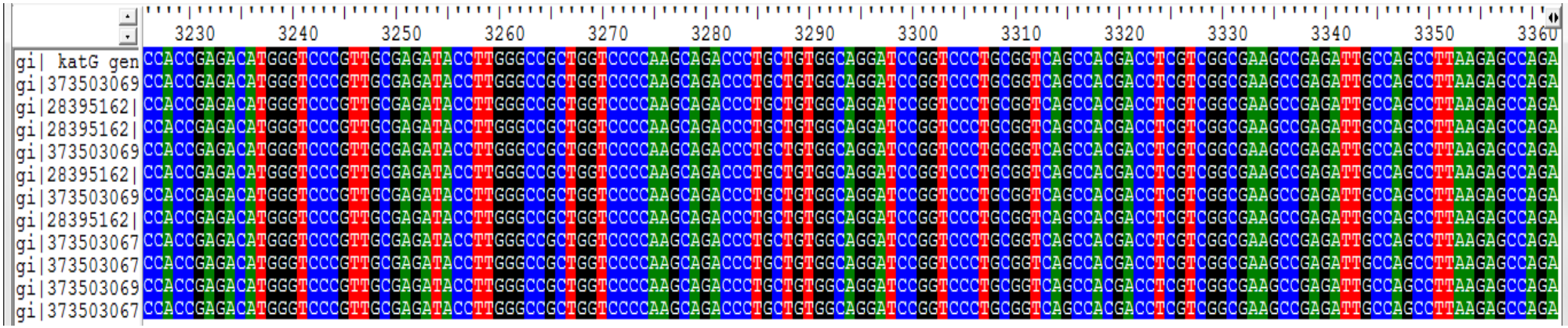
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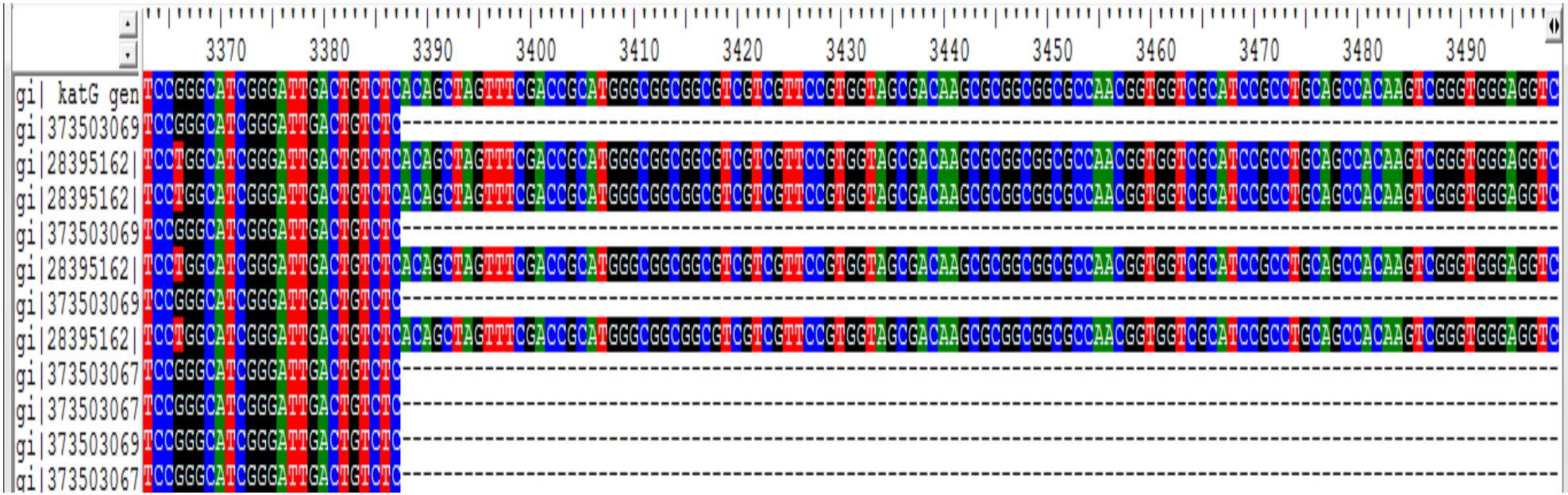
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