Cape Peninsula University of Technology

MOLECULAR CHARACTERISATION OF *MYCOBACTERIUM TUBERCULOSIS*, CLINICAL ISOLATES OBTAINED IN THE KHOMAS REGION, WINDHOEK, NAMIBIA

By

EVELYN NDINELAO BREUER

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Supervisor: Prof Sehaam Khan

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DECLARATION

I, EVELYN NDINELAO BREUER, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

12 December 2017

Signed **Date**

ABSTRACT

According to the Namibia National Tuberculosis Control Programme (NTCP) report of 2008, Namibia has one of the highest TB infection rates in the world with a case notification rate of 748/100,000. Rapid, specific and sensitive diagnosis of *Mycobacterium tuberculosis* (MTB) is needed for correct TB patient management. One of the aims of this study was thus to compare direct microscopy with two rapid molecular diagnostic tools (viz. GeneXpert MTB/RIF and Hain Genotype® MTBDR *plus* assay) for the identification of MTB from samples collected from the Khomas Region, Windhoek, Namibia. Only patients with positive TB sputum collected at the clinics and health facilities in the Khomas Region, Windhoek were eligible for the study. Three hundred and eighty-four samples were confirmed acid-fast positive by utilising the auramine staining method. The rifampicin (RIF) resistance profile detected by both molecular techniques was then compared for characterisation of the samples as drug resistant. Lastly, participants completed a survey, which included questions related to demographic and epidemiological data. Demographic data included patient age, gender, region of residence and history of treatment. The data was collected using a structured questionnaire and was captured in an Excel spreadsheet. It was then imported into Statistical Package for Social Sciences (SPSS) Version 25 for data analysis. A memorandum of understanding was also signed with the Namibia Institute of Pathology (NIP) to obtain permission to use their samples and the equipment at their site.

All sputum samples identified as acid-fast bacilli (AFB) positive (*n* = 384) were analysed using both the GeneXpert MTB/RIF and the Hain Genotype® MTBDR *plus* assay for the identification of MTB and testing for sensitivity and resistance to RIF. Since the Hain Genotype® MTBDR *plus* assay simultaneously detects isoniazid (INH), these results were also recorded using this method. The GeneXpert MTB/RIF provided a positive MTB result for all samples (*n* = 384; 100%) analysed. The results also showed that approximately 2% (7/384) were classified as rifampicin resistant MTB, while 98% (377/384) were shown to be RIF sensitive. In comparison, the Hain GenoType® MTBDR provided an overall positive MTB result for 97% of the samples (372/384) analysed. The results also indicated that 8% (29/384) of samples were classified as isoniazid resistant MTB, while 5% (20/384) were shown to be RIF resistant. The two methods showed 91% (349/384) total agreement for RIF sensitivity and 1% (4/384) total agreement for RIF resistance. Thus, in total 92% (353/384) of the

samples obtained from the two methods were in total agreement. Furthermore, 4.1% (16/384) of the samples had a disagreement of results where the Hain GenoType® MTBDR assay indicated that these samples were resistant to RIF while GeneXpert MTB/RIF assay indicated that the same samples were sensitive to RIF. Previous studies have indicated that both the GeneXpert MTB/RIF and Hain GenoType® MTBDR assays yielded similar sensitivities and specificities for the detection of MTB. In the current study both tests were able to rapidly detect the presence of *M. tuberculosis* complex DNA in most of the smear-positive cases and comparatively, our study produced a total agreement of 92% between the two methods when analysing RIF sensitivity and resistance.

A total of 382 patients were interviewed for demographic and epidemiological data and a summary of the crucial information is presented. The demographic and epidemiological data captured within our study showed that TB was more prevalent amongst women especially those between the ages of 20 to 40 years. In addition, there was a high percentage of second time TB diagnoses among respondents between the ages of 30-39 years (35.0%; 50/141), followed by respondents between the ages of 20-29 years (24.8%; 35/141). Comparatively, studies have shown that that the risk of re-infection peaked in the age group 20-30 years. Of the respondents who had been diagnosed for a second time with TB, it was interesting to note that approximately 45.5% (174/382) reported that they had completed the TB treatment course during the first infection, while 25.9% (99/382) of the respondents revealed that they did not complete the TB treatment course during the first diagnosis. Furthermore, 39.9% of the respondents were unemployed and a high percentage (63%) of the respondents were among those with limited or no skills. A study on poverty alleviation in the informal settlement communities in Windhoek, Namibia, found that on average, a dwelling had six occupants. Accordingly, research has indicated that people living in poor socio-economic conditions as well as in overcrowded dwellings have an increased risk of contracting TB. Results also showed that 62.6% (239/382) of the respondents reported that they had family members who currently had or who were previously diagnosed with TB. Finally, 38.5% (147/382) of the respondents reported that they were HIV positive while 33.5% (128/382) of the respondents revealed that they were HIV negative and 28.0% (107/382) opted not to respond to the HIV/AIDS question. Results obtained in this study thus crucially confirms that TB commonly presents as a secondary infection among people living with HIV.

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DEDICATION

THIS THESIS IS DEDICATED TO MY HUSBAND OLIVER

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GLOSSARY

AIDS	Acquired Immunodeficiency Syndrome
BCG	Bacillus Calmette-Guérin
CB DOT	Community Based Direct Observed Treatment
CPUT	Cape Peninsula University of Technology
DNA	Deoxyribonucleic Acid
DOT	Directly Observed Treatment
EMB	ethambutol
HBCs	High Burden Countries
HIV	Human Immunodeficiency Virus
INH	Isoniazid
LTBI	Latent TB Infection
MDR	Multi Drug Resistant
MGIT	Mycobacteria Growth Indicator Tube
MoHSS	Ministry of Health and Social Services
MTB	*Mycobacterium tuberculosis*
NGO	Non-Governmental Organization
NIP	Namibia Institute of Pathology
NTCP	National TB Control Programme
PCR	Polymerase Chain Reaction
PFGE	Pulsed field gel electrophoresis
PZA	pyrazinamide
RIF	Rifampicin
RNA	Ribonucleic Acid
rpoB	DNA- dependant RNA Polymerase beta subunit
TB	Tuberculosis
TPE	Tuberculous pleural effusion
TST	tuberculin skin test
UNAIDS	United Nations AIDS programme
VNTR	variable numbers of tandem repeats
WHO	World Health Organization
XDR	Extensively Drug Resistant

KEYWORDS

TB

Isoniazid

Rifampicin

Pyrazinamide

Ethambutol

Latent TB Infection

Tuberculin skin test

Antibiotic resistance genes

Antibiotic resistant bacteria

Polymerase Chain Reaction

CHAPTER 1

LITERATURE REVIEW

1.1 General Introduction

Many studies around the world have reported tuberculosis (TB) as one of the leading causes of death [Raviglione & Sulis 2016; World Health Organisation (WHO), 2013]. Tuberculosis is a contagious infectious disease caused primarily by *Mycobacterium tuberculosis* (MTB), a microorganism which is principally carried by humans. It is estimated that approximately one-third of the world's population is infected with the tuberculosis bacillus and that eight million people develop tuberculosis disease each year or die from it globally (WHO, 2015). Eighty percent of these cases have been estimated to be found in about 22 countries while the highest incidence rates were found in Africa and South-East Asia. The combination of high prevalence of the human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) epidemic and multidrug resistance to MTB as well as the deterioration of health infrastructure has worsened the situation over the past twenty years (WHO, 2015).

Mycobacterium tuberculosis is spread through inhalation of air particles from positive sputum or patients that are infected with pulmonary tuberculosis. Approximately 12% of people will develop tuberculosis after a period that ranges from a few weeks to many years after becoming infected with MTB. The risk of tuberculosis infection declines steeply with time after infection. Tuberculosis may also occur after re-infection (Jamison *et al.,* 2018).

World-wide it is estimated that at least one person becomes newly infected with the tuberculosis bacilli almost every second (WHO, 2014) which explains why the disease is considered a high public health risk. Tuberculosis is a treatable disease, but its morbidity and mortality rates remain high. In many cases, tuberculosis is almost always curable, especially when patients follow the treatment therapy without any interruptions. Despite being treatable, the elimination of tuberculosis has proven to be

challenging, especially if one looks at the number of drug-resistant (multi- and extensively-drug-resistant MTB*)* cases that are on the increase (WHO, 2016).
Many experts acknowledge that patient adherence to taking and completing the medication plays a central role in the treatment of tuberculosis and its effects. Ensuring and encouraging compliance in regular intake of treatment drugs is critical for successful cure of the disease. Furthermore, tuberculosis treatment drugs are expensive, hence there is a need to make deliberate efforts to launch special programs that can contribute to the control of tuberculosis. The World Health Organization has set up criteria for successful tuberculosis control programmes, which is based on a case detection rate of smear positive tuberculosis cases of 70% and an achievement of treatment success of 85% of the detected cases.

Accurate and early diagnosis of TB is vital as it interrupts further transmission of the disease and avoids the use of more treatment drugs and thus amplification of drug resistance and possible creation of extensively-drug-resistant tuberculosis (XDR-TB) infections. In most cases, TB can be treated effectively by using the first line drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (SM), for a period of six to nine months (American Thoracic Society, 2018; Global Tuberculosis Institute, 2018; WHO, 2010). However, multi-drug-resistant TB (MDR TB) is caused by MTB that is resistant to at least isoniazid and rifampicin, the two most potent TB drugs. Extensively-drug-resistant TB is a rare type of MDR TB that is resistant to isoniazid and rifampicin, plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e. amikacin, kanamycin or capreomycin). Studies are thus required that not only compare diagnostic tools for the detection of MTB, but also for the sensitivity and resistance towards antibiotics.

1.2 History and Current Epidemic of Tuberculosis

Evidence of TB dates back to thousands of years when Aristotle determined its contagious nature, observing that the consumptive around him has a “pernicious air” that is “disease producing” (Zücker et. al, 2016). In March 1882, *Mycobacterium tuberculosis*, known as “tubercle bacillus” was described by Robert Koch (Bunn, 2002).

During the industrial revolution TB became a major public health problem as cities became overcrowded and public health facilities became overwhelmed (Trueman,

2015). Such settings created ideal environmental conditions for the spread of TB (Ranosa & Karande, 2017). Approximately 1.8 billion people in the world per year are affected by the TB disease which is equal to one-third of the entire world population (WHO, 2006). Furthermore, MTB remains the leading cause of deaths worldwide, while African countries have the highest incidences of TB (WHO, 2017). Due to inadequate healthcare systems, the treatment of TB is also very ineffective and occurs at a very slow pace (Palmero, 2007). At the same time, Africa faces the largest funding gap of any region in the world and Namibia is no exception (WHO, 2017).

In 2005, the WHO and Africa's ministers of health declared TB a continent-wide emergency, as approximately 2.4 million TB cases occur per year in sub-Saharan Africa. High burden countries (HBCs), are those where the highest rates of TB incidence occur. More than eleven of these high burden countries are in Africa; namely Ethiopia, the Democratic Republic of Congo, Nigeria, South Africa, Tanzania, Mozambique, Sudan, Kenya, Uganda, Zimbabwe, and Namibia (**Figure 1.1**) (Global Tuberculosis report, WHO, 2015).

The Namibia National Tuberculosis Control Programme (NTCP) report of 2008 stated that Namibia has one of the highest incidence of tuberculosis infection in the world with a case notification rate of 748/100,000 [Ministry of Health and Social Services (MoHSS), 2007]. Similar to most resource limited countries in Southern Africa, Namibia is also struggling to cope with the current demands of its health sector. It is a challenge to provide care and support for the increasing number of patients that are living with HIV/AIDS. This exacerbates the problems associated with tuberculosis and the emergence of resistant forms of the MTB that cause the disease. Hospital wards are over-crowded and under resourced in terms of medication, health care workers, medical personnel and facilities (WHO, 2014). Due to these stumbling blocks, the government of Namibia, committed to eradicating tuberculosis, involved non-governmental organisations (NGOs) to assist with the control and management of TB. The strategy used to manage tuberculosis in Namibia is the Community-Based Directly Observed Therapy Strategy (CB DOTS) (WHO, 2008).

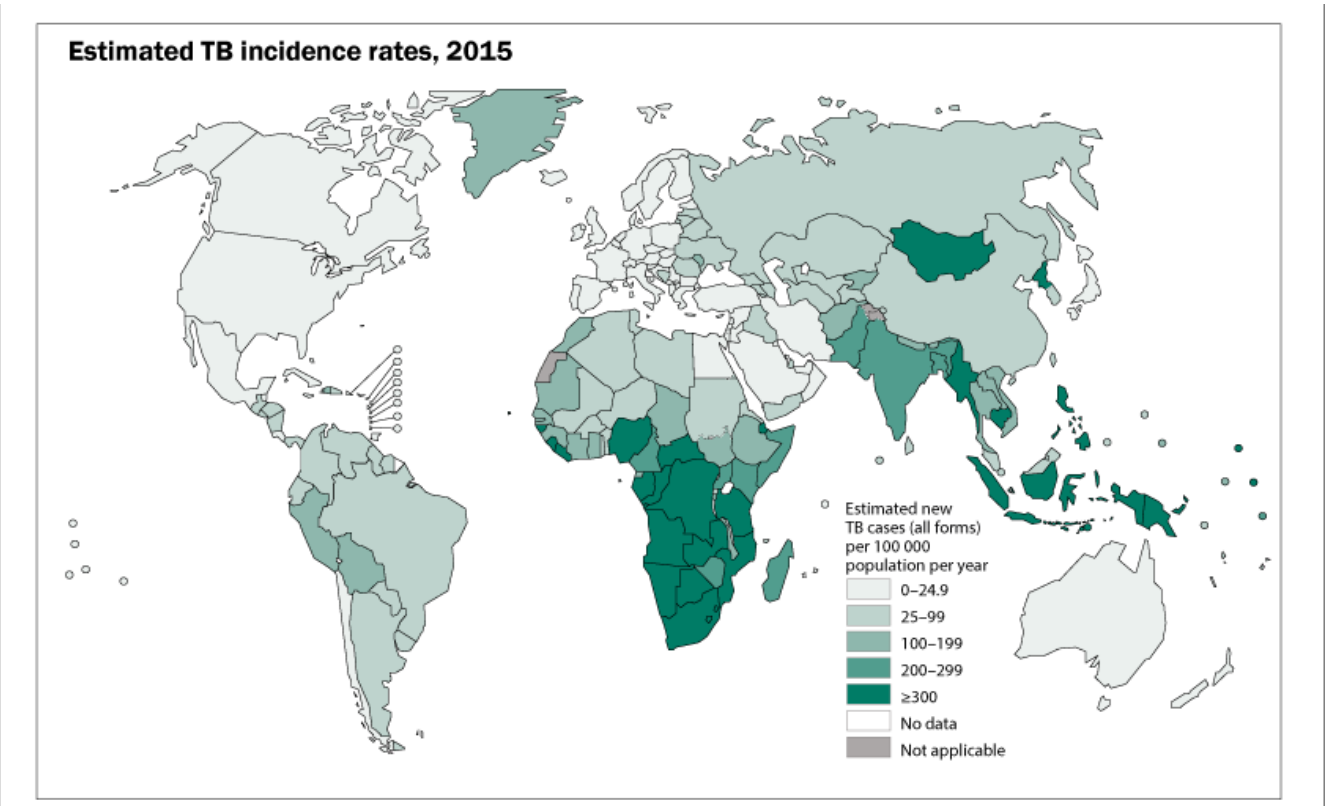

Figure 1.1: Estimated TB incidence rate (2015) (adopted from: Global Tuberculosis Report, WHO, 2016)

1.2.1 Tuberculosis and HIV/AIDS

In Africa, compared to developed countries, the high TB infection rate combined with a high HIV infection rate (combined infection rate of 19.5%) results in devastating consequences (WHO, 2007). The HIV infection is the largest single risk factor for the development of tuberculosis disease in a person who is infected with the MTB (Montales *et al.,*2015). While HIV negative persons with TB have a 5-10% lifetime risk of developing the TB disease, people who are infected with both TB and HIV have a risk of 5-10% each year of developing active TB disease (WHO, 2007). Tuberculosis in Namibia is the most prevalent cause of death in persons with HIV infection. About 50-60% of tuberculosis patients in Namibia are HIV positive and may at some point develop AIDS or HIV-related diseases (MoHSS, 2004). Since the HIV infection negatively affect cell-mediated immunity, HIV alters the pathogenesis of TB, greatly increasing the risk of disease from TB in HIV-coinfected individuals (Goozé & Daley, 2013). However, tuberculosis is curable, even in the presence of HIV/AIDS (MoHSS, 2004).

The case notification rate of TB in Namibia has averaged around 736 cases per 100,000 individuals for the last five years (748/100,000 in 2008) (MoHSS, 2015). This is nearly eight times higher than the rate obtained in 1996 and 10 times higher than the global average. This increase is enhanced by the spread of HIV/AIDS (MoHSS, 2015. In addition, Namibia's HIV prevalence was reported to be 15.6% which subsequently poses serious problems for the successful treatment of TB (MoHSS, 2015.

1.3 *Mycobacterium tuberculosis*

1.3.1 Description

Mycobacterium tuberculosis is a large, nonmotile, obligate aerobe, facultative intracellular, rod-shaped bacterium distantly related to the Actinomycetes. The rods are 2-4 µm in length and 0.2-0.5 µm in width. Species belong in the family Mycobacteriaceae and are the causative agent of tuberculosis.

The disease is caused by nine species namely MTB *sensu stricto, M. africanum, M. canetti, M. bovis, M. caprae, M. microti, M. pinnipedii, M. mungi, and M. orygis* which form the *Mycobacterium tuberculosis* complex (MTBC) (van Ingen *et al*., 2012). Some species such as MTB*, M. africanum* and *M. cannettii* are specific to humans, while *M. microti* has been found to be specific to rodents (van Ingen *et al*., 2012). Moreover, *M. bovis* and its variants have been found to be zoonotic (van Ingen *et al*., 2012).

1.3.2 Detection of Strain Variation

The MTBC is a single group consisting of different species that are genetically related and show high conservation of DNA (Frothingham *et al.,* 1998; Asiimwe *et al.,* 2008). The genome of the strain of MTB identified as H37Rv strain was described in 1998 (Cole et al., 1998). The genome size is 4 million base pairs, with 3959 genes.

Pulsed field gel electrophoresis (PFGE) was used to type MTB strains until the early 2000s. More recently however, typing of the variable number of tandem repeats (VNTR) within the genome (repeated DNA sequences within the MTB genome), which

is technically easier and allows better discrimination between strains, has now emerged as the method of choice for MTB strain typing (Hopkins *et al.,* 2007)

There are three generations of VNTR typing for MTB that have been utilised. Initially, the exact tandem repeat, which only has five loci, was used, however the resolution afforded by these five loci was not as good as PFGE. The second typing strategy (mycobacterial interspersed repetitive unit), allowed for strain discrimination equal to the PFGE technique. The third generation (mycobacterial interspersed repetitive unit - 2) typing strategy, involves the utilisation of 24 loci, which has been reported to provide a degree of resolution greater than that of PFGE and is the current standard used for typing MTB (Desikan & Narayanan, 2015).

1.3.3 Symptoms of Tuberculosis

Symptoms of tuberculosis manifest through loss of appetite, persistent cough, weight loss, constant fatigue, fever, coughing up blood and night sweats (Brosch *et al.,* 2002; Dye *et al.,* 2002; Gagneux *et al.,* 2005; Traore *et al*., 2007), while, coughing is the most common symptom of TB infection. At the onset of infection by the MTB bacteria, certain individuals may not show any signs of coughing but as the tissue becomes more necrotic and the inflammation worsens, chest pain and excessive sweating at night can be observed. Most patients infected by the TB disease will occasionally cough blood in their sputum; however, this may not only be limited to the TB infection but may also be a symptom from other cases of previous diseases other than active TB infection (Huseby & Hudson, 1976). Tuberculous pleural effusion (TPE) results from *Mycobacterium tuberculosis* infection of the pleura and is characterized by an intense chronic accumulation of fluid and inflammatory cells in pleural space (Zhai et. al, 2016). Differential diagnosis of TPE sometimes mandates more invasive procedures like medical thoracoscopy when one or more thoracenteses or/and blind needle biopsy fail to reach definite diagnosis.

1.4 Diagnostic Techniques

The WHO has recommended countries to make use of endorsed rapid methods for the early and rapid detection of the MTB by implementing the WHO's "STOP TB strategy". However, in many parts of the world TB diagnosis still relies on sputum microscopy

(acid fast bacilli – AFB) and MTB culture and identification (WHO, 2008). It is understood that the availability of standardised, easy-to-use, rapid, sensitive, specific and affordable diagnostic tools will improve the effectiveness of pulmonary TB diagnosis efforts especially the resistance to treatment drugs such as Rifampicin (RIF) (WHO, 2008). Furthermore, rapid and reliable test results will directly translate into sound patient management decisions that will assist in the rapid cure of patients and thus break the chain of TB transmission (Strohle & Bodmer, 2012). Rapid MTB confirmation and identification of drug resistance is also useful in HIV patients in whom treatment is rendered difficult by factors such as pill burden, drug interactions and the subsequent side effects and antagonism and treatment adherence issues (Nicol *et al.*, 2011).

1.4.1 Microscopy of *Mycobacterium tuberculosis*

Sputum samples from patients suspected to be infected by MTB are routinely examined using microscopy in order to diagnose infection. While many other bacteria are stained for microscopy and identification using the Gram stain, acid-fast staining of bacteria within sputum and microscopy is considered the primary tuberculosis diagnosis method. This method is effective, as the mycolic acid in the cell wall of MTB does not absorb the Gram stain. The MTB has an unusual, waxy coating on its cell surface primarily due to the presence of mycolic acid. This coating makes the cells impervious to Gram staining (Cudahy & Shenoi, 2016). When viewed under a microscope, acid fast bacilli (AFB) are red, curved rod-shaped and are often clumped together, due to the presence of fatty acids in the cell wall that stick together **(Figure 1.2)** (Cudahy & Shenoi, 2016).

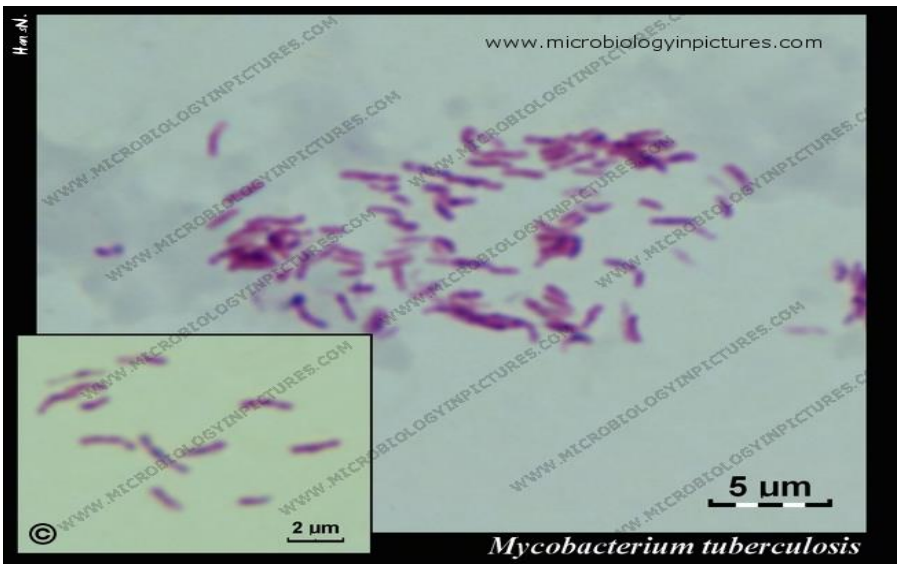

Figure 1.2: Picture illustration of *Mycobacterium tuberculosis* bacteria, Ziehl Neelsen stain. (adopted from: https://www.microbiologyinpictures.com/bacteria%20photos/mycobacterium%20tuberculosis%20photos/mycobacterium%20tuberculosis%20026.jpg)

As the technique is easy to perform and affordable, while providing rapid results, many low and middle-income countries burdened by TB employ this technique (Desikan, 2013). Microscopy offers a fast turn-around time (TAT) of 24 hours and is cheap (Miller, 2011). However, sputum smear microscopy has some restrictions with regard to its performance, especially related to the sensitivity in cases where the bacterial load is less than 10,000 bacilli/mL sputum sample (Desikan, 2013). Moreover, as the microscopy method is poorly standardised, increased inter-observer variation occurs and low assay sensitivity has been reported. The sensitivity is also dependent on the quality of the sputum specimen and the specimen concentration techniques. Also, speciation of *Mycobacteria* using microscopy is impossible. All *Mycobacteria* have the same morphological characteristics under the microscope. Sputum microscopy is also perceived to have a poor track record in the diagnosis of extra-pulmonary tuberculosis, diagnosis of paediatric tuberculosis and in patients co-infected with HIV and tuberculosis (Desikan, 2013).