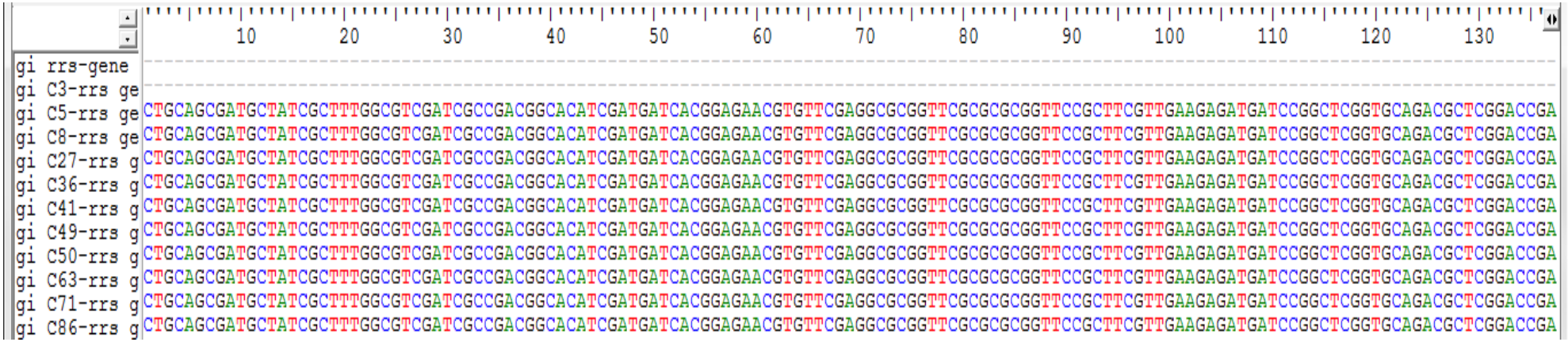
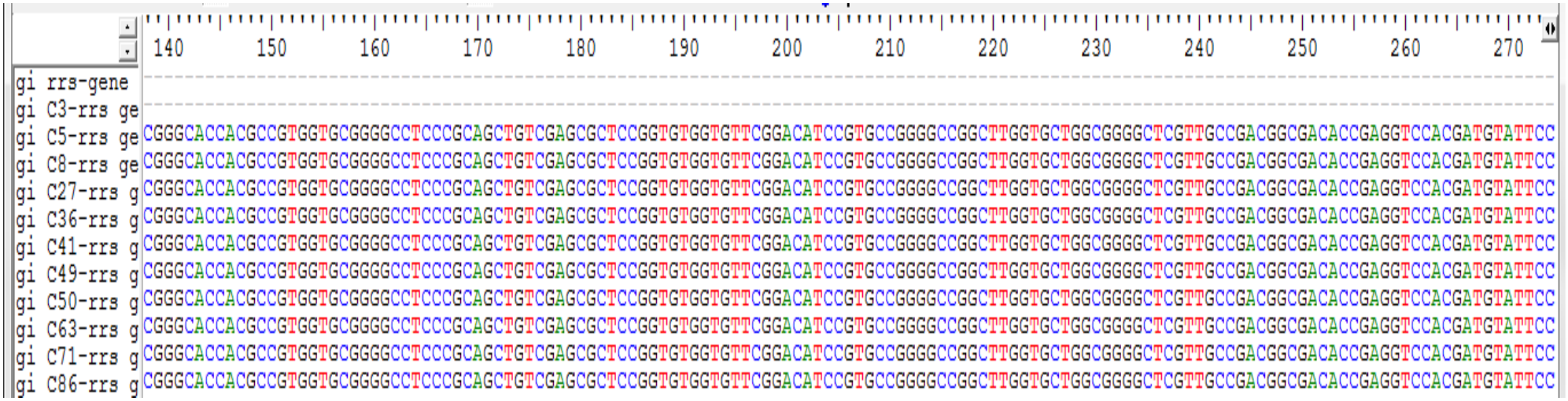
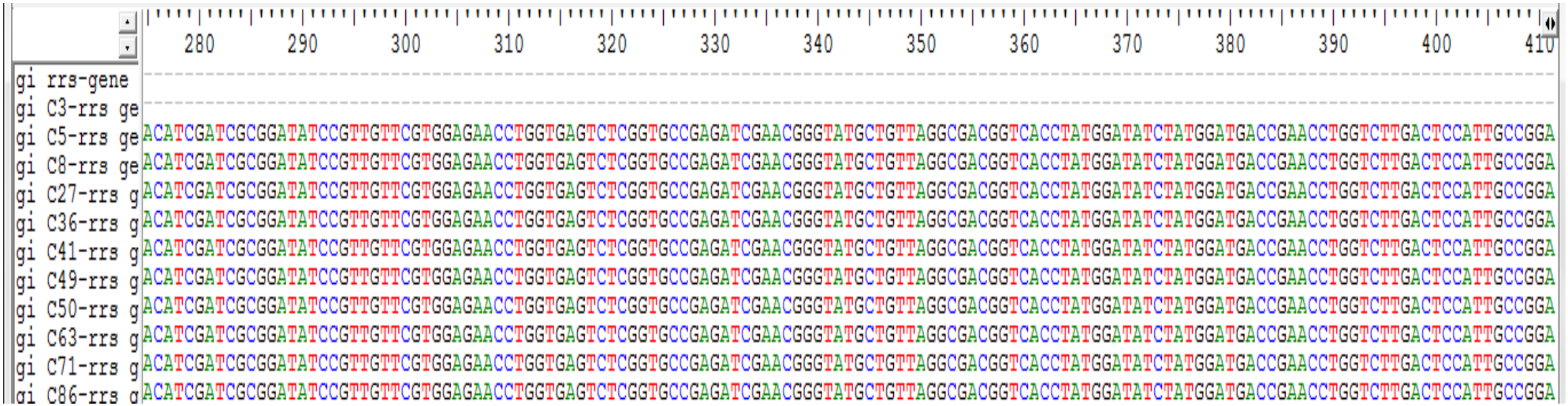
Appendix 3: *Rrs* gene alignment