In the 1930s fluorescence microscopy was introduced as an attempt to improve outcomes of smear microscopy (Desikan, 2013). This technique uses fluorochrome dyes to stain the smear and a high-pressure mercury vapour lamp or halogen is used to excite the dye and make it fluoresce. Studies comparing fluorescent and conventional microscopy found that the fluorescent microscopy performed 10% better

when it came to the sensitivity as compared to that of conventional microscopy (Mistry *et al.,* 2016). It has been concluded that the successful and widespread implementation of fluorescence microscopy may improve case finding as a result of an expected increase in sensitivity and decrease in time spent on microscopic examination (Mistry *et al.,* 2016). The cost related to this technique has however delayed the implementation thereof in lower income countries.

1.4.2 Culture of *Mycobacterium tuberculosis*

The Löwenstein–Jensen (LJ) medium, is a growth medium of choice, specially used for the culturing of *Mycobacterium* species, notably MTB (Elbir *et al*., 2008). When grown on LJ medium, MTB appears as brown, granular colonies. Culture identification and sensitivity testing may take 4 – 8 weeks due to the slow doubling time of MTB (15–20 hours) compared with other bacteria. The delay of the results availability can hinder clinical decisions especially on the initial treatment (Nicol *et al.,* 2011).

1.4.3 Molecular Diagnosis of *Mycobacterium tuberculosis*

Molecular techniques used for the diagnosis of TB are based on the decoding of the genome of MTB*,* using the commercially available nucleic acid amplification tests (NAAT) that are specific and sensitive in smear-positive samples (Palomino, 2009; Dorman, 2010). However, as a result of a lack of infrastructure and costs involved, these assays cannot be routinely performed in low-income countries for the diagnosis of TB (Conell *et al.,*2011).

The GeneXpert MTB/RIF assay detects MTB and RIF resistance by PCR amplification of the rifampicin resistance-determining region (RRDR) of the MTB *rpoB* gene and subsequent probing of this region for mutations that are associated with RIF resistance (Van Der Zanden *et al.,* 2003). The primers in the GeneXpert MTB/RIF assay amplify a portion of the *rpoB* gene containing the 81 base pair (bp) “core” region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with rifampicin resistance.

A study by Boehme *et al*. (2010) recommended the use of a molecular diagnostic method, GeneXpert MTB/RIF, for many low-income countries. The GeneXpert MTB/RIF testing method was recommended because of the fact that it has a self-contained cassette-based test making it more suitable for low-income countries as less training is required and the laboratory facilities do not need to be so advanced. Further evaluations on the real-time use for this method are still needed to determine whether the method could be considered as a potential replacement of standard smear microscopy. Based on the study and data obtained, the WHO recommended that this test should be used as the first line test in people that are suspected of having MDR-TB or HIV associated TB and as a follow-on test for smear negative samples in other patients (WHO, 2010).

The GeneXpert MTB/RIF assay has been endorsed by the WHO as a diagnostic tool (Al-Zamel, 2009; WHO, 2010; Van Rie *et al*., 2012) and has been reported in a multi-country study to have sensitivities of 98.2% among smear-positive, culture positive patients and 72.5% among smear-negative, culture positive patients on a single direct GeneXpert MTB/RIF test method compared to three smears and four culture results (Boehme *et al.,* 2010). The Namibia Institute of Pathology (NIP) laboratories adopted the implementation of the WHO (2015) published Policy framework for Implementing Tuberculosis Diagnostics and the Global Laboratory Initiative (GLI) aimed at providing technical support to TB laboratories in low- and middle-income countries, to assist with the implementation of the latest diagnostic technologies. This is the preferred algorithm for universal patient access to rapid testing to detect *MTB* and rifampicin resistance for persons being evaluated for pulmonary TB using the GeneXpert *MTB*/RIF assay (WHO, 2015).

The Hain GenoType® MTBDR *plus* assay (Hain Life science, Nehren, Germany) combines detection of MTB complex with prediction of resistance to RIF and INH, including mutations in the 81 bp hotspot region of *rpoB,* codon 315 of the *katG* gene and in the *inhA* promoter region. The Hain GenoType® MTBDR *plus* assay detects mutations associated with the *rpoB* gene for RIF resistance, *katG* genes for high level INH resistance, and the *inhA* regulatory region gene for low-level INH resistance (Hilleman *et al.,* 2007). The assay enables a rapid result from pulmonary patient specimens and from culture material. The identification of rifampicin resistance is enabled by the detection of the significant mutations within the *rpoB* gene (coding for

the β-subunit of the RNA polymerase). In order to test for high-level isoniazid resistance, the *katG* gene (coding for the catalase peroxidase) is examined, while low-level isoniazid resistance is assessed by analysing the promoter region of the *inhA* gene (coding for the NADH enoyl ACP reductase).

Figure 1.3 shows representative patterns of line probe assay (Hain GenoType® MTBDR *plus* assay) strip namely:
Lane 1, susceptible to rifampicin (RIF) and isoniazid (INH);
Lane 2, MDR- TB (*rpoB* S531L mutation and *inhA* C15T mutation);
Lane 3, rifampicin mono-resistant (mutation at rpoB530–533 gene region);
Lane 4, absence of *M. tuberculosis* complex(TUB) band;
Lane 5, isoniazid mono-resistant (*katG* S315T1 mutation);
Lane 6, DNA positive control (sensitive to rifampicin and isoniazid);
Lane 7, DNA negative control.

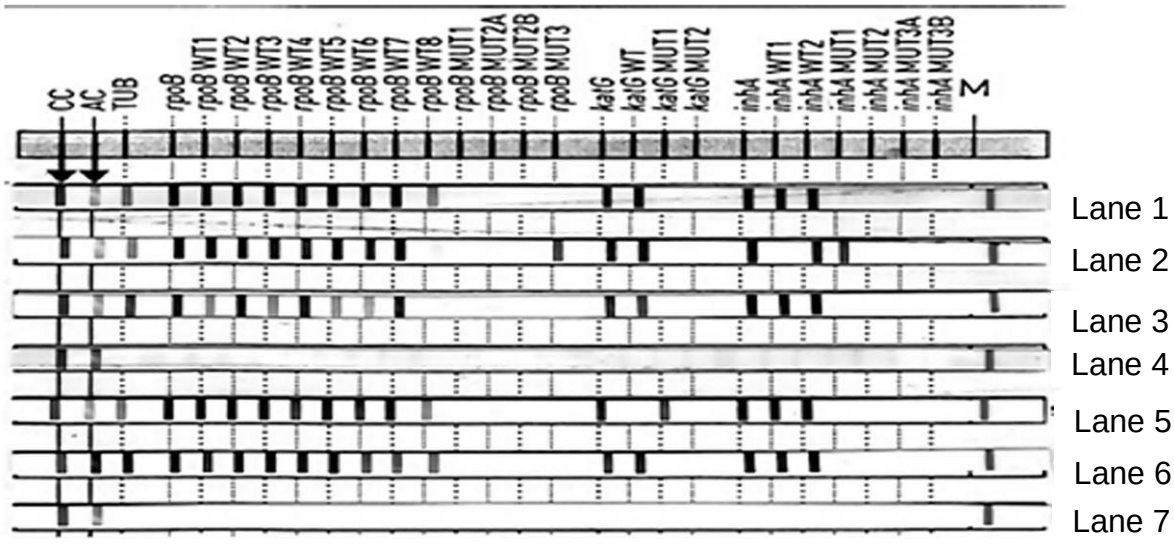

Figure 1.3: Representative patterns of the Hain GenoType® MTBDR *plus* strip (adopted from: Yadav *et al*., 2013)

Figure 3.1 shows representative DNA strip patterns obtained with the optimized Genotype MTBDR assay. The positions of the oligonucleotides are given to the top of the figure. The target genes and specific probe lines are shown from left to right

For the detection of RIF resistance, the Hain GenoType® MTBDR *plus* assay includes three more probes when compared to the GeneXpert MTB/RIF method, covering the wild-type *rpoB* sequence, which improves the sensitivity of the test. As previously

described by Ramaswamy *et al*. (2003) and Van Soolingen *et al*. (2000), the most common mutation involved in INH resistance is the S315T substitution in the katG protein, which has also been related to high levels of INH resistance. In contrast, mutations causing low levels of INH resistance are not as clearly elucidated, as they are much more complex and involve different genes; however, a firm relationship has been found between mutations in the *inhA* regulatory region and low or intermediate levels of resistance (Ramaswamy *et al.,* 2003; Brossier *et al.,* 2006). The presence of valid test results proves that the test detected the presence of bacilli in the test samples.

Line probe assays for the diagnosis of drug resistance such as Hain GenoType® MTBDR *plus* assay and the GeneXpert MTB/RIF assay reduce the time required for the diagnosis of drug resistance.

1.4.4 Radiographic Diagnosis

The presence of TB infection has been associated with chest X-ray abnormalities. However, due to HIV prevalence and the use of the subsequent prescribed medications, this is not always the case, as most patients may present normal X-ray results. Patients infected with primary tuberculosis normally show a mid or inferior lung zone penetration, often associated with ipsilateral hilar adenopathy due to the enlargement of the lymph nodes and the compression of airways commonly observed in children. Failure to treat TB in its primary stage will lead to the progression of the disease during which specific cell-mediated immunity develops and cavitation may occur (“progressive primary” tuberculosis) (Grzybowski, *et al.,* 1971).

1.4.5 Other Diagnostic Approaches

More assays such as IFN-c release assays (IGRAs) have transformed the way many people in the health care service can improve the diagnosis of Latent TB Infection (LTBI) or as sometimes called asymptomatic TB. However, these assays are also faced with some limitations. These assays are based on the same principles such as tuberculin skin test (TST), which enable them to only detect the presence of a host response to *MTB* antigens, but not to classify LTBI from active disease. The

identification of biomarkers that are able to differentiate between the different disease stages in TB is needed. These biomarkers would not only help in the differentiation of the different stages of TB, but will also help when therapeutic recommendations are needed. For example, the use of the biomarkers may indicate whether a specific treatment strategy is working or even indicate when a patient has relapsed. The research would also help in probable provision of surrogate markers of vaccine efficacy that is still needed in the fight against TB (Wallis, et al, 2009; Doherty, et al. 2009).

Another approach recommended for consideration for use in low and middle income countries was the one based on measurement of multifunctional cluster of differentiation 4 (CD4) T-cells. The HIV/AIDS program in Namibia uses the CD4 T-cell count in the control of the HIV infections through phenotyping the cell surface markers and cytokine production (Seder & Roederer, 2008). The approach is based on changes in the numbers of CD4 cells associated with bacillary load in active TB and thus their use in differentiating LTBI from active disease is being looked into and perceived to give hope for a better diagnostic method (Millington *et al*., 2007).

The idea of targeting the host genes in blood cells from patients for the diagnosis of TB was also supported by a study conducted by Mistry *et al.* (2007) in an attempt to identify a gene “signature” specific to TB so that development of a diagnostic test can be based on the findings and perhaps differentiate disease stages. The study however only derives a set of four genes which could discriminate patients with active TB from those with latent TB and those who had previously been treated (Mistry *et al.,* 2007).

In a similar fashion, another study was also conducted where a set of three distinct genes to discriminate patients with active TB from healthy individuals or those with LTBI were identified (Jacobsen *et al.,* 2007). A study based on a wider microarray approach was carried out in the Republic of South Africa in an attempt to confirm the same three genes-set (Maertzdorf *et al*., 2011). The study concluded that the three genes were unable to discriminate between all groups in this separate population. The study further reported that the three genes were only capable of creating a separate microarray profiles discriminating active TB from healthy individuals and those with latent infection. It was also found that these profiles could not discriminate LTBI from healthy individuals.

Berry *et al.* (2010), used a microarray based method to identify differential gene expression in active and latent TB and identified a 393-gene signature which discriminated active TB from healthy individuals and those with latent infection in separate groups of patients from the United Kingdom and South Africa. In this case the profiles of the genes also failed to distinguish LTBI from healthy individuals. It was also found that a separate 86-gene signature was able to differentiate patients with active TB from those with LTBI and those with other inflammatory and infectious diseases. In an effort to increase the possibility that this approach could be used in future, more studies are needed to assist in the identification of individuals at the greatest risk of developing active disease.

There is need for alternate methods that are able to test gene expression in cells which are first stimulated with *MTB* specific antigens. Another study was able to differentiate latently infected individuals from patients with active TB by measuring the expression of three genes but it must be noted that this study excluded healthy individuals as a comparison group (Wu *et al.,* 2007). Moreover, other studies revealed that measuring the ratio of the expression of levels of interleukin (IL)-4 and its splice variant IL-4d2 can correlate with extent of disease and changes in the ratio may point to changes in bacillary burden but no tangible solutions were confirmed (Demissie *et al.*, 2016; Siawaya *et al*., 2008).

1.5 Control of Tuberculosis

1.5.1 Antibiotic control

Several control and treatment strategies have been undertaken using drugs to manage the disease. The first effective drugs against MTB were Streptomycin (SM) and Paraaminosalicyclic acid (PAS), discovered in 1944 (Salyers *et al.,* 2002). Later, it was observed that a combination of the two drugs was more effective at both achieving cures and preventing acquired drug resistance. When Isoniazid (INH) was added to the treatment regimen in 1952, an efficacious triple therapy was derived.

First line anti-TB drugs are the drugs that are given to TB patients after first diagnosis of TB and the second line anti–TB drugs are those that are used to treat TB patients if first line drugs fail. Drugs considered to be first line include pyrazinamide (PZA),

Rifampicin (RIF), Streptomycin (STR), Ethambutol (EMB) and isoniazid (INH). Second line drugs include aminoglycosides (kanamycin, amikacin, capreomycin), polypeptides, fluoroquinolones (ciprofloxacin and ofloxacin), D-cycloserine and thionamides (Johnson *et al.,* 2006; WHO, 2001).

The *Mycobacterium* spp. causing TB have been shown to survive for prolonged periods of time and enter a dormant stage, which complicates effective treatment (McKinney *et al.,* 2000; Pablos-Mendez, 2000). Antibiotic treatment can find it difficult to penetrate or inhibit MTB, when the bacteria is located in pulmonary cavities, empyema pus, or solid caseous material (Elliott *et al.,* 1995). There is also a belief that there are many different populations of the TB bacteria within a single host. Numerous clinical trials in both humans and animals, affirmed the concept of many different populations of TB bacteria (Drimitrios, 2015; Wintaco, Castro & Guerrero, 2016).

Tuberculosis patients are required to use multiple drugs during treatment, as each of the drugs used against TB has a role to play and their use in combination results in a synergistic effect. The most common drugs used for therapy are for example, isoniazid which is critical at the initial stage of the therapy. Isoniazid has a bactericidal role which can reduce the sputum viable count in a fast time due to its active role against the aerobic growth of the TB organisms in pulmonary cavities (Drake, 1999). On the other hand, Pyrazinamide works optimally when the pH is low. This makes the pyrazinamide an ideal drug to kill the TB organisms when they are inside the caseous necrotic foci. Pyrazinamide is normally used for a period of two months and it is hardly recommended to the patients after the two months has lapsed since findings revealed that pyrazinamide appears to have no benefit after the second month of therapy (Drlica & Zhao, 1997),

Rifampicin is one of the most important drugs in TB therapy as it has the ability to kill the persistent organisms that are metabolizing slowly as well as keeping the patient's sputum sterile (Grumbach *et al.,* 1970; Drlica & Zhao, 1997).

1.5.2 Antibiotic Resistance

When patients do not adhere to the therapy as prescribed, organisms can develop resistance to the medication. **Figure 1.4** below demonstrates the link between

mutation rate, population size, and clinical complication in the emergence of resistance in MTB infections (Lipsitch & Levin, 1998).

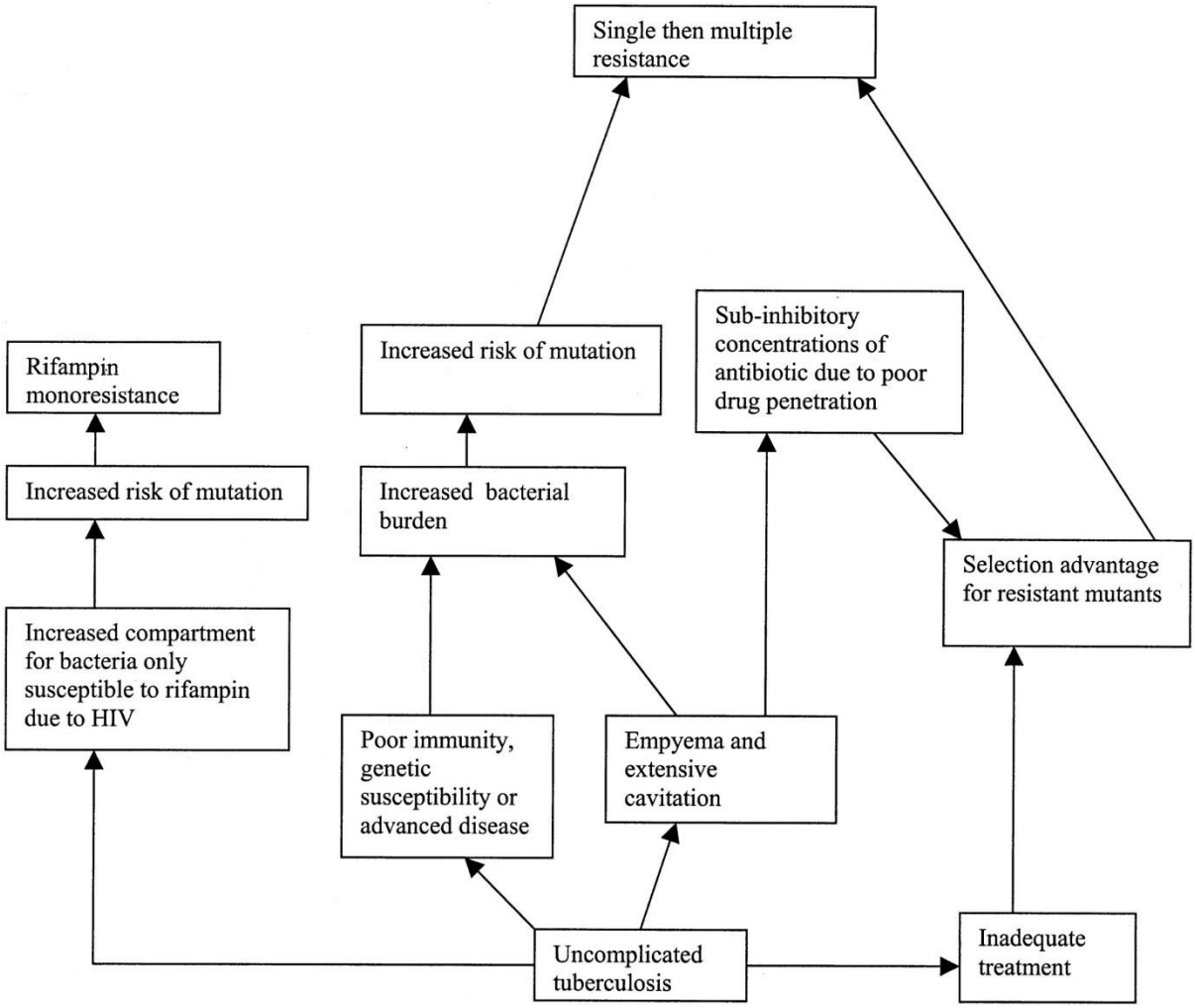

Figure 1.4: Diagrammatic representation of the link between mutation rate, population size, and clinical complication in the emergence of resistance in MTB infection. (adopted from: Lipsitch & Levin, 1998).

In recent years there has been an increase in the number of patients with Multiple Drug Resistant TB (MDR-TB). This form of TB is difficult and expensive to treat and has a high fatality rate. According to Helal *et al.* (2010) in high burdened countries, the resistance rates are around 15%. Among all reported TB cases globally, 3.6% are estimated to have MDR-TB. China and India are reported to have 50% of all TB cases worldwide (Raja *et al.,* 2011). Furthermore, MDR-TB is estimated to have caused about 150 000 deaths in 2008. According to WHO, 48% of African countries, 57% of the Americas, 38% of Eastern Mediterranean, 83% of European, 55% of South-East Asia,

52% of Western Pacific and 59% of WHO regions globally reported first-line anti-TB drug resistance (Raja *et al.,* 2011).

The report of the MoHSS (2014) indicated that about 137 cases of multi drug resistance tuberculosis (MDRTB) occurred in that year. Of these, six (6) were found to be XDR-TB (MoHSS, 2014). Even though a slight decline of MDR-TB was shown as compared to the previous year's report where 174 cases were reported, the figure remains high. In addition, the number of reported cases of rifampicin (RIF) resistant tuberculosis diagnosed using the GeneXpert MTB/RIF test increased from 89 cases in 2013 to 199 cases in 2014 (MoHSS, 2014). Namibia ranked 4th globally in terms of the per capita incidence of TB according to the same report (MoHSS, 2014).

In order to protect members of the community from being exposed to MDR-TB it is important to ensure that infected patients are hospitalised and also isolated until they are no longer infectious i.e. their sputum no longer contains the TB bacteria. Multiple Drug Resistant TB patients are required to be hospitalized for an extended period of time, which often exceeds one year. In all MDR-TB cases in the world, that emerge every year, 50% are from new TB cases while 50% are from previously treated TB patients and approximately 5-7% of these cases are expected to have Extensively Drug Resistant TB (XDR – TB) (Vijdea *et al.,* 2008).

1.5.3 Compartmentalization in Treatment of *Mycobacterium tuberculosis*

Non-adherence is not the only contributor to the development of the resistant strains of the TB organism. Compartmentalization can also contribute to the TB organism becoming resistant to the initial treatment especially in the case where the physician has prescribed an inadequate dose to a patient. This act can expose the bacteria to monotherapy, especially when the patient receives inadequate therapy (Elliott *et al.,* 1995).

In addition, association between HIV infection and multiple drug resistance has been speculated even though the reasons therefore are not known (Ridzon *et al.,* 1998). Partly this is due to circulation of multidrug-resistant tuberculosis strains in HIV-seropositive communities (Alland *et al.,* 1994). This might happen due to the fact that most HIV infected patients have their immune system suppressed making them more

vulnerable to host a large number of organisms available for mutation. This hypothesis however, needs more research before a conclusion that the large bacterial population size, was a reason for drug resistance.

1.5.4 Molecular mechanisms for the emergence of drug resistance

Over the past 10 years, discussion on the MTB resistance has taken centre stage and the understanding of some molecular mechanisms where MTB becomes resistant has been widely discussed (Hoagland, *et al.,* 2016). The MTB is often acquired early in life with acute infection and with developing immunity, granuloma formation and calcification which later forms a latent TB for a period of time, until the bacteria is triggered and activated. In addition, the bacteria will occasionally interact with other strains to exchange genetic information (Smith, 2003).

Mutations are constantly occurring due to base changes caused by exogenous agents in any prokaryotic genome. This is characterised by the DNA polymerase errors, deletions, insertions, and duplications (Drake, 1999). The mutation rate for individual genes does not always happen in a similar pattern and is influenced by many factors.

The mutation site which eventually results in rifampicin resistance was discovered by Telenti *et al.*, (1993). This study investigated how *Escherichia coli* became resistant to rifampicin through mutation in the beta subunit of the *rpoB* gene (Talenti *et al.,* 1993). The authors reported that almost all rifampicin-resistant isolates had mutations in a small region of *rpoB*. Subsequently, further clinical studies indicated that mutations are found in this region in up to 95% of resistant isolates (Ramaswamy & Musser, 1998). A similar approach has been adopted to detect mutations conferring resistance to other antibiotics. The molecular mechanism of resistance to all of the main anti-tuberculosis drugs, including isoniazid, pyrazinamide, streptomycin, ethambutol, and fluoroquinolones, has since been described (Cambau *et al.,* 1994; Hirano *et al.,* 1997). The different genes that have been associated with resistance to anti-tuberculosis agents are summarized in **Table 1.1.** below.

Table 1.1: Summary of the molecular mechanisms of anti-tuberculosis drug resistance (http://aac.asm.org/)

Drug	Associated mutated gene or mutation
Rifampicin	*rpoB*
Isoniazid	*katC*, *inhA*, *oxyR*, *ahpC*, *furA*
Streptomycin	*rrs*, *rpsL*
Pyrazinamide	*pncA*, IS*6110* insertion
Ethambutol	*embB*
Fluoroquinolones	*gyrA*, *gyrB*

Bastian and Portaels (2000) asserted that the study of the genes and mutations that cause resistance has helped to both identify the mechanisms of action of these antibiotics and to enhance our knowledge of the basic biology of the mycobacteria. Researchers have identified several ways through which drug resistance can occur as a result of mutations. One such mechanism is caused by a mutation in the gene that encodes the drug target. This is usually an enzyme, which, if inhibited, results in the toxic effects of the drug. Such mutations tend to decrease the ability of the drug to bind to the target and define the region on the protein that interacts with the drug. Other types of mutation do not change the target of the drug, but only increase its expression, ensuring that more target sites are available than that which the drug is able to inhibit. Drugs, such as INH for example, enter the bacterium as prodrugs (not active in its ingested form). In order for such drugs to be effective, they require a stage of activation which is catalysed by a bacterial enzyme. Therefore any mutations in the activator that inhibit the production of the active form of the drug will be an effective mechanism of resistance. Resistance can also result from mutations that decrease the accumulation of drug within the bacterium by either decreasing entry of a drug into the bacterium, or by increasing the rate of removal of the drug from the cell. Finally chemical modification can also cause resistance and inactivation of drugs (Spratt, 1994; Maiden, 1998).

1.6 Tuberculosis Control programs

1.6.1 The WHO Control Program

The WHO have commenced with a strategy called “End TB Strategy” (WHO, 2015; WHO, 2014). This strategy has ambitious goals to reduce TB incidence by 50% and reduce TB mortalities by 75% by 2025 (WHO, 2015).

The “End TB” Strategy, was also developed in line with the United Nations (UN) Sustainable Development Goals (SDGs), as opposed to the past global TB strategies (WHO, 2014; UN, 2015). In addition, the Direct Observed Treatment (“TB DOTS”) strategy of 1994 was also revitalised to help National TB Programs (NTPs) accelerate their anti-TB efforts (WHO, 1994). All these efforts are aimed to improve access to quality TB care in the respective countries.

The WHO framework of the post-2015 global tuberculosis strategy is presented in **Table 1.2.** below.

Table 1.2: Post-2015 Global Tuberculosis Strategy Framework (adopted from: WHO, 2014)

VISION	A world free of tuberculosis – zero deaths, disease and suffering due to tuberculosis
GOAL	End the global tuberculosis epidemic
MILESTONES FOR 2025	75% reduction in tuberculosis deaths (compared with 2015) 50% reduction in tuberculosis incidence rate (less than 55 tuberculosis cases per 100 000 population) – No affected families facing catastrophic costs due to tuberculosis
TARGETS FOR 2035	95% reduction in tuberculosis deaths (compared with 2015) 90% reduction in tuberculosis incidence rate (less than 10 tuberculosis cases per 100 000 population) – No affected families facing catastrophic costs due to tuberculosis
PRINCIPLES 1. Government stewardship and accountability, with monitoring and evaluation 2. Strong coalition with civil society organizations and communities 3. Protection and promotion of human rights, ethics and equity 4. Adaptation of the strategy and targets at country level, with global collaboration	

PILLARS AND COMPONENTS
1. **INTEGRATED, PATIENT-CENTRED CARE AND PREVENTION** A. Early diagnosis of tuberculosis including universal drug-susceptibility testing, and systematic screening of contacts and high-risk groups B. Treatment of all people with tuberculosis including drug-resistant tuberculosis, and patient support C. Collaborative tuberculosis/HIV activities, and management of comorbidities D. Preventive treatment of persons at high risk, and vaccination against tuberculosis
2. BOLD POLICIES AND SUPPORTIVE SYSTEMS A. Political commitment with adequate resources for tuberculosis care and prevention B. Engagement of communities, civil society organizations, and public and private care providers C. Universal health coverage policy, and regulatory frameworks for case notification, vital registration, quality and rational use of medicines, and infection control D. Social protection, poverty alleviation and actions on other determinants of tuberculosis
3. INTENSIFIED RESEARCH AND INNOVATION A. Discovery, development and rapid uptake of new tools, interventions and strategies B. Research to optimize implementation and impact, and promote innovations

1.6.2 Control Programs employed in Namibia

Strategies to reduce TB in Namibia have been put in place for better management and TB control (MoHSS, 2010). However, inadequate funding aimed at reducing TB remains a challenge. However, the first- and second-line medicines for TB patients are subsidised by the government and is free of charge. Eight out of every ten health-care facilities offer diagnostic services for TB patients and this is easily accessible to the patients (MoHSS, 2009).

Despite the many challenges such as insufficient diagnostic laboratories in the country as well as limited community-based DOTS providing treatment for TB and direct observation of intake of medication, Namibia still managed to have the minimum number of laboratories required to provide culture and drug susceptibility testing services in the diagnosis of TB as per WHO guidelines (WHO, 2011). The National TB programme in Namibia has achieved progress through the involvement of implementing organizations such as Advanced Community Health Care Services Namibia (CoHeNa) and Penduka (a non-governmental development organisation working with women in Namibia), which have community volunteer healthcare

providers to support and provide care to clients receiving TB treatment at designated Direct Observed Treatment (TB DOT) points (MoHSS, 2009).

All regions in the country have functional community-based DOTS which are centred around patients. This is a patient approach aimed at empowering the community and patient for the prevention of the TB disease. Experience shows that activities that foster community and patient empowerment can have a positive impact on case detection and treatment outcomes (WHO, 2009). Initiatives such as “The TB Road Show of 2011” saw two groups travelling throughout the country with a TB awareness campaign with two basic messages: 'Coughing for 2 weeks? Get TESTED for TB!' and 'On treatment for TB? COMPLETE it!' (WHO Namibia, 2011)

The WHO is committed to support the Republic of Namibia through the line ministry of the Ministry of Health and Social Service. This will be achieved through a new initiative hosted at the University of Namibia (UNAM). This initiative is looking at developing new diagnostic tools and medicines to speedup case identification and shorten the duration of treatment (WHO Namibia, 2009; UNAM, 2011). Another area for the initiative is to look into community perceptions, beliefs and practices relating to prevention, treatment and rehabilitation (MoHSS, 2009).

1.7 Objectives of Study

Accurate and early diagnosis of TB is vital as it interrupts further transmission of the disease and avoids the use of more treatment drugs and thus decrease in the development drug resistance and possible creation of extensively drug resistant-tuberculosis (XDR-TB). The diagnosis of TB is often slow because of the slow growth rate of the causative agent, *Mycobacterium tuberculosis* complex (MTBC). Furthermore, many low and middle-income countries where almost 95% of TB cases and 98% of deaths occur due to TB, consider sputum smear microscopy as the primary method for diagnosis of tuberculosis, since this technique is not complicated, and it is affordable and low turn around time to obtain the final results (Desikan, 2013).

One of the aims of this study was thus to compare direct microscopy with two rapid molecular diagnostic tools (viz. GeneXpert *MTB*/RIF and Hain Genotype® MTBDR

Plus assay) for the identification of *MTB* from samples collected from the Khomas Region, in Windhoek, Namibia.

As mentioned previously, in recent years there has been an increase in the number of patients with Multiple Drug Resistant TB (MDR-TB). Within a laboratory, many TB diagnostics detect RIF-resistance while few detect INH-mono-resistance. RIF-resistance is mainly assessed, as it has been argued that *MTB* that is resistant to RIF is more likely to have concomitant resistance to INH, making RIF resistance a surrogate marker of MDR-TB (Somoskovi *et al.,* 2001).

A second aim was thus to compare the total agreement of RIF resistance and sensitivity using GeneXpert MTB/RIF and Hain Genotype® MTBDR Plus assay.

Tuberculosis surveillance data have been globally recognised, as a fundamental data source for assessing the disease burden and epidemiological trends of TB. Public health surveillance, through the ongoing and systematic collection, analysis, interpretation and dissemination of health information, plays a critical role in informed decision-making and appropriate public health action.

Lastly, clinical, demographic and epidemiological data were collected through the interviewing of patients to assess epidemiological and clinical trends.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1. STUDY POPULATION

Ethical clearance to conduct the study was obtained from the Cape Peninsula University of Technology (CPUT) Faculty of Health Sciences Ethics Committee (Certificate number: CPUT/HW-REC 2012/H0), South Africa as well as the Ministry of Health and Social Services (MoHSS), Namibia. Furthermore, the researchers signed a memorandum of understanding with the Namibia Institute of Pathology (NIP) to further obtain permission to use the samples, and to use equipment at their site.

Patients presenting at clinics and health facilities in the Khomas region (Windhoek, Namibia) were eligible for the study. Patients of all ages, genders, etc. were selected and informed consent (**Appendix A**) was obtained from all patients prior to enrolment in the study. Where applicable, parents or legal guardians signed on behalf of minors. Sputum specimens were obtained with the assistance of nursing staff, doctors and community health workers for a period of 6 months from February to July 2014. The sputum specimens were brought to the Namibia Institute of Pathology (NIP) laboratory for acid-fast bacilli (AFB) testing (section 2.2), as this is the standard staining method utilised for presumptive identification of *Mycobacterium tuberculosis* (MTB). The TB sputum specimens were refrigerated *at* 4 °C and for cold transportation, was transported on dry ice. The Xpert MTB/RIF assay and the microscopic examination was done in Namibia while the Hain GenoType® MTBDR *plus* assay was carried out in South Africa. All experiments were completed within three months after collection. Three hundred and eighty-four samples were confirmed acid-fast positive by utilising the auramine staining method. For use in the analysis and for interpretation purposes patient sputa was given a numerical number ranging from one to 384. At least 1 mL residual volume sputum from each sample (n = 384) was utilised for subsequent testing in the current study.

2.2. DIRECT MICROSCOPY

2.2.1. Smear Preparation

The smears were directly prepared from the patient's sputum specimens by spreading sputum material in an area of approximately 2 cm^2 onto a clear glass slide. Smears were allowed to air dry completely within the biological safety cabinet. Thereafter the smear slides were heat fixed at 65-75 °C for 2 hours.

2.2.2. Auramine Staining

The auramine staining technique is used with fluorescence and light-emitting diode microscopes. Auramine is a nonspecific fluorochrome dye that has an affinity for acid-fast organisms. In the case of *Mycobacterium*, the dye can bind specifically to the mycolic acid contained in the cell wall allowing the penetration of the stain. This complex resist decolourization by acid-alcohol decolourizer solution. The counterstain, potassium permanganate helps to prevent nonspecific fluorescence, thus reducing the possibility of artefacts. When observed under the microscope with UV illumination, acid-fast cells are reddish-yellow against a dark background (Ebersole, 1992).

After heat fixing, the slides were placed on a staining rack to prevent them from touching each other. Slides were then flooded with auramine and left to stain for 20 minutes. The auramine stain was rinsed off with water in such a way that the water flowed off the edge of slide. The stained slides were then decolourized with 0.5% acid-alcohol solution for 3 minutes. The slides were again rinsed with water and the excess water was drained. Slides were flooded with 0.5% potassium permanganate counterstain, whereafter the slides were rinsed with water and the excess fluid was drained. Stained slides were then air-dried for 15 minutes at 80 °C.

2.2.3. Microscopy

2.2.3.1. Microscopic examination of smears

Acid-fast bacilli (AFB) appear reddish-yellow against the blue counterstained background. They vary greatly in shape, from short, coccoid to elongated filaments;

they can be uniformly or unevenly stained, and can even appear granular. They occur singly or in variable sized clumps and typically appear as long, slender curved rods.
The microscopic examination of the slides with an Olympus microscope (Japan) was done. Smears were examined under the high-power objective at 40 x, 60 x magnification as well as under the oil immersion objective at 100 x magnification. Reading began at the periphery of the field at the left end of the smear and ended at the centre. When the field was viewed, the slide was moved longitudinally to examine adjacent fields. The slide was then moved vertically so that a second length could be read from right to left. There were approximately 100 immersion fields in the 2 cm long axis of a smear. A total of 384 TB positive patient's sputa were identified.

2.2.3.2. Grading of sputum smear microscopy results

The information on the number of bacilli found is very important as it relates to the degree of infectivity of the patient as well as to the severity of the disease. For this reason, the report of the results of sputum smear microscopy is not only qualitative but also semi-quantitative (International Union against Tuberculosis and Lung Disease, 2000). The International Union Against Tuberculosis and Lung Disease (IUATLD) recommends the grading of results of acid-fast smear microscopy as outlined in **Table 2.1**.

Table 2.1: IUATLD-recommended grading of sputum smear microscopy results (2000)

AFB counts	Recording/reporting
No AFB in at least 100 fields	0/negative
1 to 9 AFB in 100 fields*	Actual AFB counts†
10 to 99 AFB in 100 fields‡	+
1 to 10 AFB per fields in at least 50 fields†	++
> 10 AFB per field in at least 20 fields‡	+++

* A finding of 1 to 3 bacilli in 100 fields does not correlate well with culture positivity. The interpretation of the significance of this result should be left to the National Tuberculosis Programme (NTP) and not to the microscopist. It is recommended that a new smear be prepared from the same sputum specimen and be re-examined.
† The reporting of actual AFB counts is recommended to allow a competent authority to determine whether the number fits the TB case definition of the NTP.
‡ In practice most microscopists read a few fields and confirm the finding by a quick visual scan of the remaining fields.

When MTB sputum smear microscopy is fully integrated into the general primary health care services, the real challenge is to reach a workload high enough to maintain testing proficiency. No more than 10 to12 specimens were processed at one time.

2.3. GeneXPERT MTB/RIF ASSAY

2.3.1. Expectorated Sputum Sample Procedure

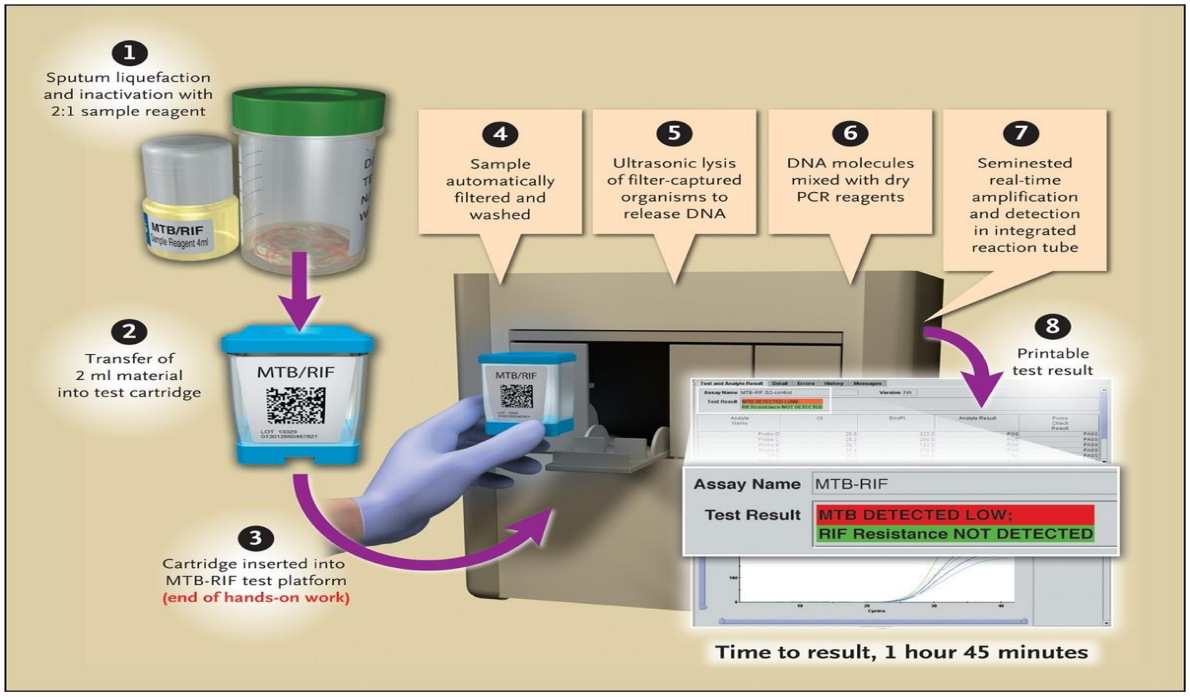

Figure 2.1: Illustrative photographs of the specialised GeneXpert equipment (retrieved from http://www.astrocyte.in/article.asp)

The GeneXpert MTB/RIF test is an automated diagnostic test that can identify MTB DNA and resistance to rifampicin (RIF) by nucleic acid amplification using the Polymerase chain reaction (PCR) (**Figure 2.1**). This analysis was performed at the Namibia Institute of Pathology (NIP) on the 384 acid-fast positive samples. Auramine stain results were then correlated to results of the GeneXpert MTB/RIF to classify a patient as either MTB negative, MTB positive and multi-drug resistant (MDR) MTB.

Each GeneXpert MTB/RIF cartridge (GeneXpert®, Cepheid, USA) was labelled with the sample identification (ID) (based on acid-fast analysis; *n* = 384). The writing was only done on the sides of the cartridge or an ID label was affixed. No labels were placed on the lid of the cartridge or cover of the existing 2D barcode of the cartridge. The lid of the sputum collection container (leak-proof) was carefully opened and 2 x the volume

of the sample reagent (SR) was combined with the sputum (2:1 dilution; SR:sputum) in the container. A 2.0 mL GeneXpert MTB/RIF Sample Reagent was added (2:1; v/v) to the expectorated sputum using a sterile transfer pipette (i.e. 2 mL GeneXpert MTB/RIF SR to 1 mL sputum). The sputum cup was vigorously shaken (10-20 times using back and forth movements in a single shake). The homogenized sample was incubated in the sputum cup for 15 minutes at room temperature. During the incubation period, the sputum cup was shaken at least once, as described above so as to liquefy the sputum sample and to ensure no visible clumps of sputum after incubation.

The treated sample was then transferred into the cartridge and the cartridge was loaded into the GeneXpert instrument (GeneXpert®, Cepheid, USA). Each GeneXpert MTB/RIF cartridge was properly labelled with the laboratory number, as labelled on the sputum cup.

Before the test commenced, the system software was checked (GX 4.0 or higher). Furthermore, the GeneXpert MTB/RIF assay definition file was imported into the software.

The steps outlined below, follow the basic steps of operation as outlined in the *GeneXpert Dx System Operator Manual* or the *GeneXpert Infinity System Operator Manual (*GeneXpert Dx System, 2012).

The GeneXpert Dx System (Cepheid, USA) uses an integrated and automated sample processing system. The system consists of an instrument, personal computer, barcode scanner and preloaded software for running tests on collected samples and viewing the results. Nucleic acid amplification and detection of the target sequences in simple or complex samples is done using real-time PCR and reverse transcriptase PCR. The primers in the GeneXpert MTB/RIF assay amplify a portion of the *rpoB* gene containing the 81 base pair “core” region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with RIF resistance. The system requires the use of single-use disposable GeneXpert cartridges that contain the PCR reagents. Given the fact that the cartridges were self-contained, cross–contamination between samples was minimised.

2.3.2. Quality Control

Each test included a Sample Processing Control (SPC) and Probe Check Control (PCC).

Sample Processing Control (SPC): It ensures that the sample is correctly processed. The SPC contains non-infectious spores in the form of a dry spore cake that is included in each cartridge to verify adequate processing of MTB. The SPC verifies that lysis of MTB occurs if the organisms are present and also verifies that specimen processing is adequate. Additionally, this control detects specimen-associated inhibition of the real-time PCR assay. The SPC should be positive in a negative sample and can be negative or positive in a positive sample. The SPC was regarded as successful if it met the validated acceptance criteria. The test result would be “invalid” if the SPC is not detected in a negative test.

Probe Check Control (PCC): Before the start of the PCR reaction, the GeneXpert Dx System measures the fluorescence signal from the probes to monitor bead rehydration, reaction-tube filling, probe integrity and dye stability. The PCC was regarded as successful if it met the assigned acceptance criteria.

2.3.2.2. Interpretation of Results

The GeneXpert Instrument system generates results from measured fluorescent signals and embedded calculation algorithms. **Table 2.2** illustrates the expected results generated from the measured fluorescent signals and embedded calculation algorithms using the GeneXpert System if MTB is detected.

Table 2.3 below illustrates the results generated from the measured fluorescent signals and embedded calculation algorithms using the GeneXpert System if MTB is not detected.

Table 2.2: Expected results (MTB detected) generated from the measured fluorescent signals and embedded calculation algorithms using the GeneXpert (adopted from: www.cepheid.com)

MTB DETECTED	**RIF Resistance DETECTED** The MTB target was present within the sample if: A mutation in the *rpoB* gene has been detected that fell within the valid delta computed tomography (Ct) setting (relative gene quantification). Probe SPC: NA (not applicable). An SPC signal was not required because MTB amplification could compete with this control Check: PASS. All probe check results passed.
	RIF Resistance NOT DETECTED The MTB target was present within the sample if: No mutation in the *rpoB* gene has been detected. SPC: NA (not applicable). An SPC signal was not required because MTB amplification could compete with this control. Probe Check: PASS. All probe check results passed.
	RIF Resistance INDETERMINATE The MTB target was present within the sample if: RIF resistance could not be determined due to insufficient signal detection. SPC: NA (not applicable). An SPC signal was not required because MTB amplification could compete with this control. Probe Check: PASS. All probe check results passed.

Table 2.3: Results (MTB Not detected) generated from the measured fluorescent signals and embedded calculation algorithms using the GeneXpert System (adopted from: www.cepheid.com)

MTB NOT DETECTED	The MTB target was not detected within the sample: SPC: PASS. The SPC met the acceptance criteria. Probe Check: PASS. All probe check results passed.
	INVALID The presence or absence of MTB could not be determined. The SPC did not meet the acceptance criteria, the sample was not properly processed, or PCR was inhibited. Repeat the test. MTB INVALID: The presence or absence of MTB DNA could not be determined. SPC: FAIL. The MTB target result is negative, and the SPC Ct was not within valid range. Probe Check: PASS. All probe check results passed.
	ERROR The presence or absence of MTB could not be determined. The test should be repeated. MTB: NO RESULT SPC: NO RESULT Probe Check: FAIL. All or one of the probe check results failed. Note: If the probe check passed, the error is caused by a system component failure
	Probe Check: NA (not applicable)

2.3.2.3. Failed Assays

The test was repeated using a new cartridge if a test result such as invalid, error and no result occurred. An INVALID result indicated that the SPC failed (**Table 2.3**). This could be due to fact that the sample was not properly processed, or that the PCR was inhibited. On the other hand, when an ERROR result occurred, this could indicate that the PCC failed, and the assay was aborted possibly due to the following: a reaction tube being filled improperly; a reagent probe integrity problem was detected; the maximum pressure limits were exceeded or a GeneXpert module failed. Finally, a NO RESULT indicated that insufficient data were collected. For example, if the operator stopped a test that was in progress.