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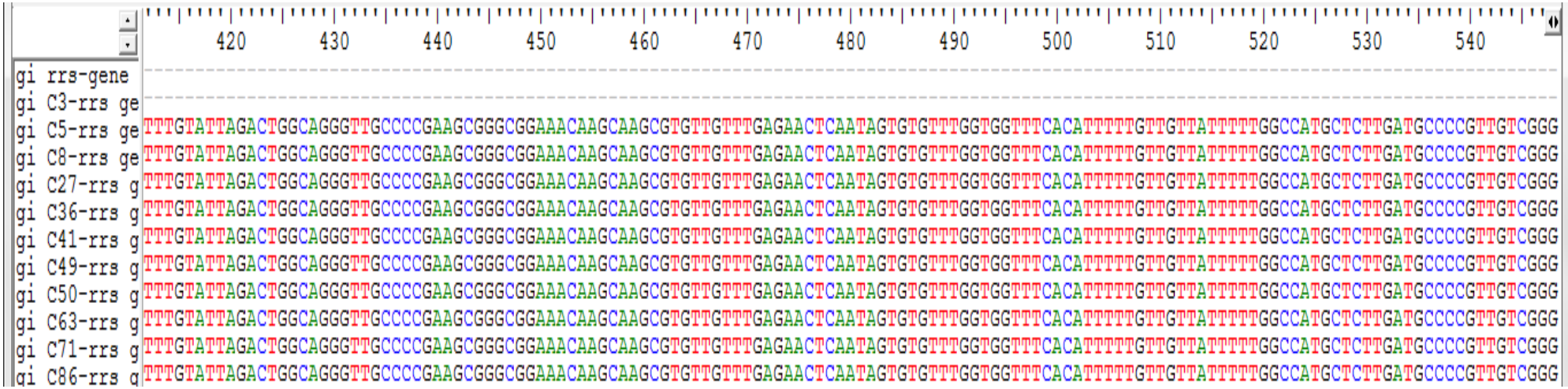
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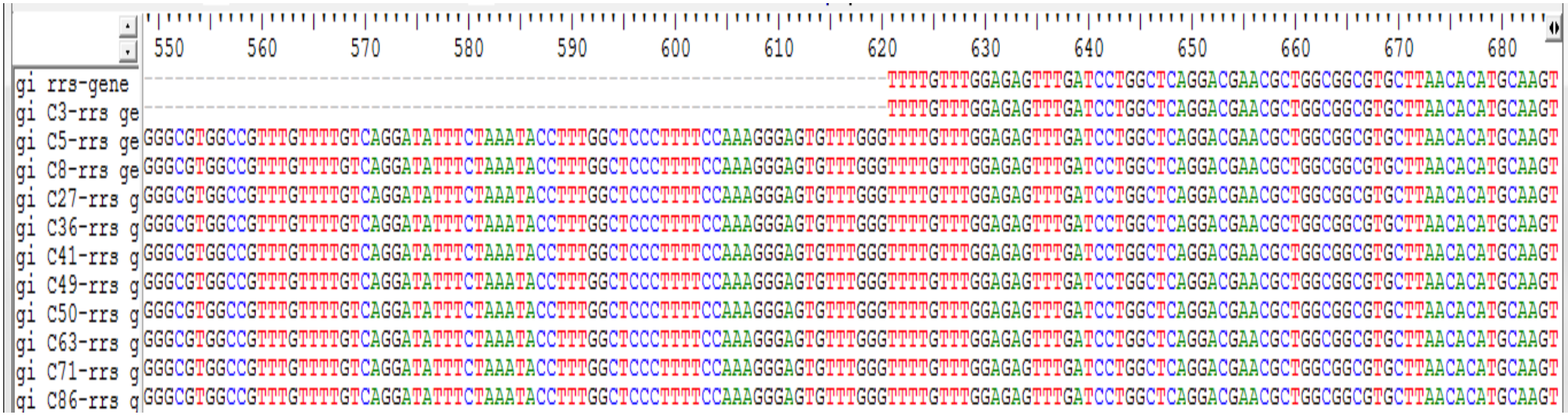
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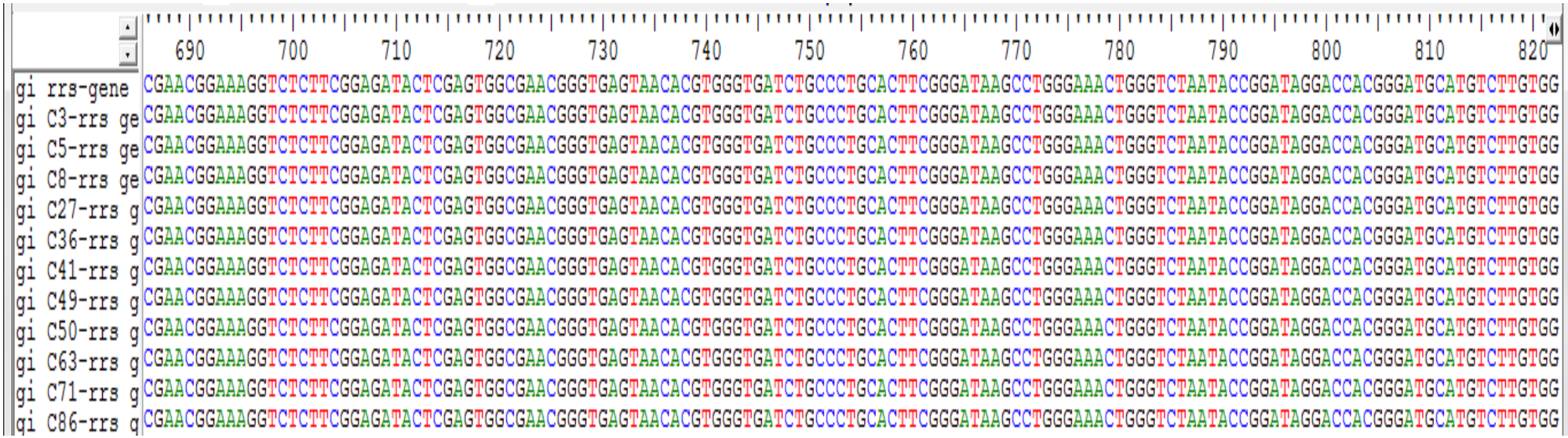