2.4. HAIN GENOTYPE® MTBDR *PLUS* ASSAY PROCEDURE

2.4.1. Principle of the Procedure

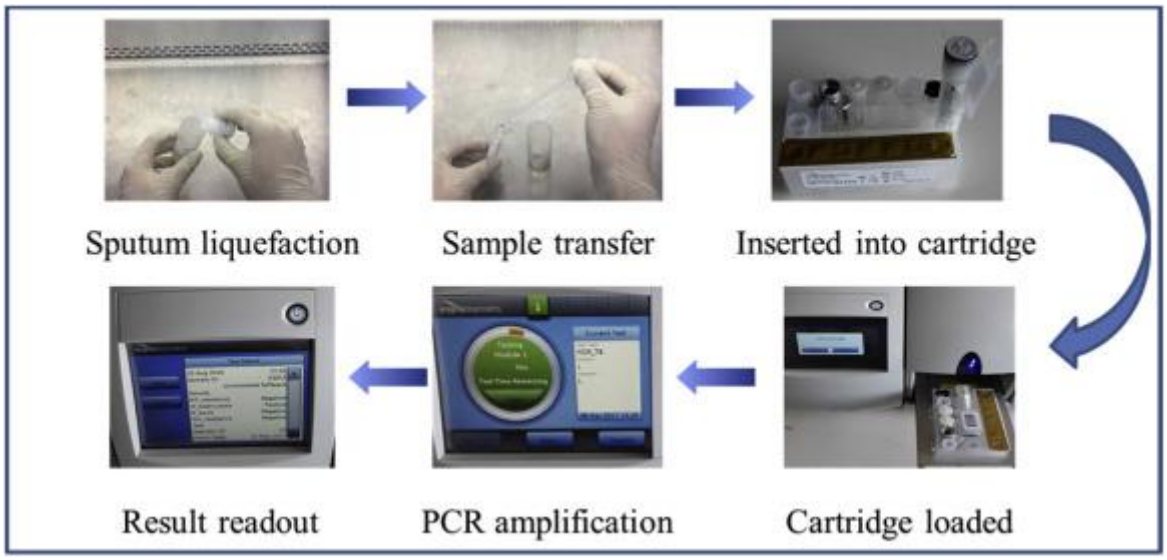

Figure 2.2: Illustrative photographs of the Hain GenoType MTBDR plus assay test kit (retrieved from: https://www.sciencedirect.com/science/article/pii/S1472979217300239)

The Hain GenoType® MTBDR *plus* assay (Hain Lifescience, Nehren, Germany) is an in vitro test for identification of the *Mycobacterium tuberculosis* complex (**Figure 2.3**). This test was conducted on all acid-fast (auramine) positive samples (*n* = 384) at the National Health Laboratory Services (NHLS), South Africa.

The Hain GenoType® MTBDR *plus* assay platform is based on DNA•STRIP® technology and permits the molecular identification of *Mycobacterium tuberculosis* complex (MTBC) species as well as the discrimination at species level within the complex. The Genotype® MTBDR *plus* assay allowed for the detection of most of the common mutations involved in drug resistances to RIF and isoniazid (INH). The methodology is DNA-based which requires DNA isolation as the first step of the procedure.

DNA could be extracted directly from biological sputum samples (*n* = 384) confirmed by microscopy to be MTB positive. The whole procedure was divided into four steps: (i) DNA extraction (from positive clinical specimen decontaminated by NALC-NaOH) (ii) A multiplex PCR amplification with biotinylated primers, (iii) reverse hybridization (the hybridization included a chemical denaturation of the amplification products,

hybridization of a single-stranded, biotin-labelled amplicons to the membrane-bound and stringent washing) and (iv) addition of a streptavidin/alkaline phosphatase (AP) conjugate and a AP mediated staining reaction.

2.4.2. Quality Control

In order to validate the correct performance of the test and the proper functioning of reagents, each MTBDRplus and MTBDRsl strip includes 5 control zones: - **Conjugate Control (CC) zone** to check the binding of the conjugate on the strip and a correct chromogenic reaction. - **Amplification Control (AC) zone** to check for a successful amplification reaction. - **MTBDRplus:** three Locus Control zones (rpoB, katG and inhA) checking the optimal sensitivity of the reaction for each of the tested gene loci. - **MTBDRsl:** three locus control zones (gyrA, rrs and embB) checking the optimal sensitivity of the reaction for each of the tested gene loci. Each time the test is performed a positive (sensitive M.tb H37RV strain) and negative (ddH2O) control is used Note: Positive control (MTBDRplus) – AC/CC/TUB positive, all 3 locus control probes positive, all wild type probes positive, all mutation probes negative, overall sensitive to rifampicin and isoniazid. **Positive control (MTBDRsl)** – AC/CC/TUB positive, all 3 locus control probes positive, all wild type probes positive, all mutation probes negative, overall sensitive to fluoroquinolones, aminoglycosides and ethambutol. **Negative control** – AC/CC positive, all other probes negative. Occasionally there may be non-specific banding patterns in the negative control. This is acceptable as long as the TUB band is absent.

2.4.3. DNA extraction from clinical specimens and MTB isolates

Acid-fast positive sputum specimens (*n* = 384) graded as scanty, 1+, 2+ and 3+ (**Table 2.1**) were prepared for the GenoType® MTBDR *plus* assay by first concentrating 500 µL of the residual processed specimen in a microcentrifuge (10 000 × g, 15 minutes, room temperature). The supernatants were decanted, and the pellet was re-suspended in 100 µL of sterile distilled water. The sample aliquot was then placed in a heating block for 20 minutes at 95 °C. Thereafter, the samples were sonicated in TECHNE-DRI-BLOD DB 2D ultrasonic bath (Techne On - Block) for 15 minutes and the supernatants were transferred to a new tube and stored for PCR analysis.

2.4.4. DNA amplification

Amplification was performed by combining 35 µL of primer nucleotide mix (PNM), (Hain Lifescience GmbH, Nehren, Germany) with 5 µL of 10 × PCR buffer (containing 15 mM $MgCl_2$), 2 µL $MgCl_2$ (25 mM MgCl2), 2.8 µL molecular grade H_2O, 0.2 µL (1 unit) Hot-Start *Taq* polymerase (QIAGEN, Hilden, Germany), and 5 µL of the bacterial suspension for a total final volume of 50 µL. The amplification profile for direct patient material as described by the manufacturer was used for all bacterial suspensions. First, the template DNA was denatured for 15 minutes at 95 °C, followed by 10 cycles consisting of 30 s at 95 °C and 2 minutes at 58 °C, with an additional 30 cycles consisting of 25 s at 95 °C, 40 s at 53 °C and 40 s at 70 °C. The final cycle consisted of an 8 minute run at 70 °C.

2.4.5. Hybridization

The procedure of hybridization and development of the assays was the same for every sample analysed.

A Twin Incubator was pre-warmed to 45 °C. Hybridization buffer (HYB, green) and stringent wash (STR, red) solutions were warmed to 37- 45 °C for 15 minutes in a water bath while the other reagents [except for Conjugate Concentrate (CON-C) and Substrate Concentrate (SUB-C) that were stored at 2-8°C till use] were left to equilibrate at room temperature. The GenoType® MTBDR *plus* assay kit (Hain Lifescience GmbH, Nehren, Germany) was used. The number of strips required for running the assay was handled with forceps and were marked on the left side of the blue/red/green line with the sample number (one to 384).

Strip trays were taken and 20 µL of denaturation (DEN) solution was dispensed on the bottom corner of each lane of the tray. Twenty microliters of PCR product were added to each DEN solution and was mixed well by pipetting. This was left to stand for 5 minutes at room temperature. One millilitre of pre-warmed HYB solution was added to each lane and shaken gently until the solution appeared evenly coloured. The corresponding strip was placed in the respective lane. Care was taken to ensure that the strip was well covered by the HYB solution. The tray was placed into the Twin Incubator and the shaking mode was turned on (frequency 300-350 rpm). The lid was closed, and the strips were left to incubate for 30 minutes at 45 °C while shaking. The

hybridization solution was completely aspirated and 1 mL of STR solution (red) was added to each lane and incubated for 15 minutes at 45 °C while shaking (300-350 rpm)

Simultaneously the Conjugate Working solution and the Substrate Working solution were prepared. The two working solutions were prepared by making a 1:100 solution of the concentrated reagents (CON-C and SUB-C) with the respective diluted reagents, now named CON-D and SUB-D, respectively.

The STR solution was removed completely by pouring off the liquid into a container and turning the tray upside down on an absorbent paper. Each strip was washed with 1 mL of RIN solution for 1 minute at room temperature shaking the tray gently. The RIN solution was removed completely by pouring off the liquid into a container and turning the tray upside down on an absorbent paper. One millilitre of Conjugate solution was added to each strip and incubated for 30 minutes at room temperature while shaking (The Twin Incubator set previously at 25 °C was used). The Conjugate solution was completely removed by pouring off the liquid into a container and turning the tray upside down on an absorbent paper. Each strip was washed twice with 1 mL RIN solution for 1 minute with gentle agitation. The solution was completely removed at the end of each step. Each strip was washed once with 1 mL distilled water, shaking gently. The water was then removed. One millilitre of Substrate solution was added to each strip and incubated for approximately 6 minutes at room temperature. The trays were covered with aluminium foil. In cases where the bands were not well developed it was possible to extend the incubation time for up to 25 minutes. The reaction was stopped by adding water and pouring off the solutions. Using forceps, each strip was placed onto absorbent paper and was left to dry.

2.4.5.1. Hybridization using the GT- Blot 20

By using the GT-Blot 20 it was possible to test 20 strips at a time. The procedure to develop HAIN Assays is automated. Volumes, incubation times and temperatures were the same as those described in manual hybridization, but a few adjustments in the procedure had to be considered at the beginning of the methodology as indicated below:

All the solutions necessary for the assay were prepared (including Conjugate and Substrate working solutions), before PCR products were prepared. Volumes were

estimated taking a "*plus* three samples" into account. The solutions were poured into specific flasks, indicated with different coloured flags (*see GTBlot Manual)*. The instruction appearing on the small display was followed so that all pumps were filled (6 in total) and ready for the assay. The "Pre-warming system" that lasted 30 minutes was activated.

All the strips necessary for a specific run were marked (up to 20) and laid out on the tray. The GT-Blot 20 specific tray was taken and 20 µL of DEN solution was dispensed on the bottom corner of each lane of the tray. Twenty microliters of PCR product were added to each DEN solution and was mixed well by pipetting. This was allowed to stand for 5 minutes at room temperature. The corresponding strip was placed in the respective lane while avoiding touching the mixed DEN-DNA solution. It was placed on the top side of the lane. The tray was placed into the GT-Blot 20 and programme "GT-45" was started. At the end of the programme, the strips were taken out and dried between two layers of absorbent papers.

2.4.6. Recording and Reporting

Once the strips were developed and dried, they were fixed onto the Worksheet included in the commercial kits. Each strip has about 22 reaction zones as illustrated in **Figure 2.3** below. For the results to be valid, conjugate control (CC) and amplification control (AC) bands must appear for every sample. The presence of a *M. tuberculosis* complex (MTBC) band indicated that *M. tuberculosis* complex was present in the sample. A mutation in the relevant gene (and resistance to the relevant drug) was signified by either an absent wild type band and/or the presence of a mutant band for each gene cluster. The *rpoB*, *katG* and *inhA* genes each have a control band which must be present in order to interpret the results. While the *rpoB* gene predicts RIF resistance, *katG* predicts high level INH resistance and *inhA* predicts low level INH resistance. For results to be valid the bands must be of intensity equal to or greater than the intensity of the AC band. For a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible (**Figure 2.3)**.

If a positive result was obtained with the negative control, the results of the entire batch must be repeated, and measures should be taken to remove amplicon contamination from all rooms and equipment.

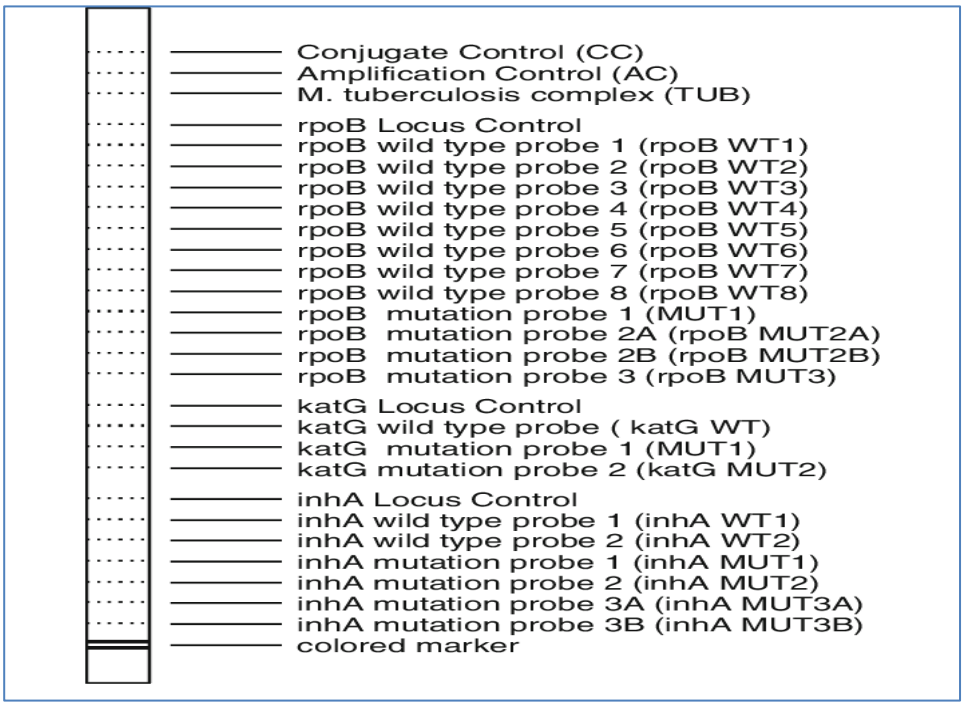

Figure 2.3: Illustration of Hain Genotype MTBDR *plus* probe arrangement on nitrocellulose strip (adopted from: Hain Lifescience, Nehren, Germany)

2.5. STATISTICAL ANALYSIS OF THE QUESTIONNAIRE

In total 382 questionnaires were completed. The data was collected using a structured questionnaire and was captured in an Excel spreadsheet. It was then imported into Statistical Package for Social Sciences (SPSS) Version 25 for data analysis (Coakes, 2005). Data management was performed whereby questionnaires were checked for completeness and variables were defined in SPSS based on the questionnaire. Edit specifications or edit rules were designed and applied in SPSS to create a final cleaned dataset for analysis.

Descriptive analysis, including demographic information and inferential analysis was performed, where necessary. The results in the form of tables and graphs were narrated in a written report and conclusions made by the researcher, is validated by the statistical results obtained.

Clinical, demographic and epidemiological data were collected through interviewing patients. A copy of the standard questionnaire utilised is outlined in **Appendix B**. The study focused primarily on patients classified with MTB using the acid-fast stain (by auramine staining)

CHAPTER 3

RESULTS AND DISCUSSION

3.1 COMPARISON OF DIRECT MICROSCOPY WITH THE GENEXPERT MTB/RIF ASSAY AND THE HAIN GENOTYPE® MTBDR *PLUS* ASSAY FOR MDR DIAGNOSIS.

As reported in many studies, the global burden of tuberculosis (TB), particularly multi-drug resistant TB (MDR TB), is increasing and has become a major health challenge (Pai *et al*., 2009; Yang *et al*., 2010; Ndjeka, 2011; WHO, 2012). The causative agent of TB is *Mycobacterium tuberculosis* (MTB) and classification as MDR TB occurs when the bacterium is resistant to two primary anti-tubercular drugs, namely, rifampicin (RIF) and isoniazid (INH). It has been argued that MTB that is resistant to RIF is more likely to have concomitant resistance to INH, making RIF resistance a surrogate marker of MDR-TB (Somoskovi *et al*., 2001). The early diagnosis of TB and the subsequent rapid detection of RIF's resistance is thus important for the proper management of drug-resistant TB (Brossier *et al*., 2006). However, despite major efforts to increase case detection, one-third of new TB cases is still not diagnosed due to the non-availability of rapid, low-cost and accurate diagnostic facilities in high-TB-burden countries (Lawn *et al*., 2013). Accurate and early diagnosis of MDR-TB is thus vital as it curtails further transmission of the disease and avoids the use of excessive drugs which could lead to the amplification of drug resistance and possibly extensively drug resistant-tuberculosis (XDR-TB).

In high-incidence TB countries, effective control is often dependant on passive case finding amongst individuals self-presenting to health care facilities, followed by either diagnosis based on clinical symptoms or laboratory diagnosis using sputum smear microscopy. While culture based methods remain the most sensitive techniques for the confirmation of MTB, the prolonged turnaround time, biosafety requirements and laboratory operational requirements (Van Kampen *et al*., 2010) limit its contribution to clinical decision making (Siddiqi *et al*., 2003). Through initiatives by the World Health Organisation (WHO), Stop TB Partnership and the Foundation for Innovative New Diagnostics partnerships (WHO, 2009), the diagnostic development process for both high-throughput and point-of-care laboratories has seen rapid innovations in the last decade (Pai *et al*., 2009). In 2009, the WHO approved the Hain GenoType® MTBDR *plus* assay (Hain Life Science, Germany) for the identification of MTB in smear-positive

specimens (WHO, 2011). Hain GenoType® MTBDR *plus* is based on the polymerase chain reaction (PCR) and the DNA•STRIP technology. *Mycobacterial* DNA is extracted from the patients specimen or cultivated material, specifically amplified via PCR and is subsequently detected on a membrane strip using reverse hybridisation and an enzymatic colour reaction. Valid results are documented by internal, conjugate and amplification controls. The Hain GenoType® MTBDR *plus* assay is efficient as the *M. tuberculosis* complex (MTBC) and its resistance to rifampicin and isoniazid are simultaneously detected in a single patient specimen. The test is therefore suitable for MDR-TB screening, for the identification of MTB complex and poly-resistance. Moreover, results are generated within five hours compared to the several months required for conventional methods such as drug susceptibility tests (DST). Furthermore, Hain GenoType® MTBDR *plus* assay is user friendly since a ready-to-use amplification mix including the *Taq* polymerase is provided with the kit. However, the assay is limited in its application as laboratory infrastructure must accommodate PCR technology, the assay is not approved for use in smear-negative cases, and in high-throughput laboratories, it can take several days from sample receipt to result reporting (Barnard *et al*., 2008).

The most recent WHO-endorsed (WHO, 2010) diagnostic tool, the GeneXpert MTB/RIF (Cepheid) assay, has been reported in a multi-country study to have sensitivities of 98.2% among smear-positive, culture positive patients and 72.5% among smear-negative, culture positive patients on a single direct GeneXpert MTB/RIF test (Boehme *et al*., 2010). The GeneXpert MTB/RIF assay is a nucleic acid amplification (NAA) test, which simultaneously detects DNA of the MTBC and mono-resistance to rifampicin (RIF) (i.e. mutation of the *rpoB* gene) in less than 2 hours. In comparison, standard culture methods can take up to two to six weeks for the MTBC to grow and conventional drug resistance tests may require and additional three weeks of analysis (Acharya, 2016). The system integrates and automates sample processing, nucleic acid amplification and the detection of the target sequences.

The current study thus aimed to assess the performance of GeneXpert MTB/RIF test and the Hain GenoType® MTBDR *plus* assay (Hain Life science), for use directly on sputum positive samples as analysed by direct microscopy for the detection of *Mycobacterium tuberculosis* and rifampicin resistance.

3.1.1 Direct microscopy of patients' sputa to detect *Mycobacterium tuberculosis*

Samples from patients presenting at clinics and health facilities in the Khomas region (Windhoek, Namibia) were eligible for the study. Sputum specimens were obtained with the assistance of nursing staff, doctors and community health workers for a period of 6 months from February to July 2014. Patients of all ages, genders, etc. were selected and informed consent (**Appendix A**) was obtained from all patients prior to enrolment in the study. The sputum specimens were brought to the Namibia Institute of Pathology (NIP) laboratory for acid-fast bacilli (AFB) testing (section 2.3), as this is the standard staining method utilised for presumptive identification of MTB in Namibia. In total, 384 AFB positive sputa were collected during the 6 month period. **Table 3.1** provides an indication of how the sputa results were observed and recorded after direct microscopy (DM). The number of AFB indicates the degree of infection hence it was necessary to record exactly what was observed.

Table 3.1: Observation and recording of direct microscopy results (adopted from: International Union against Tuberculosis and Lung Disease, 2000)

What was seen	What was recorded
No AFB in 100 fields	No AFB observed – smear negative
Less than1-9 AFB in 100 fields	Exact number of bacilli
1–9 AFB/100 fields	1+
1–9 AFB/10 fields	2+
1–9 AFB/field	3+

Table 3.1 is a standard observation table used in microbiology laboratories to categorise TB smear readings by direct microscopy (International Union against Tuberculosis and Lung Disease, 2000). Observations from the tests were as follows **(Table 3.1)**: no AFB in 100 fields were recorded as smear negative; less than 1-9 AFB in 100 fields then the exact number of bacilli were recorded; observation of 1–9 AFB/100 fields were recorded as 1+; observation of 1–9 AFB/10 fields were recorded as 2+ and the observations of 1–9 AFB/field were recorded as 3+.

A summary of the direct microscopy results is shown in **Table 3.2** below. As illustrated, of the 384 patients' AFB positive sputa samples, 200 sputa were identified as 1+, which translates to 52.1%. In addition, 79 patients' sputa were identified as 2+ (20.6%), and 105 patients' sputa were identified as 3+ (27.3%), respectively. A comprehensive list of the results for all patients' sputa is included in **Appendix C**.

Table 3.2: Summary of positive direct microscopy results

Summary of positive direct microscopy results	
Sputa identified as 1+	200 (52.1%)
Sputa identified as 2+	79 (20.6%)
Sputa identified as 3+	105 (27.3%)

Sputum smear microscopy has been the primary method for diagnosis of pulmonary tuberculosis in low and middle-income countries (WHO, 2011), which is where nearly 95% of TB cases and 98% of TB related deaths occur. It is a simple, rapid and inexpensive technique, which is highly specific in areas with a very high prevalence of TB (WHO, 2011). Hence, it has been an integral part of the global strategy for TB control. However, sputum smear microscopy has significant limitations in its performance. The sensitivity is grossly compromised when the bacterial load is less than 10,000 organisms/ml in the sputum sample. It also exhibits a poor performance record in extra-pulmonary tuberculosis, paediatric tuberculosis and in patients co-infected with HIV and tuberculosis (Perkins, 2000; Luelmo, 2004). In addition, due to the requirement for repeated sputum examinations, some patients who do not return to the medical facility are labelled as “diagnostic defaulters” (Harries *et al*., 1998). Furthermore, some patients do not return to the medical facility to collect their results and do not receive effective treatment. Research has also indicated that limited resources in combination with the large number of samples that require analysis, often reduces the observation time per slide to less than 60 seconds, which also contributes to reduction in the sensitivity of the test (Perkins, 2000; Luelmo, 2004).

3.1.2 Detection of *Mycobacterium tuberculosis* and RIF sensitivity and resistance using the GeneXpert MTB/RIF

The GeneXpert MTB/RIF assay detects MTB and RIF resistance by PCR amplification of the rifampicin resistance-determining region (RRDR) of the MTB *rpoB* gene and subsequent probing of this region for mutations that are associated with RIF resistance (van Der Zanden *et al*., 2003). The primers in the GeneXpert MTB/RIF assay amplify a portion of the *rpoB* gene containing the 81 base pair “core” region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with rifampicin resistance.

Table 3.3 shows the number of smear-positive samples analysed (*n* = 384) which were classified as MTB using the GeneXpert MTB/RIF method. The test detected the presence of bacilli in all samples. In **Table 3.3**, the column labelled "frequency" lists the actual number of specimens that yielded results viz. positive or negative and sensitive or resistant while, the column labelled "percentage" lists the actual percentages of results obtained. As indicated the GeneXpert MTB/RIF provided a positive MTB result for all samples (*n* = 384; 100%) (**Table 3.3).** The results also show that approximately 2% (7/384) were classified as rifampicin resistant MTB, while 98% (377/384) were shown to be RIF sensitive.

Table 3.3: Analysis of results using the GeneXpert MTB/RIF method

Classification	Frequency	Percentage (%)
Positive (MTB)	384	100
Negative	0	0
Total	384	100.0
RIF sensitive	377	98
RIF resistant	7	2
Total	384	100

Mycobacterium tuberculosis is part of a complex that has at least nine species that are genetically related and cause tuberculosis. These include *M. sensu stricto, M. africanum, M. canetti, M. bovis, M. caprae, M. microti, M. pinnipedii, M. mungi, and M. orygis* which form the *Mycobacterium tuberculosis* complex (Van Ingen *et al*., 2012). Since the GeneXpert MTB/RIF assay nucleic acid amplification (NAA) test detects DNA of the *Mycobacterium tuberculosis* complex (MTBC), and all sputa samples were previously identified as AFB positive by DM, the high positive MTB percentage (100%) recorded in the current study, was expected and served as a confirmation of the DM results. Furthermore, sample analysis is completed in less than 2 hours. In comparison, standard culture techniques can take up to 2 to 6 weeks for MTBC to grow, with an additional three weeks generally required for conventional drug resistance analysis (Brossier *et al*., 2006).

A study conducted in South Africa (Durban and Cape Town) (Scott *et al*., 2011) revealed that the sensitivity of the GeneXpert MTB/RIF assay (three tests performed per participant) among smear-negative, culture-positive individuals compared to standard testing (three smears and four culture results per participant) was 87% for

samples from Durban and 90% for samples from Cape Town. Other studies have also recently reported the performance and clinical role of the GeneXpert MTB/RIF test for detecting TB in extra-pulmonary specimens, with sensitivities of 69% to 85.7% for tissue specimens and up to 100% in urine and stool specimens (Hillemann *et al*., 2011; Malbruny *et al*., 2011).

Given the high sensitivity of GeneXpert MTB/RIF in detecting TB (88%), the negative predictive value (NPV) is greater than 98% both in settings with a low prevalence of TB and in those with a high prevalence of TB – that is, a negative result accurately excludes TB in most situations (WHO, 2014). Typically, in high-burden settings, between 10% and 20% of persons with respiratory symptoms will have culture-confirmed TB (WHO, 2014). In such settings the vast majority of patients with a negative result from GeneXpert MTB/RIF will not have TB. However, the ability of any diagnostic test using sputum specimens to detect TB depends on the quality of the specimen collected; therefore, an individual with a negative result from GeneXpert MTB/RIF may still have TB. An individual still suspected of having TB after a negative GeneXpert MTB/RIF test may therefore require further clinical management and another diagnostic test, including a repeated GeneXpert MTB/RIF test using a different sputum specimen (WHO, 2014). The specificity of GeneXpert MTB/RIF for detecting TB is however, very high (99%), and false-positive results are likely to be linked to the detection by GeneXpert MTB/RIF of dead MTB bacilli that would not be detected by culture based analysis, which is the current reference standard (WHO, 2014).

As stated, the GeneXpert MTB/RIF assay has been endorsed by the WHO (WHO, 2010; Al-Zamel, 2009; Van Rie *et al.*, 2012) as a diagnostic tool, and has been reported in a multi-country study to have sensitivities of 98.2% among smear-positive, culture positive patients and 72.5% among smear-negative, culture positive patients on a single direct GeneXpert MTB/RIF test compared to three smears and four culture results (Boehme *et al*., 2010). The Namibia Institute of Pathology (NIP) laboratories adopted the implementation of the WHO (2015) published Policy framework for Implementing Tuberculosis Diagnostics and the Global Laboratory Initiative (GLI) aimed at providing technical support to TB laboratories in low- and middle-income countries, to assist with the implementation of the latest diagnostic technologies. The GeneXpert MTB/RIF test is then the preferred algorithm for universal patient access to

rapid testing and is utilised to detect MTB and rifampicin resistance in persons being evaluated for pulmonary TB (WHO, 2015).

3.1.3 Detection of *Mycobacterium tuberculosis* and RIF and INH sensitivity and resistance using the Hain GenoType® MTBDR-*plus* assay

The Hain GenoType MTBDR *plus* enables a rapid analysis of pulmonary patient specimens and isolates from culture based analysis. The assay combines the detection of the MTB complex with the prediction of resistance to RIF and INH, including mutations in the 81 bp hotspot region of *rpoB,* codon 315 of the *katG* gene and in the *inhA* promoter region. For the detection of RIF resistance, the Hain GenoType MTBDR *plus* assay includes three more probes when compared to the GeneXpert MTB/RIF method, covering the wild-type *rpoB* sequence in order to improve the sensitivity of the test. As has previously been described by among others Ramaswamy *et al*. (2003) and Van Soolingen *et al*. (2000), the most common mutation involved in INH resistance is the S315T substitution in the katG protein, which has also been related to high levels of INH resistance. In contrast, mutations causing low levels of INH resistance are not as clearly elucidated, as they are much more complex and involve different genes; however, a firm relationship has been found between mutations in the *inhA* regulatory region and low or intermediate levels of resistance (Brossier *et al*., 2006; Ramaswamy *et al*., 2003). It thus detects mutations associated with the *rpoB* gene (coding for the β-subunit of the RNA polymerase) for RIF resistance, *katG* genes (coding for the catalase peroxidase) for high level INH resistance and the *inhA* regulatory gene region (coding for the NADH enoyl ACP reductase) for low-level INH resistance (Hilleman *et al*., 2007).

The Hain GenoType MTBDR *plus* line probe assay (LPA) was performed according to the manufacturer's (Hain Life science, Nehren, Germany) instructions. Three steps for the LPA test included, DNA extraction, multiplex PCR amplification and reverse hybridisation. **Table 3.4** includes the analysis of results using the Hain GenoType MTBDR *plus* assay among smear-positive samples analysed (*n* = 384) which were classified as MTB by Direct Microscopy (DM). The test did not detect the presence of bacilli in 12 (3%) samples as the results were recorded as negative or non-reactive. The Hain GenoType® MTBDR thus provided an overall positive MTB result for 97% of the samples (372/384) (**Table 3.4).** The results also indicated that 8% (29/384) of the samples were classified as isoniazid resistant MTB, while 5% (20/384) were shown to

be RIF resistant. A total of 352 samples (92%) were thus classified as rifampicin sensitive while 343 (89%) samples were classified as isoniazid sensitive.

Table 3.4: Analysis of results using the Hain GenoType® MTBDR *plus* assay

Classification	Frequency	Percent (%)
Positive (MTB)	372	97
Negative/Non-reactive	12	3
Total	384	100.0
RIF sensitive	352	92
RIF resistant	20	5
Non-reactive	12	3
Total	384	100
INH sensitive	343	89
INH resistant	29	8
Non-reactive	12	3
Total	384	100

No further investigation was carried out to determine why the 12 samples yielded a negative reaction using the Hain GenoType MTBDR *plus* method of testing whilst yielding a positive reaction using the GeneXpert MTB/RIF and the DM methods of testing and screening. It should however, be noted that when analysing the 12 samples by direct microscopy, all 12 samples were reported as +1 smear positive. Since the 12 samples were +1 positive, the number of bacilli present was observed as 1–9 AFB/100 fields. Previous studies on the MTB/ RIF assay have reported test sensitivities of 98 to 100% in cases of smear-positive, culture-positive pulmonary tuberculosis (Arzu *et al*., 2011). When MTB is not detected; it could imply that the MTB target DNA is not present particularly if both controls, sample processing control (SPC) and probe check control (PCC), meet the assigned acceptance criteria.

A summary of the Hain GenoType MTBDR *plus* assay results is provided in **Figure 3.1**, which illustrates that overall 88% (338/384) of the smear-positive samples analysed (*n* = 384) were classified as INH sensitive and RIF sensitive, 3% (12/384) yielded negative (non-reactive) results for both INH and RIF, 4% (15/384) were classified as INH and RIF resistant, 4% (14/384) were classified as INH resistant and RIF sensitive and 1% (5/384) were classified as INH sensitive and RIF resistant.

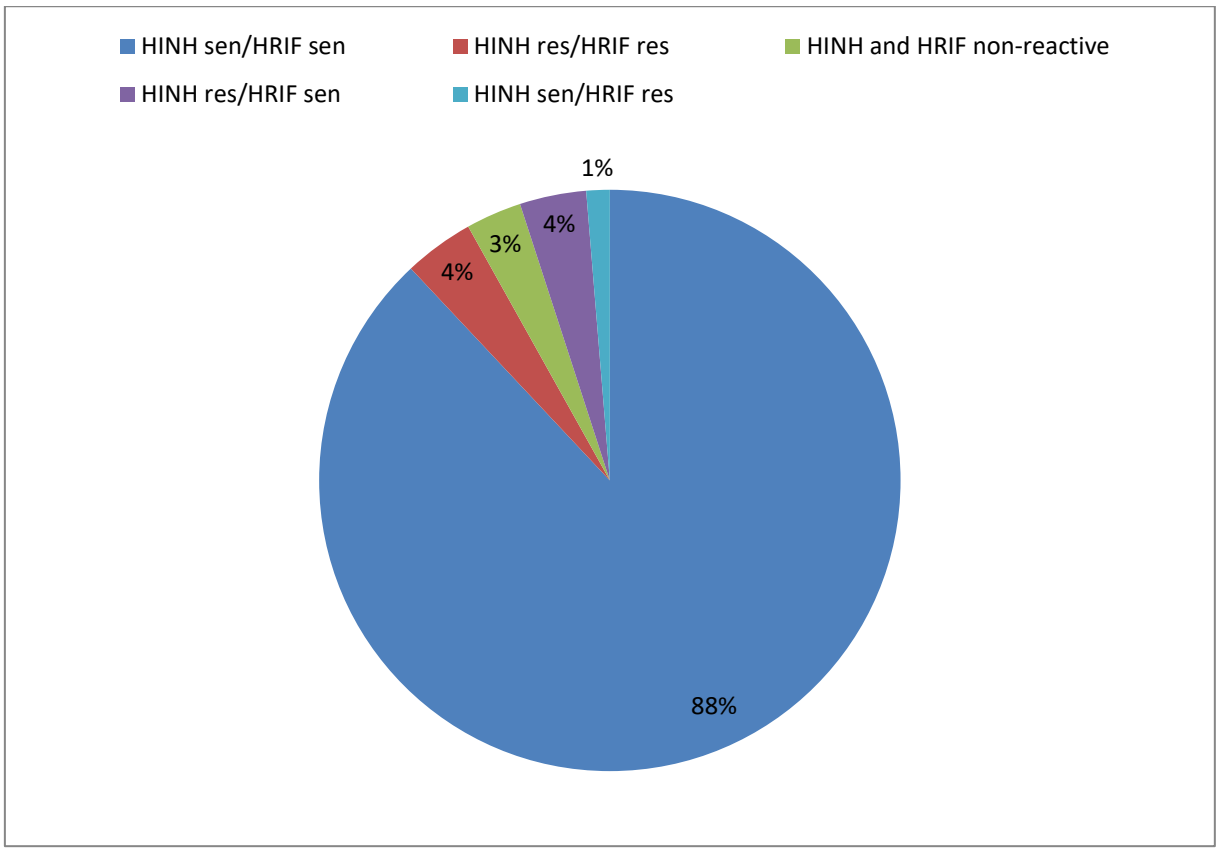

Figure 3.1: Graphical representation of results for Hain GenoType® MTBDR *plus* assay

Earlier studies have shown that the sensitivity of the Hain GenoType MTBDR *plus* assay for the detection of RIF resistance was found to vary from 95% to 99% (Rahman *et al.,*2016; Lacoma *et al.,*2008). This high rate of detection can be explained by the fact that the mutations responsible for RIF resistance are mainly located in the 81-bp hot-spot region and that mutations outside this location are rare and are associated with low-level resistance (Heep *et al*., 2001; Hillemann *et al*., 2005). Mutations in the upstream region of *inhA* have also been described to be responsible for resistance in INH strains, especially those with low-level resistance (Brossier *et al.*, 2006; Ramaswamy *et al.*, 2003). To that effect, the Hain GenoType MTBDR-*plus* assay, includes six more probes aimed at the promoter region of *inhA*: two are for the wild type and four cover mutations at different positions [nucleotides -8 (two probes), -15, and -16].

Various studies (Brossier *et al*., 2006: Cavusoglu *et al*., 2006 and Ramaswamy *et al*., 2003) documented similar trends regarding sensitivity of detection of treatment resistance. Brossier *et al*., 2006 reported that the assay had a sensitivity of 67.3%

(64/95) for the detection of INH resistance, though when only strains with high-level resistance were considered, this value increased to 89.4% (59/66). A similar trend was found by Cavusoglu *et al*., 2006, whose sensitivity values rose from 72.9% to 87.1% when only the strains above the critical concentration of minimum inhibitory concentration (MIC) “high-level resistance” were considered.

Mutations in the upstream region of *inhA* have also been described to be responsible for resistance in *INH* strains, especially those with the MIC equal to or slightly above the critical concentration “low-level resistance” (Ramaswamy *et al*., 2003). However, it should be noted that in the current study the MIC of both first-line and second-line drugs for the 384 multidrug-resistant *Mycobacterium tuberculosis* isolates was not analysed.

3.1.4 Comparison of results for Direct Microscopy, GeneXpert MTB/RIF and Hain GenoType MTBDR-*plus* assay for the detection of MTB

A total of 384 specimens were assayed for TB detection by Direct Microscopy (DM), GeneXpert MTB/RIF (GE) and Hain GenoType MTBDR-*plus* (HAIN) as illustrated in **Figure 3.2** below.

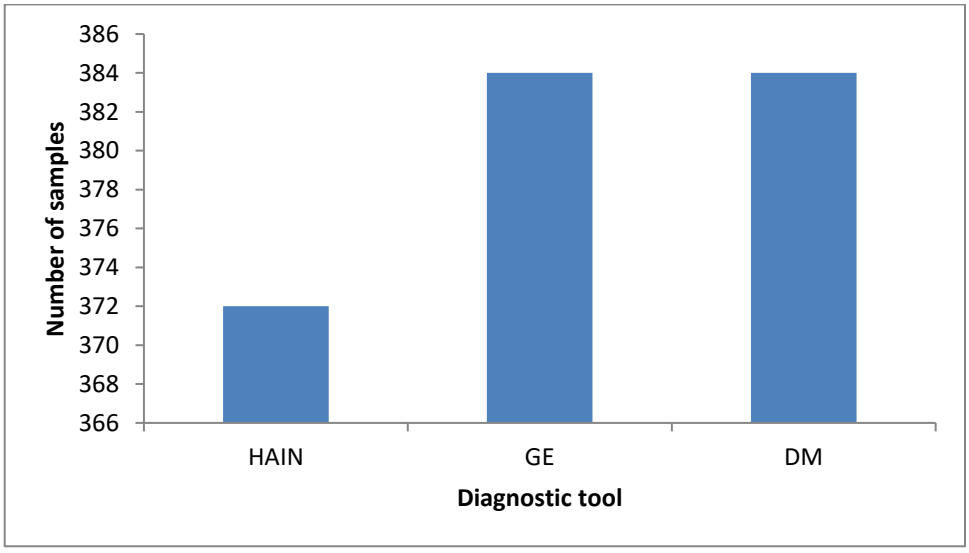

Figure 3.2: Diagnostic efficacy Comparison of Hain GenoType MTBDR *plus* vs GeneXpert vs Direct Microscopy

A confirmed positive culture of MTBC was used as a reference standard. Out of the 384 specimens, 384 (100%) were positive for *M. tuberculosis* by direct microscopy and

the GeneXpert MTB/RIF, while 372 (97%) were positive with Hain GenoType MTBDR *plus* method as12 samples yielded negative (non-reactive) results for both INH and RIF using this technique. Complete comparison between the molecular techniques and the conventional method, and agreement between the techniques are presented in **Appendix C and Appendix D,** respectively.

The results obtained relate closely to a comparison of the assays' performances for the diagnosis of drug-resistant TB carried out by Talenti (1997) and Herrera *et al*. (2003) which showed that GeneXpert MTB/RIF can detect mutations in the *rpoB* gene which occur in 95%–99% of RIF-resistant isolates and is considered a good indicator for MDR TB.

Table 3.5 provides information on the total agreement and total disagreement of the three methods for the identification of *M. tuberculosis*. Of the 384 specimens tested, there was a 100% agreement between the DM and GE positive samples. However, when comparing the HAIN method to the DM screening method, a 97% (372/384) agreement on samples that had a positive result (reaction) was recorded. The same result was found when the HAIN method of testing was compared to the GE method and the two methods were in agreement for 97% of specimens that were found to have positive results. When comparing the Hain vs GE and the Hain vs DM, a total disagreement of 3% (12/384) was thus recorded. This was expected, as 12 samples exhibited a non-reactive result when analysed with the Hain GenoType MTBDR-*plus* line probe assay. On average, the results showed 98% average agreement on the MTB presence for all three methods used.

Table 3.5: Total agreement and Total disagreement of three methods vs. each other for identification of *M. tuberculosis*

Pairwise comparison of diagnostic tools	**Total Agreement**	**Total Disagreement**
	+/+	**-/+**
HAIN vs GE	372/384 (97%)	12/384 (3%)
HAIN vs DM	372/384 (97%)	12/384 (3%)
GE vs DM	384/384 (100%)	-

+/+ indicates that both tests showed a positive result
-/- indicates that both tests showed a negative result
+/- indicates that the first test was positive while the latter test was negative
-/+ indicates that the first test was negative while the latter test was positive

It is however, difficult to conclude which of the three methods were more sensitive for the identification of *M. tuberculosis*. As mentioned previously, the 12 samples that yielded the non-reactive/negative result when using the Hain GenoType MTBDR-*plus* method were +1 positive, indicating that the number of bacilli present was observed as 1–9 AFB/100 fields. Direct microscopy was carried out first, followed by GE and lastly the HAIN method. Since a small number of bacilli were detected in the 12 specimens, it could be speculated that the bacilli could have degraded by the time the HAIN method was used as the quality of the specimens is important when using the Hain GenoType MTBDR-*plus* assay.

In an extensive validation study that was conducted by the WHO (2008) at the National Health Laboratory Service (NHLS) in Cape Town, South Africa, the efficacy and effectiveness of HAIN GenoType MTBDR *plus* assay in detecting MDR TB screening was demonstrated. The HAIN test is designed to detect *M. tuberculosis* bacterial complexes, and genetic mutations that are linked to isoniazid and rifampicin resistance in one day. The validation study stated that approximately 97% of the tests retained interpretable results detecting *M*. *tuberculosis.* In addition, with regard to sensitivity, specificity, and positive and negative predictive values, the study documented impressively high accuracy levels of 99%, 100%, 100% and 100% respectively. These results were documented in the American Journal of Respiratory and Critical Care Medicine. Based on the results obtained in the study, the HAIN GenoType MTBDR *plus* assay approach also demonstrated superiority regarding capacity to handle huge volumes of tests at a lower cost relative to conventional screening methods (WHO, 2008).

According to the study of Marouane *et al*. (2014), the results of the evaluation of GeneXpert sensitivity, specificity, positive predictive value and negative predictive value were 90%, 60.6%, 19.6% and 98.3%, respectively. Furthermore, Barnard *et al* (2012) showed that the GeneXpert MTB/RIF and Hain GenoType MTBDR *plus* (v2.0) LPA diagnostic modalities showed sensitivities of 71.2% and 73.1%, respectively and similar specificities of 100%, which compared well with previous studies using the GeneXpert MTB/RIF (Boehme *et al*. 2010; Crudu *et al*. 2012.; Hillemann *et al*., 2011). Similar to the observations in the current research, both tests were able to rapidly detect the presence of *M. tuberculosis* complex DNA in the smear-positive cases.

3.2 COMPARISON OF THE RIF RESULTS FOR THE GENE XPERT MTB/RIF AND HAIN GENOTYPE MTBDR-*PLUS* ASSAY

Since the GeneXpert MTB/RIF detects mono-resistance to RIF and the Hain GenoType MTBDR-*plus* assay detects poly-resistance to RIF and INH, the two assays could only be compared for their ability to detect RIF sensitivity and resistance. **Table 3.6** shows a comparison for the 384 samples analysed for RIF sensitivity and resistance using the GeneXpert MTB/RIF and Hain GenoType® MTBDR-*plus* methods of testing. Using the GeneXpert MTB/RIF method of testing, 2% (7/384) were classified as RIF resistant, while 5% (20/384) were classified as RIF resistant using the Hain GenoType® MTBDR *plus* method. Accordingly, the GeneXpert MTB/RIF classified 98% (377/384) as RIF sensitive, while the Hain GenoType® MTBDR-*plus* classified 92% (352/384) as sensitive to RIF. None were classified as non-reactive using the GeneXpert MTB/RIF method, however the Hain GenoType® MTBDR-*plus* classified 3% (12/384) of the specimens as non-reactive. Agreement for antibiotic sensitivity and resistance between the two molecular techniques for all samples are shown in **Appendix D**.

Table 3.6: Comparison of results for GeneXpert MTB/RIF and Hain GenoType® MTBDR *plus* methods

Classification		**Method**	
		GeneXpert MTB/RIF	**Hain GenoType® MTBDR *plus***
RIF	Sensitive (Count % of Total)	377 (98%)	352 (92%)
	Resistant (Count % of Total)	7 (2%)	20 (5%)
	Non-Reactive (Count % of Total)	0 (0.0%)	12 (3%)
Total	Count (% of Total)	384 (100.0%)	384 (100.0%)

Table 3.7 illustrates the total agreement and total disagreement for both RIF sensitivity and RIF resistance of *M. tuberculosis* using the two methods of testing (GE and HAIN) respectively. As indicated, the two methods showed 91% (349/384) total agreement for RIF's sensitivity and 1% (4/384) total agreement for RIF's resistance. Thus, in total 92% (353/384) of the samples obtained from the two methods were in total agreement.

Table 3.7: Total agreement and total disagreement of two methods vs. each other for RIF sensitivity and RIF resistance of *M. tuberculosis*

Testing Methods	**Total Agreement**		**Total Disagreement**	
	+/+	**-/-**	**+/-**	**-/+**
HAIN Sen vs GE Sen	349 (91%)		-	
HAIN Res vs GE Res		4 (1%)	-	
HAIN Sen vs GE Res			3 (0.8%)	
HAIN Res vs GE Sen				16 (4.1%)
HAIN NR vs GE Sen				12 (3.1%)
Total:	353 (92%)		31 (8%)	

+/+ indicates that both tests showed a positive (sensitive) result
-/- indicates that both tests showed a negative (resistant) result
+/- indicates that the first test was positive (sensitive) while the latter test was negative (resistant)
-/+ indicates that the first test was negative (resistant) while the latter test was positive (sensitive)

A total of 12 (3.1%) samples were classified as negative (non-reactive) using the Hain GenoType® MTBDR-*plus* whereas no negative classification was detected using the GeneXpert MTB/RIF method of testing. There was thus a disagreement of 8% (31/384) between the two methods of testing. This indicates that the first test viz. HAIN showed that 0.8% (3/384) of the samples were sensitive to RIF, while the second test viz. GE showed that these samples were resistant to RIF. Furthermore, 4.1% (16/384) of the samples had a disagreement of results where the HAIN methods indicated that these samples were resistant to RIF, while the GE indicated that the same samples were sensitive to RIF.

GeneXpert MTB/RIF and Hain Genotype MTBDR *plus* assay are two WHO endorsed probe based molecular drug susceptibility testing (DST) methods utilised for the rapid diagnosis of drug resistant tuberculosis. Both methods target the same 81 bp

Rifampicin Resistance Determining Region (RRDR) of bacterial RNA polymerase β subunit (*rpoB*) for detection of rifampicin (RIF) resistance associated mutations using DNA probes. Both methods are also highly sensitive and specific in the detection of RIF's resistance (Rahman *et al*., 2016).

The Hain Genotype MTBDR *plus* assay, is a multiplex PCR in combination with reverse hybridisation-based technique that employs 8 wild-type (WT) probes and 4 mutation specific probes (MUT), that target the RRDR (**Figure 2.3**). Non-hybridisation of WT probes or hybridisation of MUT bands indicates RIF's resistance. Furthermore, unlike the GeneXpert, the Hain Genotype MTBDR *plus* assay can provide specific information on four of the most prevalent mutations (viz. S531L, H526Y, H526D and D516V) (Rahman *et al*., 2016). In addition, INH resistance is conferred by mutations within the catalase-peroxidase enzyme gene *inhA*, while RIF resistance is conferred by mutation within *katG* gene (Rahman *et al*., 2016).

Research has however, also shown that the GeneXpert MTB/RIF assay is a novel integrated diagnostic device that performs sample processing and semi-nested real-time PCR analysis in a single hands-free step for the diagnosis of tuberculosis and the rapid detection of RIF's resistance in clinical specimens (Helb *et al*., 2010). The GeneXpert MTB/RIF assay detects *M. tuberculosis* and RIF's resistance by PCR amplification of the 81-bp fragment of the *M. tuberculosis rpoB* gene and subsequent probing of this region for mutations that are associated with RIF resistance.