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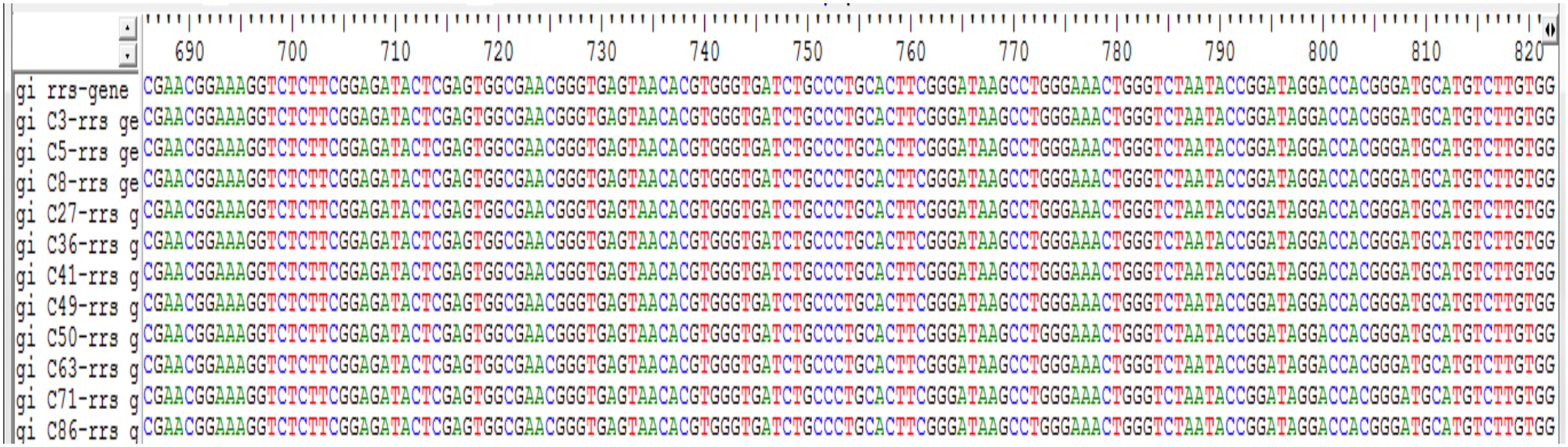
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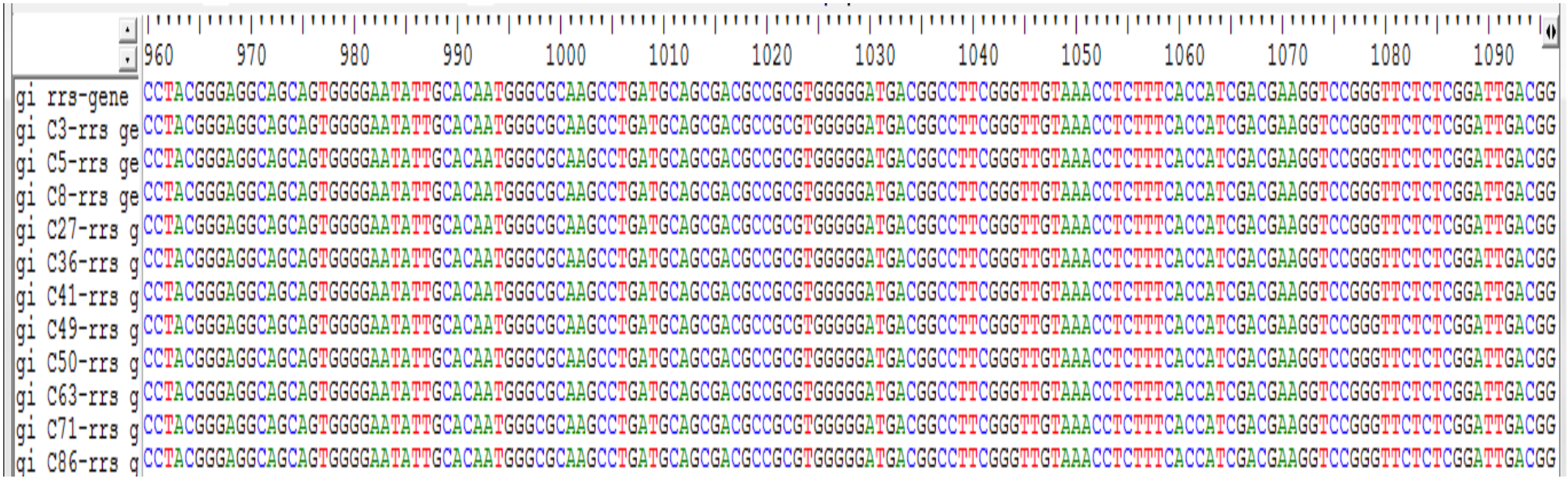
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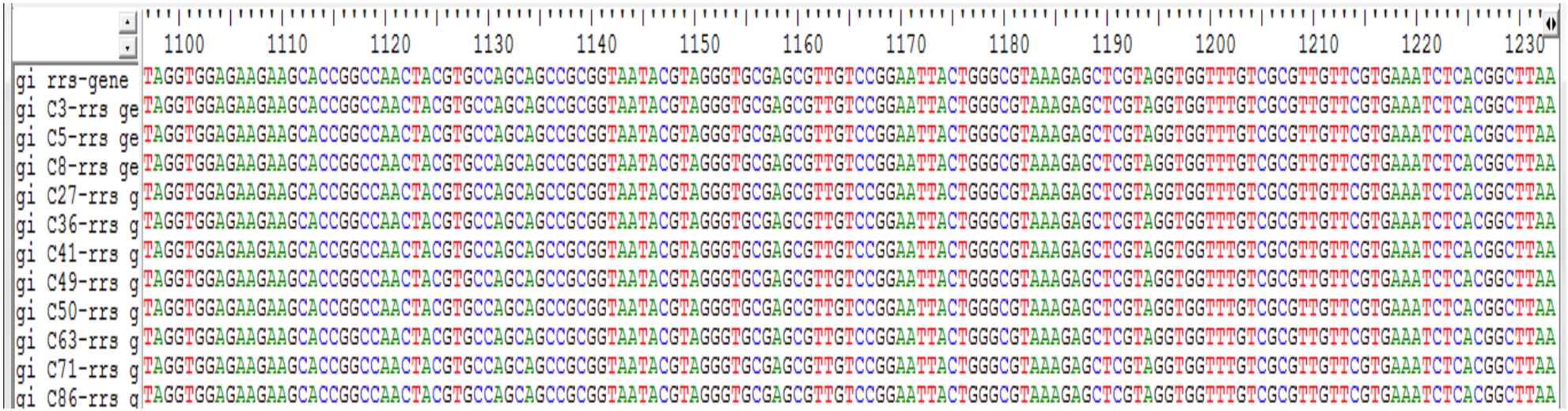
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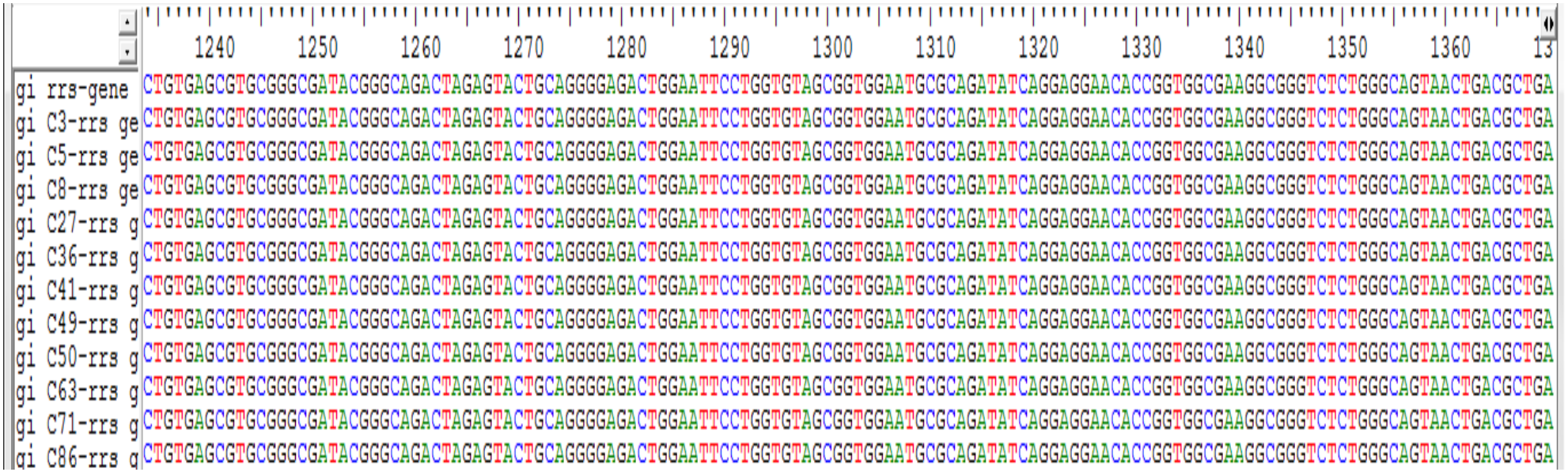