According to various studies, the two molecular methods, GeneXpert MTB/RIF and Hain GenoType MTBDR *plus* assay yield a relatively good agreement for the resistance and sensitivity profiles of *M. tuberculosis* (Rufai *et al*., 2014; Steingart *et al*. 2015; Rahman *et al*., 2016). This result was corroborated in the current study where an overall total agreement of 92% (RIF sensitivity/resistance) was obtained between the two methods.

3.3 ANALYSIS OF THE DEMOGRAPHIC QUESTIONNAIRE DATA

During the sample collection period, a total of 384 patients' sputa test positive for acid-fast bacilli. However, only 382 patients consented and subsequently participated in the demographic questionnaire. Patients' sputa were allocated unique identifier codes that tracked patient demographics such as age and gender. Each patient's sputum sample

was allocated only one unique code which allowed for the result of the coded sputa to be linked to a specific patient. The average demographics related to gender and age of the patients from which the sputa were collected are shown in **Figure 3.3** and **Table 3.8**.

Of the 382 TB positive sputa patients' surveyed, 62.6% (239/382) were female and 36.6% (140/382) patients were male. The gender of 0.8% respondents were not recorded (**Figure 3.3**) since three patients did not state whether they were females or males when completing the questionnaire. The World Health Organization encourages gender specific comparisons in TB rates in order to determine whether the incidence of TB in women is frequently diagnosed (first time and second time), reported and treated (Jiménez-Corona *et al*., 2006). The gender profile of the TB diagnosis in Windhoek, Khomas region (current study) is similar to TB studies conducted in Asia and Europe where the prevalence of TB was more than 10% higher in women than in men for both the first and second diagnosis of TB (Andrew *et al*., 2011; Holmes *et al*., 1999).

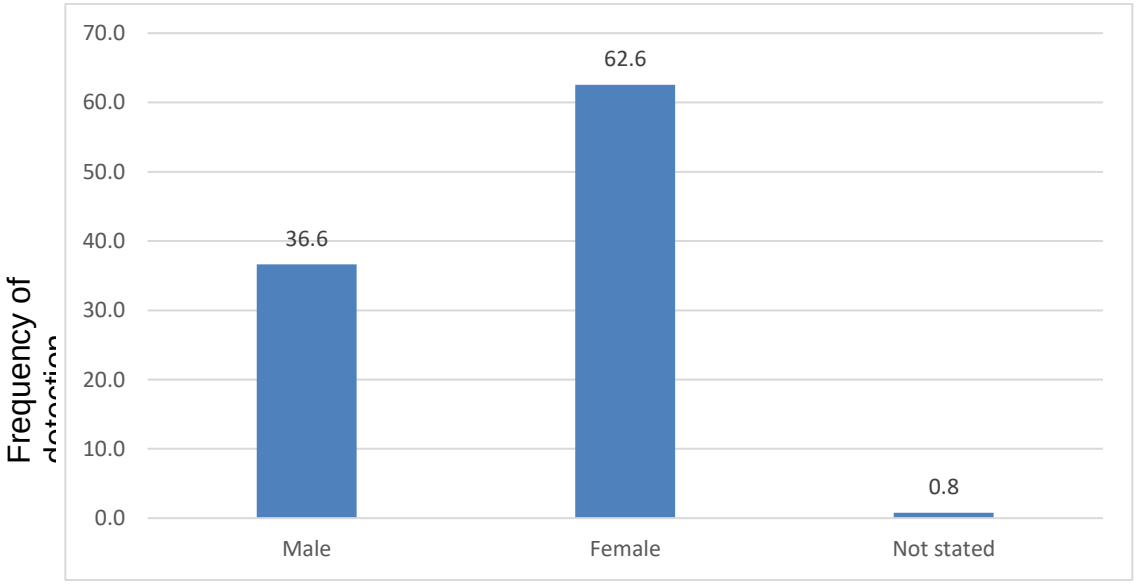

Figure 3.3: Demographic distribution of the respondents by gender

The age categories of the respondents (*n* = 382) were segmented as shown in **Table 3.8**. It should be mentioned that out of the 382 patients surveyed, 9 patients did not complete the age category on the survey document. Frequency of respondents age categorisation (*n* = 373) indicated a normal distribution where 32.2% (120/373) and 27.0% (101/373) of the TB positive sputa patients were in the age groups of 30- 39

years and 20 – 29 years , respectively. This was followed by the age group of 40 – 49 years that had a percentage distribution of 21.6% (81/373). For the age groups of 50 – 59 years, 60+ years and less than 20 years, the percentage distribution was 9.2% (34/373), 6.5% (24/373) and 3.5% (13/373), respectively.

LaCourse *et al*., (2016) reported that TB was one of the leading infectious causes of morbidity and mortality among women of childbearing age and it has severe consequences for women, especially during their reproductive years and pregnancy. Our study, revealed a higher percentage of females (62.6%, 239/382) (**Figure 3.3**) who were TB sputa positive, with a combined total percentage of 57.7% (**Table 3.8**) for women who fell between the ages of 20 – 40 years, which could be categorised as of childbearing age.

Table 3.8: Distribution of TB positive patient's sputa by age and gender

Age group	**Male**	**Female**	**Total (*n* = 373)**
	%	**%**	**%**
Less than 20 years	2.3	4.2	3.5
20-29 years	32.8	23.8	27.0
30-39 years	29.0	33.9	32.2
40-49 years	18.3	23.4	21.6
50-59 years	9.2	9.2	9.2
60+ years	8.4	5.4	6.5
Total	100.0	100.0	100.0

Figure 3.4 shows that 45.5% (174/382) of the respondents reported that it was the first time that they had been diagnosed with TB. Approximately 36.9% (141/382) of the respondents revealed that it was the second time that they had been diagnosed with TB, while 17.5% (67/382) did not state how often they have been diagnosed.

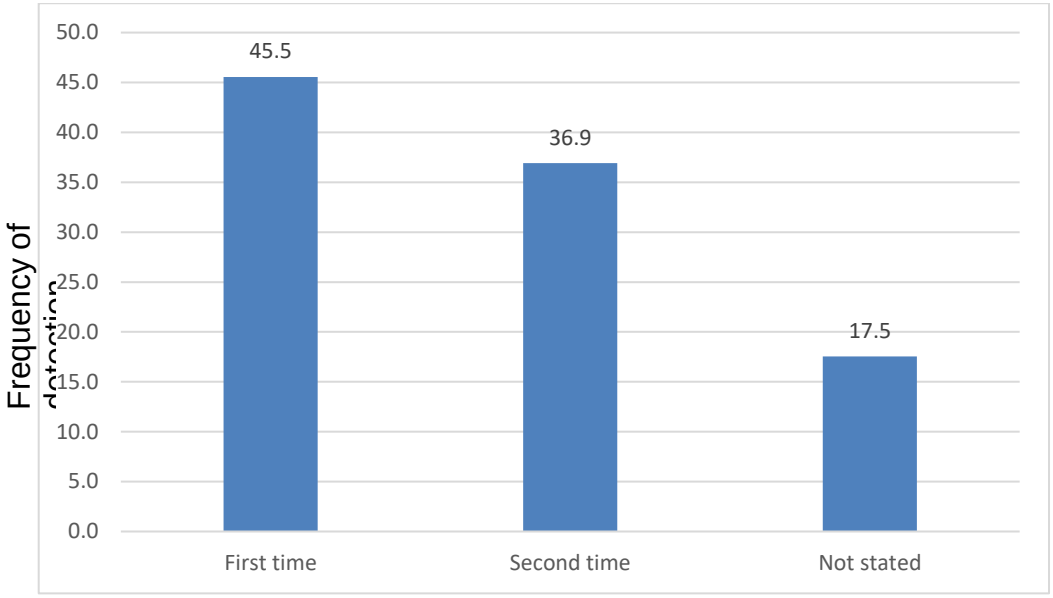

Figure 3.4: Number of TB diagnoses for the respondent

Thirty-one-point six percent of the males (55/174) respondents and 68.4% of female respondents (119/174) indicated that this was their first TB infection (**Table 3.9**). In addition, a higher percentage of females (58%; 82/141) were diagnosed with TB for the second time in comparison to their male counterparts (42%; 59/141).

Table 3.9: The number of times the respondents have been diagnosed with TB by gender

Gender of respondent	Time Diagnosed with TB	
	First time	Second time
	%	%
Male	31.6	42.0
Female	68.4	58.0
Total	100.0	100.0

As shown in **Table 3.10**, the current study revealed that there was a high percentage of second time TB diagnoses among respondents between the ages of 30-39 years (35.0%; 50/141), followed by respondents between the ages of 20-29 years (24.8%; 35/141). A percentage of re-infection of 23.4% (33/141) was obtained for respondents between the ages of 40-49 years. For the age groups of 60+ years and 50 – 59 years, the percentage distribution was 6.6% (9/141) and 5.8% (8/141), respectively. It should be noted that while 4.4% (6/141) of respondents were less than 20 years of age, only one respondent included in this study was under the age of two years.

Table 3.10: Re-infection with TB by age

Age group	Second time (%)
Less than 20 years	4.4
20-29 years	24.8
30-39 years	35.0
40-49 years	23.4
50-59 years	5.8
60+ years	6.6
Total	100.0

The Global Tuberculosis report (WHO, 2016), reported that the risk of MTB (*Mycobacterium tuberculosis*) re-infection was highest in infants (younger than 4 years) compared to other age groups. It was also found that the risk of re-infection declines in the age group of 5-10 years, increased in the adolescence age group of 15-19 years and peaks in the age group of 20-30 years. The variations in the risk of re-infection after primary infection is linked to firstly differences in response to infection and secondly to clinical features of the disease. Newton *et al*. (2008), also reported that children have a higher risk not only of progression to the TB disease but also of extra pulmonary dissemination and deaths. Infants have a particularly high morbidity and mortality from TB. While many factors including host genetics, microbial virulence and underlying conditions that impair immune competence (e.g. malnutrition and HIV infection) determine the outcome of infection, it was reported that the high rate of progressive TB seen in young children was largely a reflection on the immaturity of the immune response. Thus while only one TB positive sputa respondent was below the age of two years and no direct conclusion can be drawn to the risk of re-infection amongst infants, in the current study females had the higher incidence of first and re-infection and it is hypothesised that as the primary caregivers, they would be repeatedly exposed to infection when in contact with TB positive family members.

Figure 3.5 shows the distribution of respondents by level of education and gender. In the survey (**Appendix B**) “below matric“ referred to those who never obtained the final year of high school qualification, while “Matric” denotes those who obtained the final year of high school qualification. The “tertiary level” refers to those who attained an education level following the completion of high school education either from a

university or from institutions that teach specific capacities of higher learning such as colleges, technical training institutes etc.

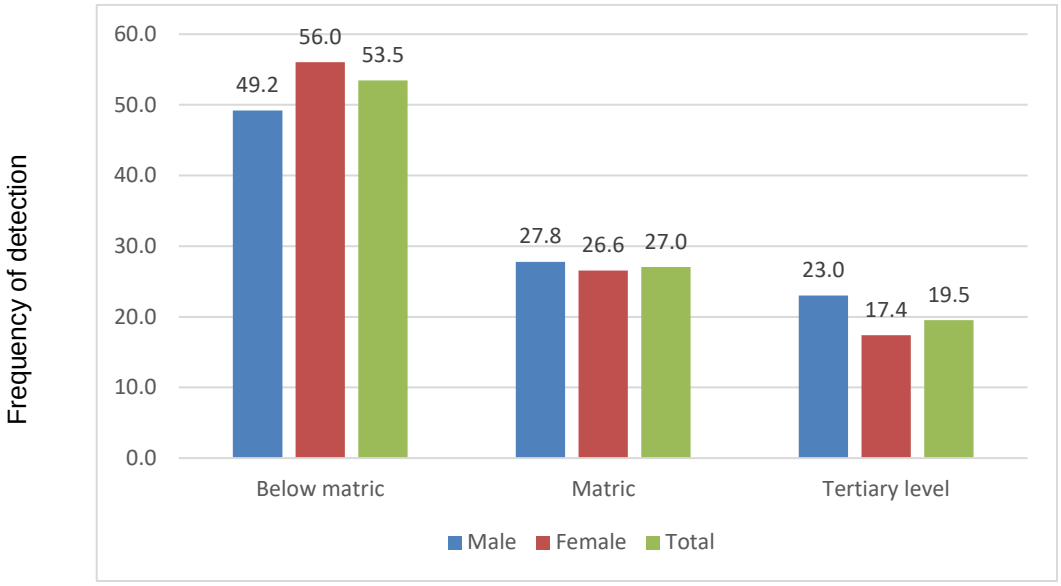

Figure 3.5: Distribution of respondents by level of education and gender

It should be noted that 46 respondents did not specify their education level on the survey. Based on the survey analysis, a total of 53.5% (180/336) of the respondents were below matric level, 27.0% (90/336) were at matric level and 19.5% (66/336) were at tertiary level. In terms of gender, for the female respondents the percentage of below matric level was 56.0% (188/336), 26.6% (89/336) for matric and 17.4% (59/336), for tertiary education, respectively. In comparison, for the male respondents 49.2% (165/336); 27.8% (94/336) and 23.0% (77/336) were below matric, matric and has tertiary education, respectively. A study by Dean and Fenton (2010) on social determinants of health in the prevention and control of HIV/AIDS and Tuberculosis confirmed that there was an association between education and TB diagnosis. This study specifically showed that there was an inverse associated relationship between education and TB diagnosis. A similar result was obtained in the current study as 19.5% of the TB positive sputa respondents had some form of tertiary level education, while 53.5% of respondents with TB positive sputa were categorised as below matric level.

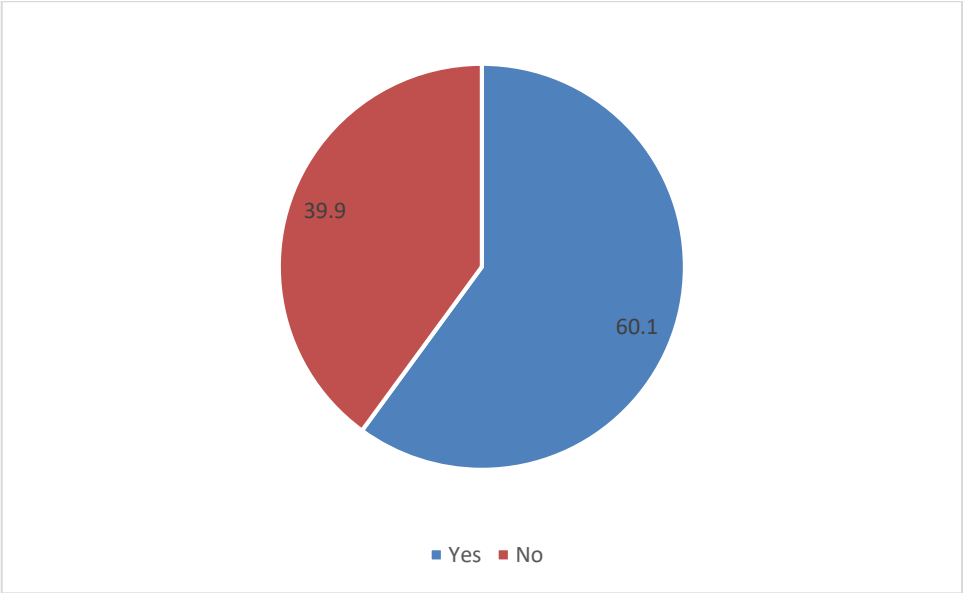

Figure 3.6: Distribution of respondents by employment status

Figure 3.6 shows the distribution of respondents by employment status. Employment refers to respondents who were employed in any sector formal or informal and earning an income on a monthly basis. Of the 382 respondents, 16 did not state their employment status. Data revealed that 60.1% (219/366) occupied some sort of employment while 39.9% (147/366) were unemployed. A study conducted by Przybylski *et al*. (2014) also compared groups of employed and unemployed TB patients. The aim of their study was to determine whether unemployment was a factor that could influence the incidence and course of TB. Results from the study indicated that approximately 54% (606/1130) of the TB patients interviewed were unemployed and it was concluded that unemployment was a serious problem amongst TB positive patients. In the current study 39.9% of the respondents were unemployed **(Figure 3.6)** and while 60.1% of the respondents indicated that they were employed, most fell into the job category of unskilled and not permanent labour (**Figure 3.7**).

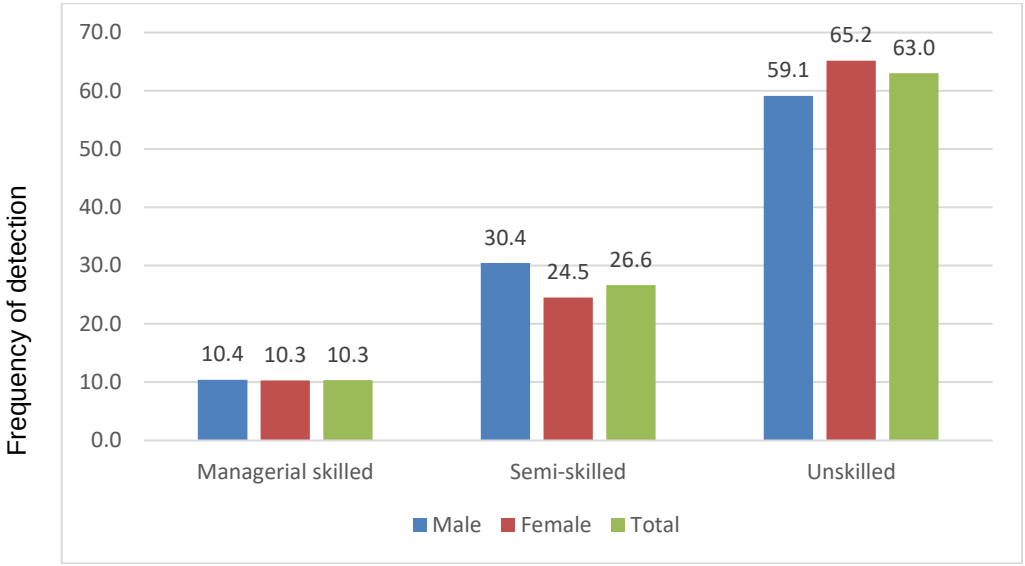

Figure 3.7: Distribution of respondents by occupation and gender

Figure 3.7 shows the distribution of respondents by occupation and gender. Unskilled refers to workers who did not have any special skill or training, semi-skilled refers to workers who had or required less training than a skilled worker and required more skills and training to reach the level of those who were skilled managerial, while the skilled managerial were those workers who were knowledgeable about a specific skill or trade thus requiring no or limited training. Sixty respondents did not indicate their occupation. The survey revealed that there was a high total percentage of 63% (203/322) of unskilled respondents, while 26.6% (86/322) were semi-skilled and 10.3% (33/322) were managerial skilled respondents. In terms of gender, the percentage of unskilled, semi-skilled and managerial skilled for females was 65.2% (219/336), 24.5% (82/336) and 10.3% (35/336) respectively. In comparison, 59.1% (199/336), 30.4% (102/336) and 10.4% (35/336) of the male respondents indicated their occupation as unskilled, semi-skilled and managerial skilled, respectively. According to Namibia's skills deficit report (2010), Namibia as a country is experiencing a severe skills shortage or scarcity. This coupled with the unemployment rate that stood at 51.2% by the year 2008, it is likely that the burden of TB among the socially disadvantaged communities may increase (Namibia Skills Deficit Report, 2010). A study by Nugussie *et al*., (2017), confirmed that TB had a high prevalence among the uneducated as well as among those with limited or no skills. This was corroborated in the current study as 63% of the respondents fell into the category of unskilled.

Figure 3.8 shows respondent results by completion of TB treatment after the initial infection. Approximately 45.5% (174/382) of the respondents reported that they

completed the TB treatment course after the initial diagnoses, while 25.9% (99/382) of the respondents revealed that they did not complete the TB treatment course after the diagnoses. Approximately 0.5% (2/382) of the respondents indicated that they did not know (indicated by “DK”), while 28.0% (107/382) did not state whether they completed the TB treatment and did not complete the question (indicated as “Not stated”). The reason for this could be that the respondents did not know the importance of completion of the medication.

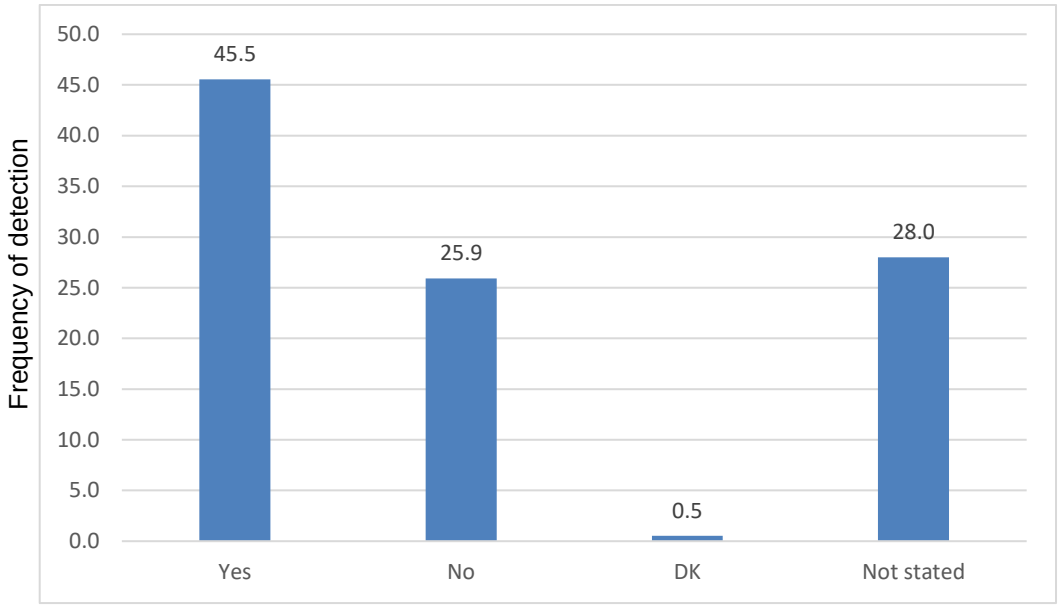

Figure 3.8: Respondents completion of TB treatment

Furthermore, as illustrated in **Table 3.11**, 62.1% (108/174) of the female respondents and 36.8% (64/174) of the male respondents indicated that they completed their treatment. One-point-one percent (2/174) of those respondents who completed their course of treatment, did not indicated their gender. More females (60.6%; 60/99) had not completed their TB treatment course as compared to the males (38.4%; 38/99) (**Table 3.11**). Both respondents who had indicated that they did not know whether they had completed the treatment course, were females (100%). In addition, a total of 107 patients both male (35.5%; 38/107) and female (64.5%; 69/107) did not state whether they completed the TB treatment.

Table 3.11: Completion of first TB treatment by gender

Gender of respondent	Completion of course during first treatment			
	Yes	No	DK	Not stated
	%	%	%	%
Male	36.8	38.4	0.0	35.5
Female	62.1	60.6	100.0	64.5
Not stated	1.1	1.0	0.0	0.0
Total	100.0	100.0	100.0	100.0

A study by Dooley *et al*. (2011) which included a population of 291 patients revealed that patients with TB required retreatment if they failed or defaulted from their initial treatment and that retreatment was most frequent among patients with initial treatment default (57%). As mentioned above, in the current study 36.9% (141/382) of the respondents were diagnosed with TB for a second time (**Figure 3.4**). Of these, only 48 respondents indicated that they did not complete their TB treatment during the initial infection. This included 25 females (52.1%), 22 males (45.8%) and one respondent (2.1%) who did not indicate gender.

Figure 3.9 shows the respondents by family member having (currently infected) or who had been infected with TB (past infection). It can be observed from **Figure 3.9** that 62.6% (239/382) of the respondents reported that they had family members who currently have or who were infected with TB. Approximately 24.9% (95/382) of the respondents reported that they did not have family members who currently has or who had TB, while 0.3% (1/382) did not know and 12.3% (47/382) did not state whether they had family members who were infected with TB. It is generally accepted that people living in poor socio-economic conditions as well as in overcrowded dwellings have an increased risk of contracting TB. A study on poverty alleviation in the informal settlement communities in Windhoek, Namibia (Sem, 2010), found that on average a dwelling had 6 occupants which could lead to an increased risk of infection as TB has been associated with environmental factors such as indoor air pollution.