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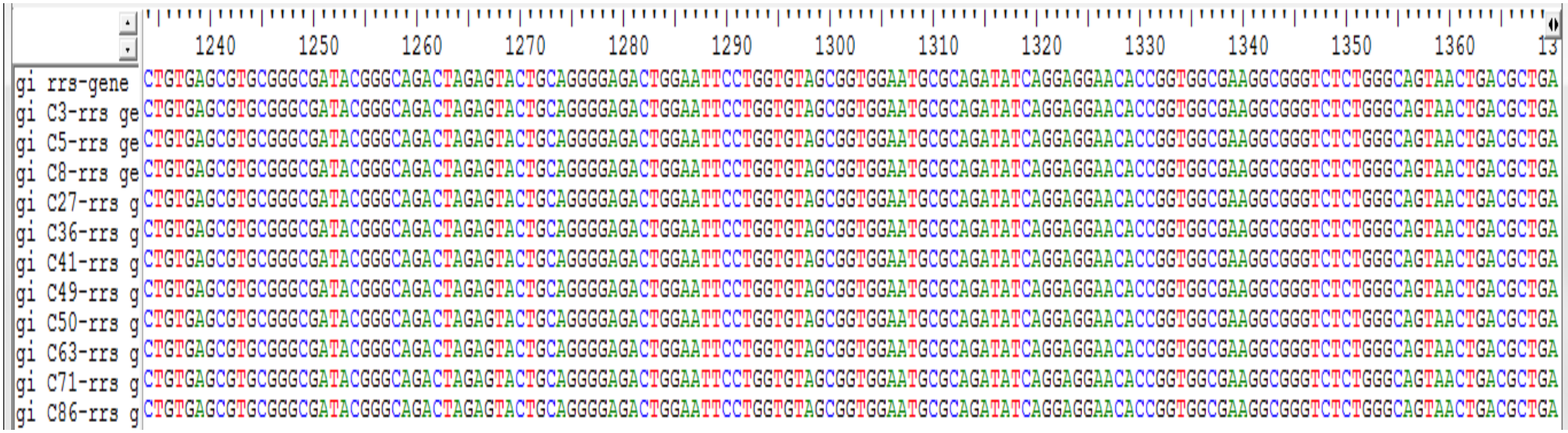
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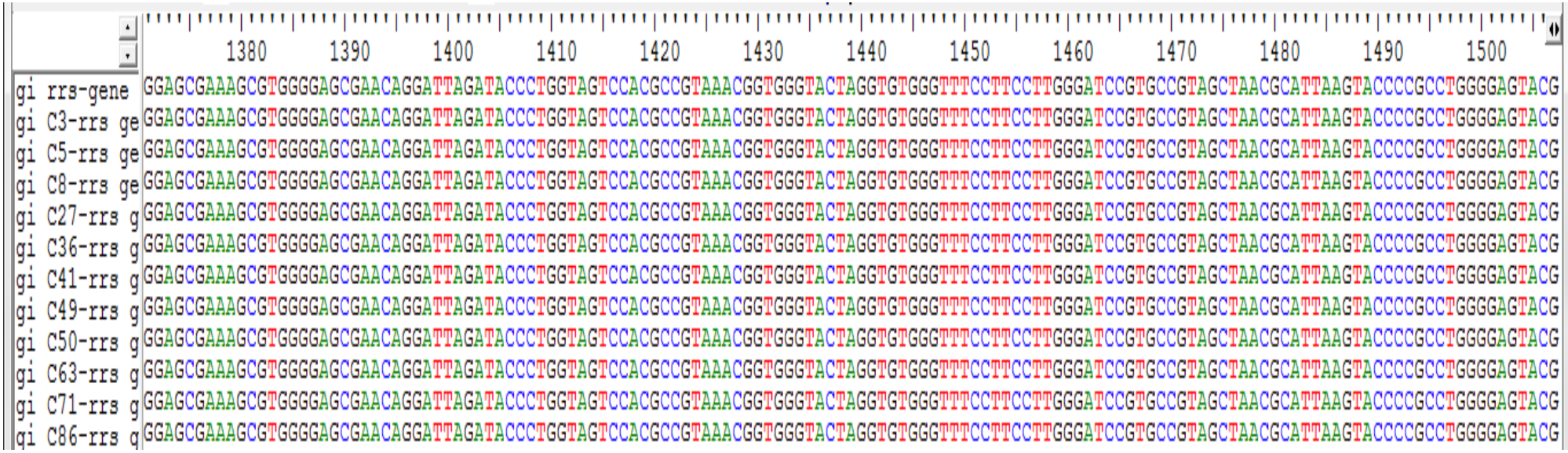
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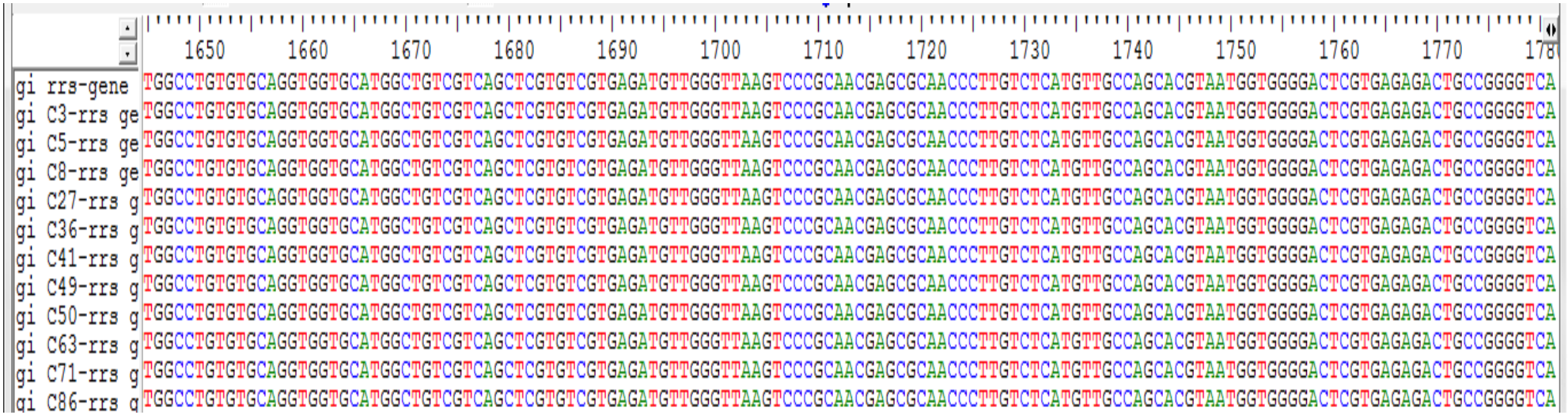
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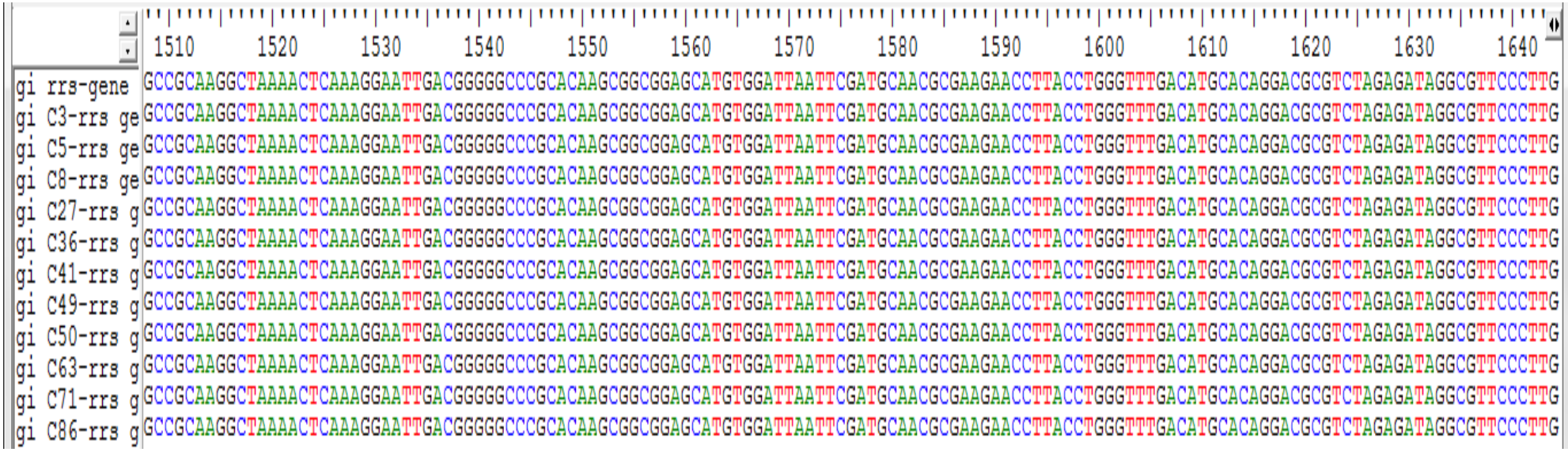
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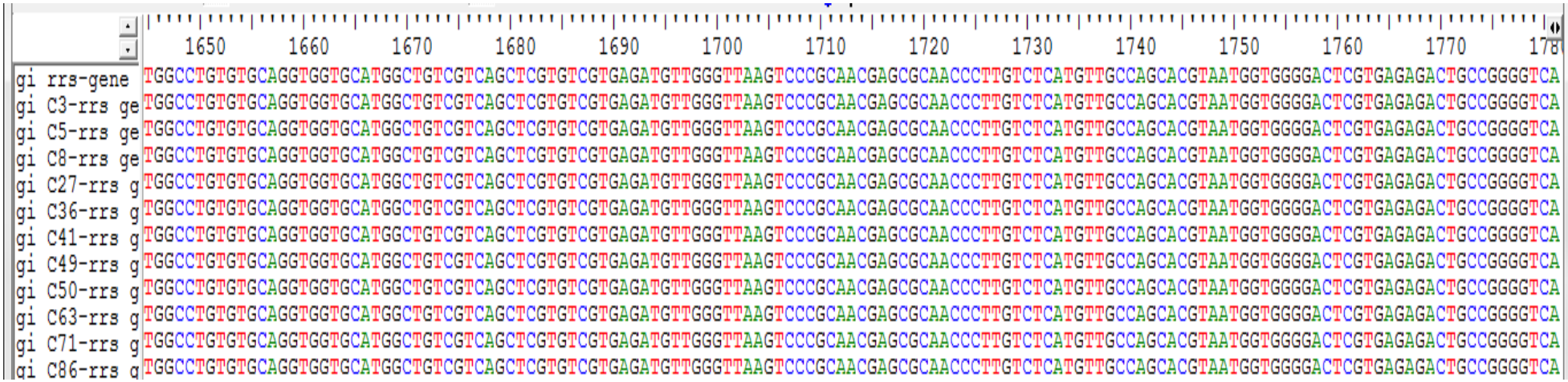
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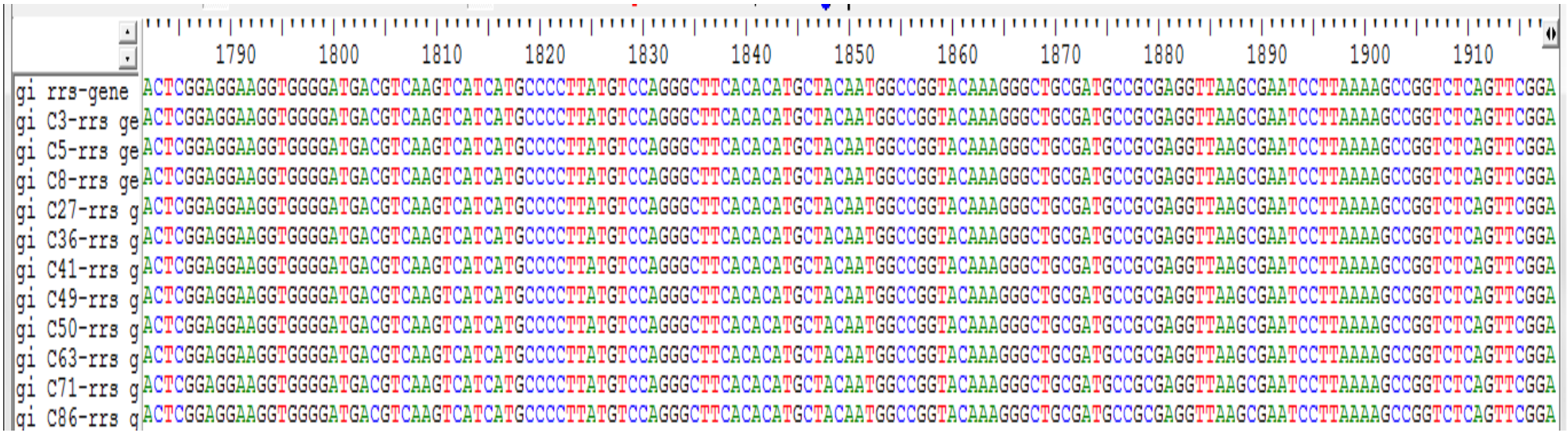