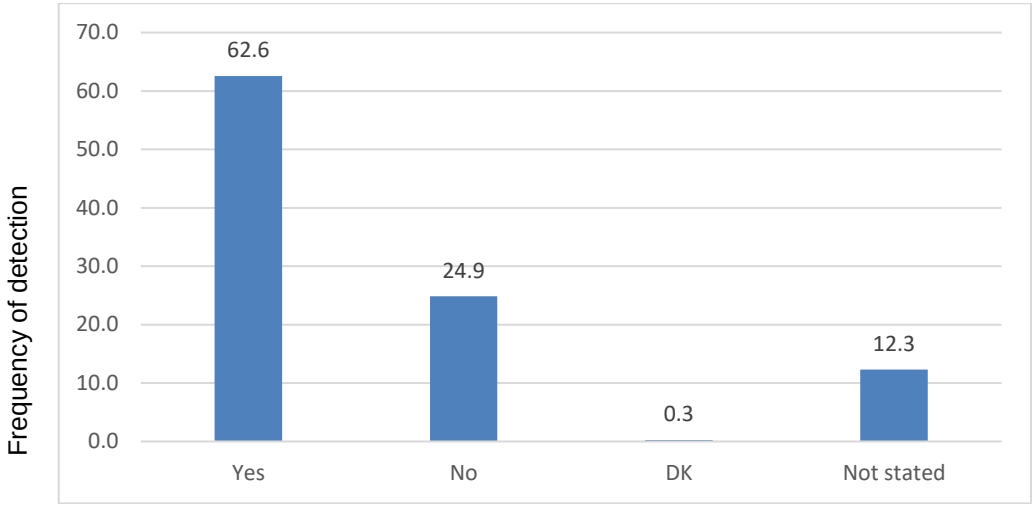

Figure 3.9: Respondents with family members who have or were infected with TB

Figure 3.10 represents the data of the respondents by HIV/AIDS status and indicates that 38.5% (147/382) of the TB positive sputa sample respondents were HIV positive. Approximately 33.5% (128/382) of the respondents revealed that they were HIV negative while 28.0% (107/382) opted not to respond to the HIV/AIDS question. As the HIV and AIDS question was optional a high percentage of "missing" data is indicated in **Figure 3.10**. This limited the researcher's ability to have a complete analysis of the HIV and AIDS status of the respondents in this study. However, tuberculosis is the most common presenting illness among people living with HIV, including those taking antiretroviral treatment, and it is the major cause of HIV-related deaths (WHO, 2013). According to Elliott *et al*. (2009), people living with HIV are 16-27 times more likely to develop TB than persons without. Furthermore, the high prevalence of HIV in patients with tuberculosis suggests that an epidemic of reactivating tuberculosis infections is increased in those who are infected with HIV.

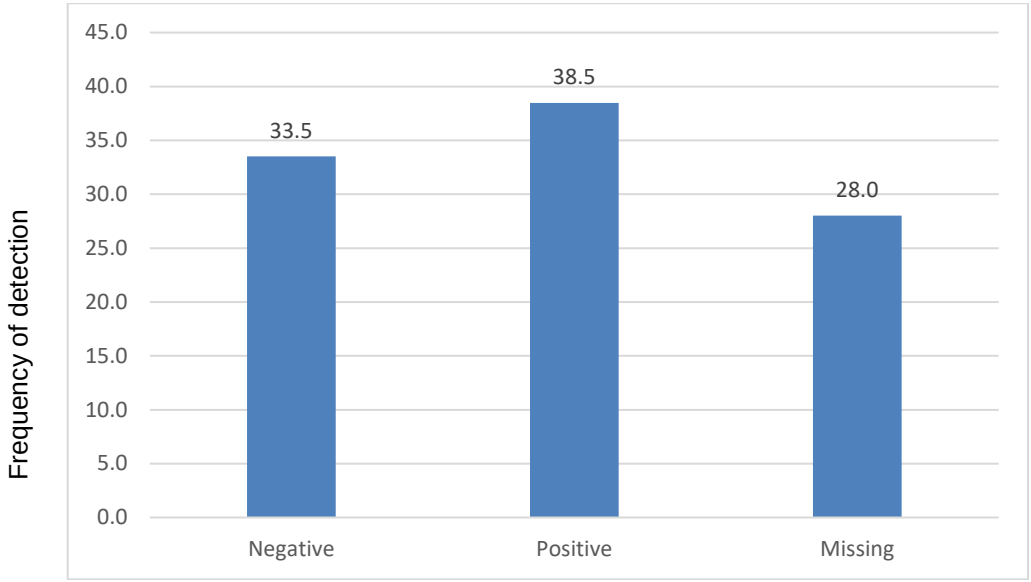

Figure 3.10: Respondents by HIV/AIDS status

CHAPTER 4

CONCLUSION

It is estimated that approximately one-third of the world's population is infected with MTB, and that eight million people globally develop tuberculosis disease each year or die from it (WHO report, 2015). Even though TB is treatable, elimination of it has proven to be almost impossible especially looking at the number of increased drug-resistant cases. There are approximately 2.4 million TB cases occurring per year in sub-Saharan Africa and 80% of the world's TB cases occur in 23 countries worldwide (MoHSS report, 2007). These countries together are known as "high burden countries" (HBCs). Eleven of these HBCs are in Africa; and include Nigeria, South Africa, Ethiopia, the Democratic Republic of Congo, Tanzania, Kenya, Uganda, Zimbabwe, Mozambique, Sudan and Namibia. Furthermore, African countries have the highest rates of TB and the worst treatment outcomes globally, driven by weak health systems and fuelled by the Tuberculosis-Human Immunodeficiency Virus (TB-HIV) co-epidemics (Palmero, 2007). At the same time, Africa faces the largest funding gap of any region in the world and Namibia is no exception.

Accurate and early diagnosis of TB is vital as it interrupts further transmission of the disease and avoids the use of more drugs and thus amplification of drug resistance and possible creation of extensively drug resistant-tuberculosis (XDR-TB). The diagnosis of TB often is slow because of the slow growth rate of the causative agent, *Mycobacterium tuberculosis* complex (MTBC). Furthermore, many low and middle-income countries where almost 95% of TB cases and 98% of deaths occur due to TB, consider sputum smear microscopy as the primary method for diagnosis of tuberculosis (Desikan, 2013), since this technique is not complicated, and it is affordable and fast (Desikan, 2013).

One of the aims of this study was thus to compare direct microscopy with two rapid molecular diagnostic tools (viz. GeneXpert MTB/RIF and Hain Genotype® MTBDR Plus assay) for the identification of MTB from samples collected from the Khomas Region, in Windhoek, Namibia. On average, the results showed 98% average agreement on the MTB presence for all three methods used. It was found that when comparing both Hain vs GE and Hain vs DM, the only disagreement was for the 3%

(12/384) of samples which GE and DM showed as positive while HAIN showed the result as negative/non-reactive. From the results obtained in this research it has been observed that sputum smear microscopy is a simple, rapid and inexpensive technique which is highly specific in areas with a very high prevalence of tuberculosis. The technique can easily facilitate the identification of patients who are infectious. The low-cost nature of sputum smear microscopy allows it to be easily applicable especially in the low to medium economies where TB is most prevalent. However, compared to the GeneXpert MTB/RIF assay and the Hain Genotype® MTBDR Plus assay, sputum smear microscopy has a major drawback in that it has a very low sensitivity especially in situations where bacterial loads are low (below 10,000 organisms/ml) and in TB patients that are also infected with HIV. The sensitivity is grossly compromised when the bacterial load is less than 10,000 organisms/ml in a sputum sample. It also has a poor track record in extra-pulmonary tuberculosis, paediatric tuberculosis and in patients co-infected with HIV and tuberculosis. Unlike the other two techniques which provide instant complete diagnosis, serial sputum microscopy examinations may be required resulting in the possibility of some patients not coming back for repeat examinations thus compromising diagnosis and possible treatment (Desikan, 2013).

The approach to chemotherapy for tuberculosis is very different from that for other bacterial infections. The TB bacteria can live long and has a capacity to be dormant when its low metabolic activity makes it a difficult therapeutic target (McKinney, et al., 2000; Pablos-Mendez, 2000). In addition, *M. tuberculosis* may be located in pulmonary cavities, empyema pus, or solid caseous material, where penetration of antibiotics is difficult, or the pH is sufficiently low to inhibit the activity of most antibiotics (Elliott, et al., 1995). Most times TB can be treated effectively by using the first line drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (SM) for up to six to nine months. Multidrug-resistant TB (MDR TB) is caused by MTB that is resistant to at least isoniazid and rifampicin, the two most potent TB drugs. Extensively drug-resistant TB (XDR TB) is a rare type of MDR TB that is resistant to isoniazid and rifampicin, plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e. amikacin, kanamycin or capreomycin).

Within a laboratory, many TB diagnostics detect RIF-resistance while few detect INH-monoresistance. This said, since it has been argued that MTB that is resistant to RIF is more likely to have concomitant resistance to INH, making RIF resistance a

surrogate marker of MDR-TB (Somoskovi et al., 2001). Within our study, all sputum samples identified as acid-fast bacilli (AFB) positive (*n* = 384) were also analysed using both the GeneXpert MTB/RIF and the Hain Genotype® MTBDR Plus assay for sensitivity and resistance to RIF. Since the Hain Genotype® MTBDR Plus assay simultaneously detects isoniazid (INH), these results were also recorded using this method. The GeneXpert MTB/RIF showed that approximately 2% (7/384) were classified as rifampicin resistant MTB, while 98% (377/384) were shown to be RIF sensitive. In comparison, the Hain GenoType® MTBDR results indicated that 8% (29/384) of samples were classified as isoniazid resistant MTB, while 5% (20/384) were shown to be RIF resistant. The two methods showed 91% (349/384) total agreement for RIF sensitivity and 1% (4/384) total agreement for RIF resistance. Thus, in total 92% (353/384) of the samples obtained from the two methods were in total agreement. The GeneXpert MTB/RIF assay thus identified 2% of the samples as MDR-TB while Hain GenoType® MTBDR indicated that 5% of the samples were MDR-TB (based on using RIF as a surrogate marker). The two methods ultimately agreed that 1% of the samples were RIF resistant. This study saw a close similarity between GeneXpert MTB/RIF assay and the Hain Genotype® MTBDR Plus assay in terms of MDR-TB detection related to RIF. Finding in this research have however further provided evidence for the WHO recommendation of the implementation of a universal molecular test. Recently, newer line probe assays (LPAs) such as the Nipro non-tuberculous mycobacteria (NTM)+MDR-TB have been developed to enable rapid drug-susceptibility testing for rifampicin (RIF) and isoniazid (INH) resistance in the detection of *Mycobacterium tuberculosis*. A recent study using this test showed sensitivity and specificity among smear positive isolates were 98.8% for both, respectively (Nathavitharana et al., 2017).

Tuberculosis surveillance data have been the globally recognized, as a fundamental data source for assessing the disease burden and epidemiological trends of TB. Public health surveillance, through the ongoing and systematic collection, analysis, interpretation and dissemination of health information, plays a critical role in informed decision-making and appropriate public health action. Furthermore, surveillance was one of the five core components in the original World Health Organization (WHO) Framework for Effective Tuberculosis Control (the WHO DOTS strategy) established in 1994.

The demographic and epidemiological data captured within our study showed that TB was more prevalent amongst women especially those between the ages of 20 to 40 years. In addition, there was a high percentage of second time TB diagnoses among respondents between the ages of 30-39 years (35.0%; 50/141), followed by respondents between the ages of 20-29 years (24.8%; 35/141). Of the respondents who had been diagnosed for a second time with TB, it was interesting to note that approximately 45.5% (174/382) reported that they had completed the TB treatment course during the first infection, while 25.9% (99/382) of the respondents revealed that they did not complete the TB treatment course during the first diagnosis. As acknowledged by many experts, the patient plays a central role since adherence to drug regime completion is vital to control the TB disease.

A study on poverty alleviation in the informal settlement communities in Windhoek, Namibia, found that on average, a dwelling had six occupants. Accordingly, research has indicated that people living in poor socio-economic conditions as well as in overcrowded dwellings have an increased risk of contracting TB. Within our study, 39.9% of the respondents were unemployed and a high percentage (63%) of the respondents were among those with limited or no skills. Results also showed that 62.6% (239/382) of the respondents reported that they had family members who currently had or who were previously diagnosed with TB.

Finally, 38.5% (147/382) of the respondents reported that they were HIV positive while 33.5% (128/382) of the respondents revealed that they were HIV negative and 28.0% (107/382) opted not to respond to the HIV/AIDS question. Results obtained in this study thus confirms that TB commonly presents as a secondary infection among people living with HIV. The study also confirmed that epidemiological data can reveal high-risk groups who are vulnerable to the disease.

In conclusion, further analysis of clinical and epidemiological data can help identify programmatic gaps, prioritize interventions and research topics and allocate resources for subsequent action. Tuberculosis control cannot be carried out without setting up an effective system to define the course of the epidemic and assess the impact of control measures on the disease.

CHAPTER 5

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APPENDIX A:
CONSENT FORM FOR THE RESEARCH STUDY

Confidential

Title of Project: Identification and Molecular characterization of MDR-TB clinical isolates obtained in the Khomas Region Windhoek, Namibia

1. Name of researcher **Evelyn Ndinelao Breuer**

2. Name /Code _____

3. Date: |__|__|__|__|__|__|__|__|

(DD/ MM/ YY)

4. Study Number |__|__|__|__|

5. Health Centre _____

I confirm that I have read and understand the information sheet for the above study.

I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from Ministry Of Health and Social Services (MoHSS), from regulatory authorities or from the Cape Peninsula University of Technology (CPUT), NIP, where it is relevant to my taking part in this research. I give permission for these individuals/bodies to have access to my records.

I agree to take part in the above research study.

_____	_____	_____
Name of Patient (optional)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

When complete, 1 copy for patient: 1 copy for researcher site file: 1 (original) to be kept in research study notes.

APPENDIX B

STANDARD QUESTIONNAIRE

Confidential

Name /Code _____

Date |__|__|__|__|__|__|__|__|
(DD/ MM/ YY)

Study Number |__|__|__|__|

Sex (please Tick) Female ☐ Male ☐

Home address _____

_____

_____

Date of Birth |__|__|__|__|__|__|__|__|

Age(years) |__|__|__|

Marital Status: (please Tick) Married ☐ Single ☐ Divorced ☐

Widowed ☐

Usual place of residence _____

Now residing in _____

_____

_____

Weight (Kg). |__|__|__|

Height (cm) |__|__|__|

Highest level of education Reached: (please Tick) Below Matric ☐

Matric ☐ Tertiary Level ☐

Employed (please Tick) Yes ☐ No ☐

Salary per month (N$) |__|__|__|__|__|__|

Occupation (please Tick) Managerial Skilled ☐ Semi-Skilled ☐

Unskilled ☐

Husband/Wife Employed Yes ☐ No ☐

No of people living with you |__|__|__|

Any of the people living with you has or had TB Yes ☐ No ☐

When were you diagnosed with TB |__|__|__|

How long are you on TB treatment (No. of Months/Days)? |__|__|__|

Is it the first time or ☐ s☐nd time Diagnosed with TB ☐

Did you finish the whole course during first treatment? Yes ☐ No ☐

Which Regime (only if known) _____

Is this the first TB infection Yes ☐ No ☐

How many times did you get infected with TB?

Once ☐ twice ☐ three t☐ or more ☐

Any member of the family who has or had TB Yes ☐ No ☐

What is your HIV (Not Compulsory) Positive ☐ Negative ☐

When were you diagnosed with HIV (Not Compulsory) |__|__|__|

Are you on ARV's (Not Compulsory) Yes ☐ No ☐

Name of ARV (Not Compulsory) _____

Since when are you on ARV's (Not Compulsory) |__|__|__|

APPENDIX C

RESULTS OF PATIENTS SPUTA

PT. Code	Hain GenoType®		GeneXpert	DM
	INH	RIF	RIF	
1.	S	S	S	3+
2.	S	S	S	2+
3.	S	S	S	1+
4.	S	S	S	1+
5.	S	S	S	1+
6.	S	S	S	2+
7.	S	S	S	1+
8.	S	S	S	1+
9.	S	S	S	1+
10.	S	S	S	1+
11.	S	S	S	2+
12.	S	S	S	3+
13.	S	S	S	3+
14.	S	S	S	1+
15.	S	S	S	1+
16.	S	S	S	2+
17.	S	S	S	3+
18.	S	S	S	2+
19.	S	S	S	1+
20.	S	R	S	3+
21.	S	S	S	2+
22.	R	R	S	3+
23.	S	S	S	3+
24.	S	S	S	1+
25.	S	S	S	3+
26.	R	R	R	1+
27.	S	S	S	1+
28.	S	S	S	3+
29.	S	S	S	2+
30.	S	S	S	1+
31.	S	S	S	3+
32.	S	S	S	1+
33.	S	S	S	1+
34.	S	S	S	2+
35.	S	S	S	3+
36.	R	S	S	3+
37.	S	S	S	1+
38.	R	S	S	3+
39.	S	S	S	2+
40.	S	S	S	3+
41.	NEG	NEG	S	1+

42.	NEG	NEG	S	1+
43.	S	S	S	2+
44.	S	S	S	2+
45.	NEG	NEG	S	1+
46.	NEG	NEG	S	1+
47.	R	S	S	1+
48.	S	S	S	1+
49.	S	S	S	2+
50.	S	S	S	1+
51.	R	R	R	1+
52.	NEG	NEG	S	1=
53.	S	S	S	1+
54.	S	S	S	3+
55.	S	S	S	1+
56.	R	S	S	3+
57.	R	R	R	3+
58.	S	S	S	3+
59.	S	S	S	2+
60.	S	S	S	1+
61.	NEG	NEG	S	1+
62.	S	S	S	3+
63.	S	S	S	1+
64.	R	R	S	2+
65.	R	R	S	1+
66.	R	R	S	1+
67.	S	S	S	3+
68.	S	S	S	3+
69.	S	S	S	3+
70.	S	S	S	1+
71.	S	S	S	2+
72.	S	S	S	3+
73.	S	S	S	2+
74.	S	S	S	1+
75.	S	S	S	1+
76.	S	S	S	1+
77.	S	S	S	3+
78.	S	S	S	3+
79.	S	S	S	1+
80.	S	S	S	3+
81.	S	S	S	1+
82.	S	S	S	1+
83.	S	S	S	2+
84.	S	S	S	3+
85.	S	S	S	2+
86.	S	S	S	2+
87.	S	S	S	3+

88.	S	S	S	1+
89.	S	S	S	3+
90.	S	S	S	1+
91.	S	S	S	3+
92.	S	S	S	1+
93.	S	S	S	2+
94.	NEG	NEG	S	1+
95.	S	S	S	3+
96.	S	S	S	1+
97.	S	S	S	3+
98.	S	S	S	2+
99.	S	S	S	2+
100.	NEG	NEG	S	1+
101.	S	S	S	1+
102.	S	S	S	3+
103	S	S	S	3+
104.	S	S	S	1+
105.	S	S	S	1+
106.	S	S	S	1+
107.	S	S	S	3+
108.	S	S	S	2+
109.	NEG	NEG	S	1+
110.	S	S	S	2+
111.	S	S	S	3+
112.	S	S	S	1+
113.	S	S	S	1+
114.	S	S	R	2+
115.	S	S	S	3+
116.	S	S	S	1+
117.	S	S	S	1+
118.	S	S	S	3+
119.	S	S	S	2+
120.	S	S	S	3+
121.	S	S	S	2+
122.	S	S	S	2+
123.	S	S	S	1+
124.	S	S	S	2+
125.	S	S	S	2+
126.	S	S	S	3+
127.	S	S	S	3+
128.	S	S	S	3+
129.	S	S	S	1+
130.	S	S	S	2+
131.	S	S	S	1+
132.	S	S	S	1+
133.	S	S	S	1+

134.	S	S	S	1+
135.	S	S	S	1+
136.	S	S	S	1+
137.	S	S	S	3+
138.	S	S	S	2+
139.	S	S	S	1+
140.	S	S	S	3+
141.	S	S	S	2+
142.	S	S	S	3+
143.	S	S	S	1+
144.	S	S	S	1+
145.	S	S	S	1+
146.	S	S	S	3+
147.	S	S	S	2+
148.	S	S	S	1+
149.	S	S	S	1+
150.	S	S	S	1+
151.	S	S	S	1+
152.	S	S	S	3+
153.	S	S	S	1+
154.	S	S	S	3+
155.	S	S	S	1+
156.	S	S	S	1+
157.	S	S	S	1+
158.	S	S	S	1+
159.	R	R	S	1+
160.	S	S	S	3+
161.	S	S	S	1+
162.	S	S	S	1+
163.	S	S	S	1+
164.	S	S	S	3+
165.	S	S	S	2+
166.	R	S	S	1+
167.	S	S	S	1+
168.	S	S	S	2+
169.	R	S	S	2+
170.	S	S	S	1+
171.	S	S	S	3+
172.	S	S	S	3+
173.	S	S	S	3+
174.	S	R	S	1+
175.	S	S	S	2+
176.	S	S	S	1+
177.	S	S	S	3+
178.	S	R	S	2+
179.	S	S	S	1+

180.	R	S	S	1+
181.	S	S	S	1+
182.	R	S	S	1+
183.	S	S	S	3+
184.	R	S	S	1+
185.	S	S	S	2+
186.	S	S	S	1+
187.	S	S	S	1+
188.	S	S	S	1+
189.	R	R	S	1+
190.	R	S	S	1+
191.	R	S	S	1+
192.	S	S	S	1+
193.	S	S	S	1+
194.	S	S	S	2+
195.	S	S	S	2+
196.	NEG	NEG	S	1+
197.	S	S	R	3+
198.	S	S	S	3+
199.	S	S	S	2+
200.	S	S	S	3+
201.	R	S	S	1+
202.	S	R	S	1+
203.	S	S	S	2+
204.	R	S	R	2+
205.	R	R	S	1+
206.	R	R	S	2+
207.	S	S	S	1+
208.	NEG	NEG	S	2+
209.	S	S	S	1+
210.	S	S	S	3+
211.	R	R	R	3+
212.	S	S	S	1+
213.	R	S	S	2+
214.	R	R	S	1+
215.	S	S	S	2+
216.	S	S	S	3+
217.	R	R	S	3+
218.	S	S	S	1+
219.	S	S	S	1+
220.	S	S	S	1+
221.	S	S	S	3+
222.	S	S	S	1+
223.	S	S	S	1+
224.	S	S	S	1+
225.	S	S	S	1+

226.	S	S	S	1+
227.	S	S	S	3+
228.	R	R	S	1+
229.	S	S	S	3+
230.	S	S	S	1+
231.	S	S	S	3+
232.	S	S	S	1+
233.	S	S	S	3+
234.	S	S	S	2+
235.	S	S	S	2+
236.	S	S	S	2+
237.	S	S	S	1+
238.	S	S	S	3+
239.	S	S	S	1+
240.	S	S	S	3+
241.	NEG	NEG	S	1+
242.	S	S	S	1+
243.	S	S	S	1+
244.	S	S	S	1+
245.	S	S	S	1+
246.	S	S	S	1+
247.	S	S	S	3+
248.	S	S	S	3+
249.	S	S	S	3+
250.	S	S	S	1+
251.	S	S	S	1+
252.	S	S	S	3+
253.	S	S	S	2+
254.	S	S	S	1+
255.	S	S	S	1+
256.	S	S	S	2+
257.	S	S	S	3+
258.	S	S	S	1+
259.	S	S	S	2+
260.	S	S	S	3+
261.	S	S	S	3+
262.	S	S	S	3+
263.	S	S	S	2+
264.	S	S	S	1+
265.	S	S	S	2+
266.	S	S	S	3+
267.	S	S	S	2+
268.	S	S	S	1+
269.	S	S	S	1+
270.	S	S	S	2+
271.	S	S	S	3+