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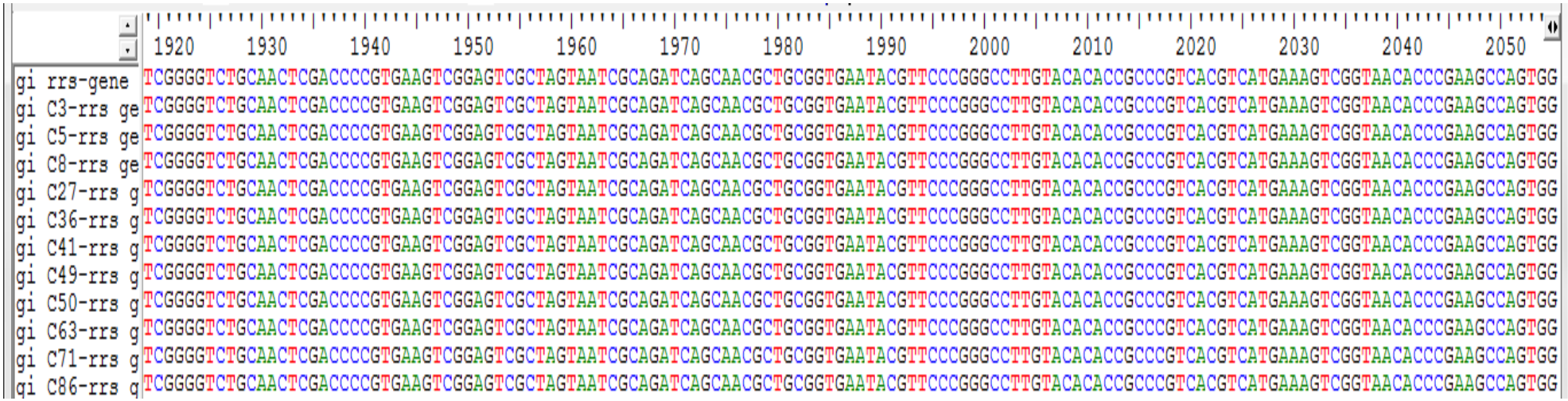
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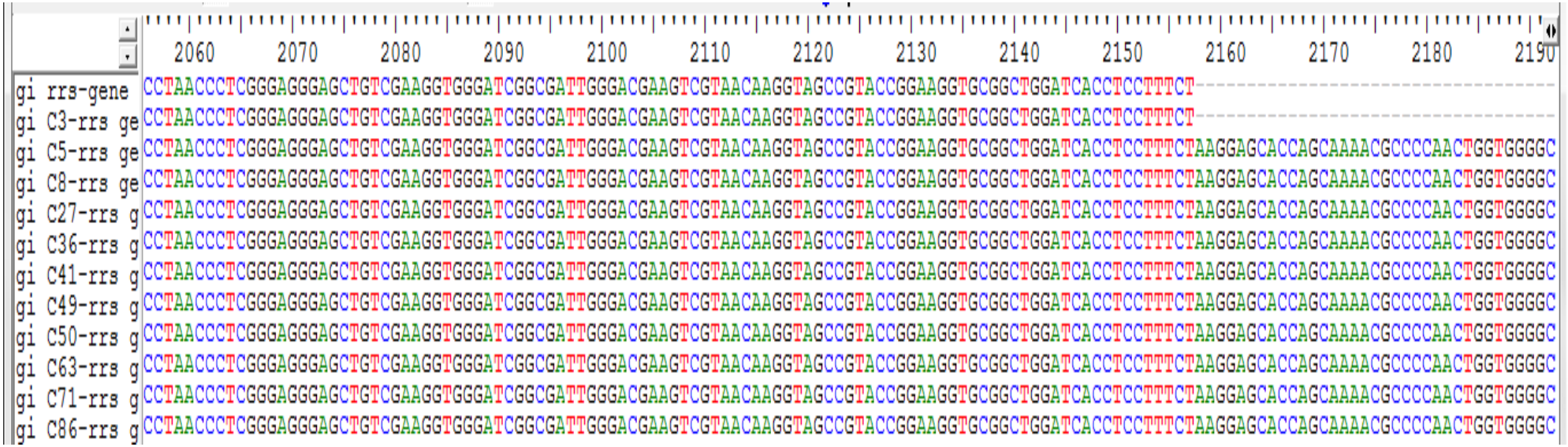
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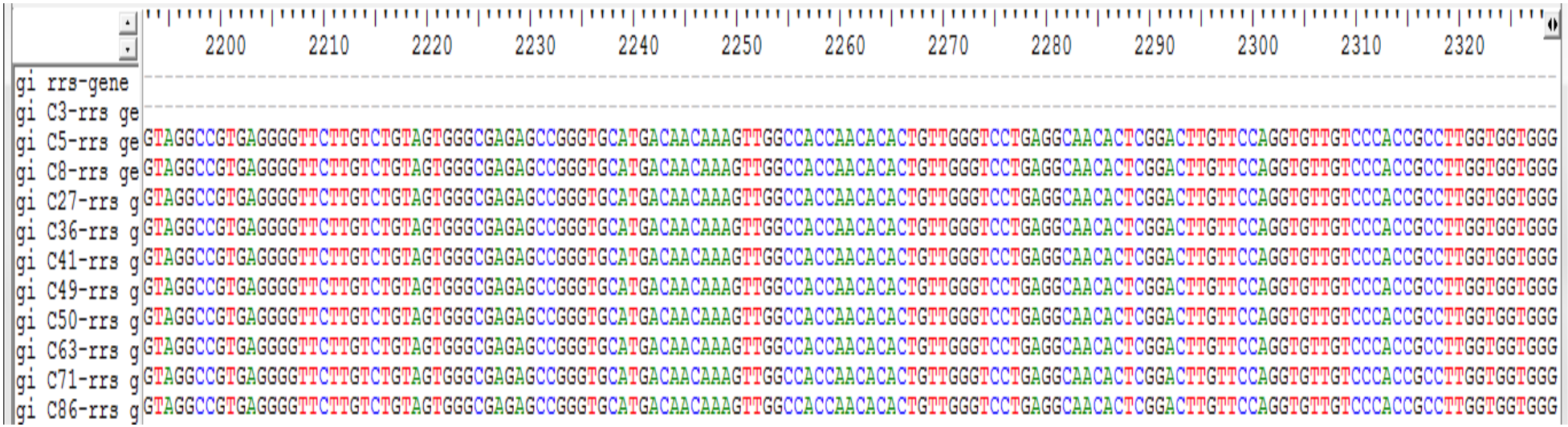
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REFERENCES

Abdool Karim S.S, Churchyard G.J, Abdool Karim Q & Lawn S.D (2009). HIV infection and tuberculosis in South Africa: an urgent need to escalate the public health response. Lancet. 374:921-933.

Angela D.P, Giuseppina C, Tony F.V, Bijo B, Fatmira S & Giuseppina T (2006). Detection of *Mycobacterium tuberculosis* complex in milk using polymerase chain reaction (PCR). Food Control. 17:776-780.

Aranaz A, Cousins D, Mateos A & Dominguez L (2003). The elevation of *Mycobacterium tuberculosis* subsp. caprae Aranaz et al. 1999 to species rank as *Mycobacterium caprae* comb nov., sp. Nov. International Journal of Systematic Evolution of Microbiology. 53:1785-1789.

Bernatowska E, Wolska-Kusnierz B, Pac M, Kurenko-Deptuch M, Zwolska Z, Casanova J. L.*et al.* (2007). Disseminated Bacillus Calmette-Guerin infection and immunodeficiency. Emerging. Infectious Disease. 13:799–801.

Brosman S.A (1992). Bacillus Calmette-Guerin immunotherapy. Urologic Clinics of North America. 19:557-564.

Burst J.C, Gandhi N.R, Carrara H, Osburn G & Padayatchi N (2010). High treatment failure and default rates for patients with multidrug-resistant tuberculosis in KwaZulu-Natal, South Africa, 2000-2003. International Journal of Tuberculosis and Lung Diseas. 14**:** 413-419.

Duet E.L, Domingos M, Amado A & Botelho A (2008). Spoligotyping diversity of *Mycobacterium caprae* animal isolates. Veterinary Microbiology. 130:415-421.

Flne P.E.M (1995). Variation ion protection by BCG: implications of and for heterogous immunity. The lancet. 346:1330-1345.

Gibson G.R, Probert H.M, Van loo J, Rastall, R.A & Roberfroid M.B (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition Research Reviews 17:259-275.

Heller T, Lessells R.J, Wallrauch C.G, Barnighausen T, Cooke, G.S, Mhlongo L. *et al.* (2010). Community-based treatment for multidrug-resistant tuberculosis in rural KwaZulu-Natal, South Africa. International Journal of Tuberculosis and Lung Disease. 14**:**420-426.

Lamm D.L, van der Meijden A.P.N, Morales A, Brodin P, Buchriser C, Eiglmeir K. *et al*. (1992). Incidence and treatment of complications of bacillus Calmette-Guerin intravesical therapy in superficial bladder cancer. Journal of Urology. 147:596-600.

Lotte A, Wasz-Hockert O & Poisson P (1988). Second IUATLD study on complications induced by intradermal BCG-vaccination. Bull. International Union Against Tuberculosis and Lung Disease. 63:47-59.

Lugosi L (1992) Theoretical and methodological aspects of BCG vaccine from the discovery of Calmette and Guerin to molecular biology. A review. Tubercle and Lung Disease. 73:252-261.

Mlambo C.K, Warren R.M, Poswa X, Victor T.C, Duse A.G & Marais E (2008). Genotypic diversity of extensively drug-resistant tuberculosis (XDR-TB) in South Africa. International Journal of Tuberculosis and Lung Disease. 12:99-104.

Mokrousov I, Filliol I, Legrand E, Sola C, Otten T, Vyshnevskaya E. *et al.* (2002). Molecular characterization of multiple-drug-resistant *Mycobacterium tuberculosis* isolates from northwestern Russia and analysis of rifampin resistance using RNA/RNA mismatch analysis as compared to the line probe assay and sequencing of the *rpoB* gene. Resistance Microbiology. 153:213–219. doi: 10.1016/S0923-2508(02)01311-6.

Onifade D.A, Bayer A.M, Montoya R, Haro M, Alva J, Franco J. *et al.* (2010). Gender-related factors influencing tuberculosis control in Shantytowns: a qualitative study. BMC Public Health. 10:381.

Padayatchi N, Stiefvater E, Naidoo K, Ndungu T & Abdool Karim Q (2009). Expanding HIV surveillance to include TB patients in resource-limited settings with a generalized epidemic. International Journal of Tuberculosis and Lung Disease. 13:1447-1449.

Parkash O, Singh B.P & Pai M (2009). Regions of Differences Encoded Antigens as Targets for Immunodiagnosis of Tuberculosis in Humans. Scandinavian Journal of Immunology. 1365-3083.

Pavlik I, Dvorska L, Bartos M, Barmova I, Meliciiarek I, Jesenska A. *et al*. (2002). Molecular epidemiology of *bovine tuberculosis* Czech Republic and Slovakia in the period 1965-2001 studied by Spoligotyping. Veterinary medicine (Praha). 47: 181-194.

Somoskovi A, Parsons L.M & Salfinger M (2001). The molecular basis of resistance to isoniazid, rifampin and pyrazinamide in *Mycobacterium tuberculosis*. Respiratory Research. 2(3):164-168.

Talbot E.A.S, Perkins M.D, Silva S.F.M & Frothingham R (1996). Disseminated BCG disease after vaccination: case report and review of the literature. Clinical Infectious Disease., in press.

Warren R.M, Gey van Pittius N.C, Barnard M, Hesseling A, Engelke E, de Kock M. *et al*. (2006). Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. International Journal of Tuberculosis and Lung Disease. 10:818-822.

World Health Organization (2012). Global Tuberculosis Control. Geneva, 38 www.who.int/tb/publications/globalreport/. (Accessed 28 May 2013).

World Health Organisation (2013) http://www.who.int/tb/challenges/gender/. (Accessed 11 December 2013).