272.	S	S	S	1+
273.	S	S	S	1+
274.	S	S	S	3+
275.	S	S	S	1+
276.	S	S	S	1+
277.	S	S	S	3+
278.	S	S	S	1+
279.	S	S	S	1+
280.	S	S	S	1+
281.	S	S	S	2+
282.	S	S	S	1+
283.	S	S	S	3+
284.	S	S	S	2+
285.	S	S	S	1+
286.	S	S	S	1+
287.	S	S	S	1+
288.	S	S	S	1+
289.	S	S	S	1+
290.	S	S	S	3+
291.	S	S	S	3+
292.	S	S	S	1+
293.	S	S	S	1+
294.	S	S	S	1+
295.	S	S	S	1+
296.	S	S	S	1+
297.	S	S	S	3+
298.	S	S	S	3+
299.	S	S	S	3+
300.	S	S	S	1+
301.	S	S	S	1+
302.	S	S	S	1+
303.	S	S	S	1+
304.	S	S	S	1+
305.	S	S	S	3+
306.	S	S	S	1+
307.	S	S	S	2+
308.	S	S	S	2+
309.	S	S	S	3+
310.	S	S	S	1+
311.	S	S	S	1+
312.	S	S	S	3+
313.	S	S	S	3+
314.	S	S	S	1+
315.	S	S	S	2+
316.	S	S	S	1+
317.	S	S	S	2+

318.	S	S	S	1+
319.	S	S	S	1+
320.	S	S	S	1+
321.	S	S	S	3+
322.	S	S	S	3+
323.	S	S	S	1+
324.	S	S	S	1+
325.	S	S	S	1+
326.	S	S	S	1+
327.	S	S	S	1+
328.	S	S	S	3+
329.	S	S	S	1+
330.	S	S	S	1+
331.	S	S	S	3+
332.	S	S	S	2+
333.	S	S	S	1+
334.	S	S	S	2+
335.	S	S	S	2+
336.	S	S	S	3+
337.	S	S	S	1+
338.	S	S	S	1+
339.	S	S	S	3+
340.	S	S	S	3+
341.	S	S	S	1+
342.	S	S	S	1+
343.	S	S	S	3+
344.	S	S	S	2+
345.	S	S	S	1+
346.	S	S	S	1+
347.	S	S	S	1+
348.	S	S	S	1+
349.	S	S	S	3+
350.	S	S	S	1+
351.	S	S	S	1+
352.	S	S	S	3+
353.	S	S	S	1+
354.	S	S	S	3+
355.	S	S	S	2+
356.	S	S	S	1+
357.	S	S	S	2+
358.	S	S	S	1+
359.	S	S	S	2+
360.	S	S	S	1+
361.	S	S	S	2+
362.	S	S	S	1+
363.	S	S	S	1+

364.	S	S	S	1+
365.	S	S	S	1+
366.	S	S	S	2+
367.	S	S	S	1+
368.	S	S	S	1+
369.	S	S	S	1+
370.	S	S	S	2+
371.	S	S	S	1+
372.	S	S	S	2+
373.	S	S	S	1+
374.	S	S	S	2+
375.	S	S	S	2+
376.	S	S	S	1+
377.	S	S	S	1+
378.	S	S	S	3+
379.	S	S	S	1+
380.	S	S	S	1+
381.	S	S	S	2+
382.	S	S	S	3+
383.	S	S	S	3+
384.	S	R	S	1+

APPENDIX D

DATA GENERATED FROM THE HAIN, GENEXPERT AND DM RESULTS

. CODE	HAIN	GE	DM	ALL	%Agreement	3 agree	2 agree	HAIN RIF		GE RIF		Agreement RIF	
								Sen	Res	Sen	Res	2 Agree Sen	1 Agree Sen
1	1	1	1	3	100%	1	0	+	-	+	-	1	0
2	1	1	1	3	100%	1	0	+	-	+	-	1	0
3	1	1	1	3	100%	1	0	+	-	+	-	1	0
4	1	1	1	3	100%	1	0	+	-	+	-	1	0
5	1	1	1	3	100%	1	0	+	-	+	-	1	0
6	1	1	1	3	100%	1	0	+	-	+	-	1	0
7	1	1	1	3	100%	1	0	+	-	+	-	1	0
8	1	1	1	3	100%	1	0	+	-	+	-	1	0
9	1	1	1	3	100%	1	0	+	-	+	-	1	0
10	1	1	1	3	100%	1	0	+	-	+	-	1	0
11	1	1	1	3	100%	1	0	+	-	+	-	1	0
12	1	1	1	3	100%	1	0	+	-	+	-	1	0
13	1	1	1	3	100%	1	0	+	-	+	-	1	0
14	1	1	1	3	100%	1	0	+	-	+	-	1	0
15	1	1	1	3	100%	1	0	+	-	+	-	1	0
16	1	1	1	3	100%	1	0	+	-	+	-	1	0
17	1	1	1	3	100%	1	0	+	-	+	-	1	0

18	1	1	1	3	100%	1	0	+	-	+	-	1	0
19	1	1	1	3	100%	1	0	+	-	+	-	1	0
20	1	1	1	3	100%	1	0	-	+	+	-	0	1
21	1	1	1	3	100%	1	0	+	-	+	-	1	0
22	1	1	1	3	100%	1	0	-	+	+	-	0	1
23	1	1	1	3	100%	1	0	+	-	+	-	1	0
24	1	1	1	3	100%	1	0	+	-	+	-	1	0
25	1	1	1	3	100%	1	0	+	-	+	-	1	0
26	1	1	1	3	100%	1	0	-	+	-	+	1	0
27	1	1	1	3	100%	1	0	+	-	+	-	1	0
28	1	1	1	3	100%	1	0	+	-	+	-	1	0
29	1	1	1	3	100%	1	0	+	-	+	-	1	0
30	1	1	1	3	100%	1	0	+	-	+	-	1	0
31	1	1	1	3	100%	1	0	+	-	+	-	1	0
32	1	1	1	3	100%	1	0	+	-	+	-	1	0
33	1	1	1	3	100%	1	0	+	-	+	-	1	0
34	1	1	1	3	100%	1	0	+	-	+	-	1	0
35	1	1	1	3	100%	1	0	+	-	+	-	1	0
36	1	1	1	3	100%	1	0	+	-	+	-	1	0
37	1	1	1	3	100%	1	0	+	-	+	-	1	0
38	1	1	1	3	100%	1	0	+	-	+	-	1	0
39	1	1	1	3	100%	1	0	+	-	+	-	1	0
40	1	1	1	3	100%	1	0	+	-	+	-	1	0

41	0	1	1	3	67%	0	1	NR	NR	+	-	0	1
42	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
43	1	1	1	3	100%	0	1	+	-	+	-	1	0
44	1	1	1	3	100%	1	0	+	-	+	-	1	0
45	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
46	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
47	1	1	1	3	100%	1	0	+	-	+	-	1	0
48	1	1	1	3	100%	1	0	+	-	+	-	1	0
49	1	1	1	3	100%	1	0	+	-	+	-	1	0
50	1	1	1	3	100%	1	0	+	-	+	-	1	0
51	1	1	1	3	100%	1	0	-	+	-	+	1	0
52	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
53	1	1	1	3	100%	1	0	+	-	+	-	1	0
54	1	1	1	3	100%	1	0	+	-	+	-	1	0
55	1	1	1	3	100%	1	0	+	-	+	-	1	0
56	1	1	1	3	100%	1	0	+	-	+	-	1	0
57	1	1	1	3	100%	1	0	-	+	-	+	1	0
58	1	1	1	3	100%	1	0	+	-	+	-	1	0
59	1	1	1	3	100%	1	0	+	-	+	-	1	0
60	1	1	1	3	100%	1	0	+	-	+	-	1	0
61	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
62	1	1	1	3	100%	1	0	+	-	+	-	1	0

63	1	1	1	3	100%	1	0	+	-	+	-	1	0
64	1	1	1	3	100%	1	0	-	+	+	-	0	1
65	1	1	1	3	100%	1	0	-	+	+	-	0	1
66	1	1	1	3	100%	1	0	-	+	+	-	0	1
67	1	1	1	3	100%	1	0	+	-	+	-	1	0
68	1	1	1	3	100%	1	0	+	-	+	-	1	0
69	1	1	1	3	100%	1	0	+	-	+	-	1	0
70	1	1	1	3	100%	1	0	+	-	+	-	1	0
71	1	1	1	3	100%	1	0	+	-	+	-	1	0
72	1	1	1	3	100%	1	0	+	-	+	-	1	0
73	1	1	1	3	100%	1	0	+	-	+	-	1	0
74	1	1	1	3	100%	1	0	+	-	+	-	1	0
75	1	1	1	3	100%	1	0	+	-	+	-	1	0
76	1	1	1	3	100%	1	0	+	-	+	-	1	0
77	1	1	1	3	100%	1	0	+	-	+	-	1	0
78	1	1	1	3	100%	1	0	+	-	+	-	1	0
79	1	1	1	3	100%	1	0	+	-	+	-	1	0
80	1	1	1	3	100%	1	0	+	-	+	-	1	0
81	1	1	1	3	100%	1	0	+	-	+	-	1	0
82	1	1	1	3	100%	1	0	+	-	+	-	1	0
83	1	1	1	3	100%	1	0	+	-	+	-	1	0
84	1	1	1	3	100%	1	0	+	-	+	-	1	0

85	1	1	1	3	100%	1	0	+	-	+	-	1	0
86	1	1	1	3	100%	1	0	+	-	+	-	1	0
87	1	1	1	3	100%	1	0	+	-	+	-	1	0
88	1	1	1	3	100%	1	0	+	-	+	-	1	0
89	1	1	1	3	100%	1	0	+	-	+	-	1	0
90	1	1	1	3	100%	1	0	+	-	+	-	1	0
91	1	1	1	3	100%	1	0	+	-	+	-	1	0
92	1	1	1	3	100%	1	0	+	-	+	-	1	0
93	1	1	1	3	100%	1	0	+	-	+	-	1	0
94	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
95	1	1	1	3	100%	1	0	+	-	+	-	1	0
96	1	1	1	3	100%	1	0	+	-	+	-	1	0
97	1	1	1	3	100%	1	0	+	-	+	-	1	0
98	1	1	1	3	100%	1	0	+	-	+	-	1	0
99	1	1	1	3	100%	1	0	+	-	+	-	1	0
100	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
101	1	1	1	3	100%	1	0	+	-	+	-	1	0
102	1	1	1	3	100%	1	0	+	-	+	-	1	0
103	1	1	1	3	100%	1	0	+	-	+	-	1	0
104	1	1	1	3	100%	1	0	+	-	+	-	1	0
105	1	1	1	3	100%	1	0	+	-	+	-	1	0
106	1	1	1	3	100%	1	0	+	-	+	-	1	0
107	1	1	1	3	100%	1	0	+	-	+	-	1	0

108	1	1	1	3	100%	1	0	+	-	+	-	1	0
109	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
110	1	1	1	3	100%	1	0	+	-	+	-	1	0
111	1	1	1	3	100%	1	0	+	-	+	-	1	0
112	1	1	1	3	100%	1	0	+	-	+	-	1	0
113	1	1	1	3	100%	1	0	+	-	+	-	1	0
114	1	1	1	3	100%	1	0	+	-	-	+	0	1
115	1	1	1	3	100%	1	0	+	-	+	-	1	0
116	1	1	1	3	100%	1	0	+	-	+	-	1	0
117	1	1	1	3	100%	1	0	+	-	+	-	1	0
118	1	1	1	3	100%	1	0	+	-	+	-	1	0
119	1	1	1	3	100%	1	0	+	-	+	-	1	0
120	1	1	1	3	100%	1	0	+	-	+	-	1	0
121	1	1	1	3	100%	1	0	+	-	+	-	1	0
122	1	1	1	3	100%	1	0	+	-	+	-	1	0
123	1	1	1	3	100%	1	0	+	-	+	-	1	0
124	1	1	1	3	100%	1	0	+	-	+	-	1	0
125	1	1	1	3	100%	1	0	+	-	+	-	1	0
126	1	1	1	3	100%	1	0	+	-	+	-	1	0
127	1	1	1	3	100%	1	0	+	-	+	-	1	0
128	1	1	1	3	100%	1	0	+	-	+	-	1	0
129	1	1	1	3	100%	1	0	+	-	+	-	1	0
130	1	1	1	3	100%	1	0	+	-	+	-	1	0

131	1	1	1	3	100%	1	0	+	-	+	-	1	0
132	1	1	1	3	100%	1	0	+	-	+	-	1	0
133	1	1	1	3	100%	1	0	+	-	+	-	1	0
134	1	1	1	3	100%	1	0	+	-	+	-	1	0
135	1	1	1	3	100%	1	0	+	-	+	-	1	0
136	1	1	1	3	100%	1	0	+	-	+	-	1	0
137	1	1	1	3	100%	1	0	+	-	+	-	1	0
138	1	1	1	3	100%	1	0	+	-	+	-	1	0
139	1	1	1	3	100%	1	0	+	-	+	-	1	0
140	1	1	1	3	100%	1	0	+	-	+	-	1	0
141	1	1	1	3	100%	1	0	+	-	+	-	1	0
142	1	1	1	3	100%	1	0	+	-	+	-	1	0
143	1	1	1	3	100%	1	0	+	-	+	-	1	0
144	1	1	1	3	100%	1	0	+	-	+	-	1	0
145	1	1	1	3	100%	1	0	+	-	+	-	1	0
146	1	1	1	3	100%	1	0	+	-	+	-	1	0
147	1	1	1	3	100%	1	0	+	-	+	-	1	0
148	1	1	1	3	100%	1	0	+	-	+	-	1	0
149	1	1	1	3	100%	1	0	+	-	+	-	1	0
150	1	1	1	3	100%	1	0	+	-	+	-	1	0
151	1	1	1	3	100%	1	0	+	-	+	-	1	0
152	1	1	1	3	100%	1	0	+	-	+	-	1	0

153	1	1	1	3	100%	1	0	+	-	+	-	1	0
154	1	1	1	3	100%	1	0	+	-	+	-	1	0
155	1	1	1	3	100%	1	0	+	-	+	-	1	0
156	1	1	1	3	100%	1	0	+	-	+	-	1	0
157	1	1	1	3	100%	1	0	+	-	+	-	1	0
158	1	1	1	3	100%	1	0	+	-	+	-	1	0
159	1	1	1	3	100%	1	0	-	+	+	-	0	1
160	1	1	1	3	100%	1	0	+	-	+	-	1	0
161	1	1	1	3	100%	1	0	+	-	+	-	1	0
162	1	1	1	3	100%	1	0	+	-	+	-	1	0
163	1	1	1	3	100%	1	0	+	-	+	-	1	0
164	1	1	1	3	100%	1	0	+	-	+	-	1	0
165	1	1	1	3	100%	1	0	+	-	+	-	1	0
166	1	1	1	3	100%	1	0	+	-	+	-	1	0
167	1	1	1	3	100%	1	0	+	-	+	-	1	0
168	1	1	1	3	100%	1	0	+	-	+	-	1	0
169	1	1	1	3	100%	1	0	+	-	+	-	1	0
170	1	1	1	3	100%	1	0	+	-	+	-	1	0
171	1	1	1	3	100%	1	0	+	-	+	-	1	0
172	1	1	1	3	100%	1	0	+	-	+	-	1	0
173	1	1	1	3	100%	1	0	+	-	+	-	1	0
174	1	1	1	3	100%	1	0	-	+	+	-	0	1

175	1	1	1	3	100%	1	0	+	-	+	-	1	0
176	1	1	1	3	100%	1	0	+	-	+	-	1	0
177	1	1	1	3	100%	1	0	+	-	+	-	1	0
178	1	1	1	3	100%	1	0	-	+	+	-	0	1
179	1	1	1	3	100%	1	0	+	-	+	-	1	0
180	1	1	1	3	100%	1	0	+	-	+	-	1	0
181	1	1	1	3	100%	1	0	+	-	+	-	1	0
182	1	1	1	3	100%	1	0	+	-	+	-	1	0
183	1	1	1	3	100%	1	0	+	-	+	-	1	0
184	1	1	1	3	100%	1	0	+	-	+	-	1	0
185	1	1	1	3	100%	1	0	+	-	+	-	1	0
186	1	1	1	3	100%	1	0	+	-	+	-	1	0
187	1	1	1	3	100%	1	0	+	-	+	-	1	0
188	1	1	1	3	100%	1	0	+	-	+	-	1	0
189	1	1	1	3	100%	1	0	-	+	+	-	0	1
190	1	1	1	3	100%	1	0	+	-	+	-	1	0
191	1	1	1	3	100%	1	0	+	-	+	-	1	0
192	1	1	1	3	100%	1	0	+	-	+	-	1	0
193	1	1	1	3	100%	1	0	+	-	+	-	1	0
194	1	1	1	3	100%	1	0	+	-	+	-	1	0
195	1	1	1	3	100%	1	0	+	-	+	-	1	0
196	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
197	1	1	1	3	100%	1	0	+	-	-	+	0	1

198	1	1	1	3	100%	1	0	+	-	+	-	1	0
199	1	1	1	3	100%	1	0	+	-	+	-	1	0
200	1	1	1	3	100%	1	0	+	-	+	-	1	0
201	1	1	1	3	100%	1	0	+	-	+	-	1	0
202	1	1	1	3	100%	1	0	-	+	+	-	0	1
203	1	1	1	3	100%	1	0	+	-	+	-	1	0
204	1	1	1	3	100%	1	0	+	-	-	+	0	1
205	1	1	1	3	100%	1	0	-	+	+	-	0	1
206	1	1	1	3	100%	1	0	-	+	+	-	0	1
207	1	1	1	3	100%	1	0	+	-	+	-	1	0
208	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
209	1	1	1	3	100%	1	0	+	-	+	-	1	0
210	1	1	1	3	100%	1	0	+	-	+	-	1	0
211	1	1	1	3	100%	1	0	-	+	-	+	1	0
212	1	1	1	3	100%	1	0	+	-	+	-	1	0
213	1	1	1	3	100%	1	0	+	-	+	-	1	0
214	1	1	1	3	100%	1	0	-	+	+	-	0	1
215	1	1	1	3	100%	1	0	+	-	+	-	1	0
216	1	1	1	3	100%	1	0	+	-	+	-	1	0
217	1	1	1	3	100%	1	0	-	+	+	-	0	1
218	1	1	1	3	100%	1	0	+	-	+	-	1	0
219	1	1	1	3	100%	1	0	+	-	+	-	1	0

220	1	1	1	3	100%	1	0	+	-	+	-	1	0
221	1	1	1	3	100%	1	0	+	-	+	-	1	0
222	1	1	1	3	100%	1	0	+	-	+	-	1	0
223	1	1	1	3	100%	1	0	+	-	+	-	1	0
224	1	1	1	3	100%	1	0	+	-	+	-	1	0
225	1	1	1	3	100%	1	0	+	-	+	-	1	0
226	1	1	1	3	100%	1	0	+	-	+	-	1	0
227	1	1	1	3	100%	1	0	+	-	+	-	1	0
228	1	1	1	3	100%	1	0	-	+	+	-	0	1
229	1	1	1	3	100%	1	0	+	-	+	-	1	0
230	1	1	1	3	100%	1	0	+	-	+	-	1	0
231	1	1	1	3	100%	1	0	+	-	+	-	1	0
232	1	1	1	3	100%	1	0	+	-	+	-	1	0
233	1	1	1	3	100%	1	0	+	-	+	-	1	0
234	1	1	1	3	100%	1	0	+	-	+	-	1	0
235	1	1	1	3	100%	1	0	+	-	+	-	1	0
236	1	1	1	3	100%	1	0	+	-	+	-	1	0
237	1	1	1	3	100%	1	0	+	-	+	-	1	0
238	1	1	1	3	100%	1	0	+	-	+	-	1	0
239	1	1	1	3	100%	1	0	+	-	+	-	1	0
240	1	1	1	3	100%	1	0	+	-	+	-	1	0
241	0	1	1	2	67%	0	1	NR	NR	+	-	0	1
242	1	1	1	3	100%	1	0	+	-	+	-	1	0

243	1	1	1	3	100%	1	0	+	-	+	-	1	0
244	1	1	1	3	100%	1	0	+	-	+	-	1	0
245	1	1	1	3	100%	1	0	+	-	+	-	1	0
246	1	1	1	3	100%	1	0	+	-	+	-	1	0
247	1	1	1	3	100%	1	0	+	-	+	-	1	0
248	1	1	1	3	100%	1	0	+	-	+	-	1	0
249	1	1	1	3	100%	1	0	+	-	+	-	1	0
250	1	1	1	3	100%	1	0	+	-	+	-	1	0
251	1	1	1	3	100%	1	0	+	-	+	-	1	0
252	1	1	1	3	100%	1	0	+	-	+	-	1	0
253	1	1	1	3	100%	1	0	+	-	+	-	1	0
254	1	1	1	3	100%	1	0	+	-	+	-	1	0
255	1	1	1	3	100%	1	0	+	-	+	-	1	0
256	1	1	1	3	100%	1	0	+	-	+	-	1	0
567	1	1	1	3	100%	1	0	+	-	+	-	1	0
258	1	1	1	3	100%	1	0	+	-	+	-	1	0
259	1	1	1	3	100%	1	0	+	-	+	-	1	0
260	1	1	1	3	100%	1	0	+	-	+	-	1	0
261	1	1	1	3	100%	1	0	+	-	+	-	1	0
262	1	1	1	3	100%	1	0	+	-	+	-	1	0
263	1	1	1	3	100%	1	0	+	-	+	-	1	0
264	1	1	1	3	100%	1	0	+	-	+	-	1	0
265	1	1	1	3	100%	1	0	+	-	+	-	1	0

266	1	1	1	3	100%	1	0	+	-	+	-	1	0
267	1	1	1	3	100%	1	0	+	-	+	-	1	0
268	1	1	1	3	100%	1	0	+	-	+	-	1	0
269	1	1	1	3	100%	1	0	+	-	+	-	1	0
270	1	1	1	3	100%	1	0	+	-	+	-	1	0
271	1	1	1	3	100%	1	0	+	-	+	-	1	0
272	1	1	1	3	100%	1	0	+	-	+	-	1	0
273	1	1	1	3	100%	1	0	+	-	+	-	1	0
274	1	1	1	3	100%	1	0	+	-	+	-	1	0
275	1	1	1	3	100%	1	0	+	-	+	-	1	0
276	1	1	1	3	100%	1	0	+	-	+	-	1	0
277	1	1	1	3	100%	1	0	+	-	+	-	1	0
278	1	1	1	3	100%	1	0	+	-	+	-	1	0
279	1	1	1	3	100%	1	0	+	-	+	-	1	0
280	1	1	1	3	100%	1	0	+	-	+	-	1	0
281	1	1	1	3	100%	1	0	+	-	+	-	1	0
282	1	1	1	3	100%	1	0	+	-	+	-	1	0
283	1	1	1	3	100%	1	0	+	-	+	-	1	0
284	1	1	1	3	100%	1	0	+	-	+	-	1	0
285	1	1	1	3	100%	1	0	+	-	+	-	1	0
286	1	1	1	3	100%	1	0	+	-	+	-	1	0
287	1	1	1	3	100%	1	0	+	-	+	-	1	0
288	1	1	1	3	100%	1	0	+	-	+	-	1	0

289	1	1	1	3	100%	1	0	+	-	+	-	1	0
290	1	1	1	3	100%	1	0	+	-	+	-	1	0
291	1	1	1	3	100%	1	0	+	-	+	-	1	0
292	1	1	1	3	100%	1	0	+	-	+	-	1	0
293	1	1	1	3	100%	1	0	+	-	+	-	1	0
294	1	1	1	3	100%	1	0	+	-	+	-	1	0
295	1	1	1	3	100%	1	0	+	-	+	-	1	0
296	1	1	1	3	100%	1	0	+	-	+	-	1	0
297	1	1	1	3	100%	1	0	+	-	+	-	1	0
298	1	1	1	3	100%	1	0	+	-	+	-	1	0
299	1	1	1	3	100%	1	0	+	-	+	-	1	0
300	1	1	1	3	100%	1	0	+	-	+	-	1	0
301	1	1	1	3	100%	1	0	+	-	+	-	1	0
302	1	1	1	3	100%	1	0	+	-	+	-	1	0
303	1	1	1	3	100%	1	0	+	-	+	-	1	0
304	1	1	1	3	100%	1	0	+	-	+	-	1	0
305	1	1	1	3	100%	1	0	+	-	+	-	1	0
306	1	1	1	3	100%	1	0	+	-	+	-	1	0
307	1	1	1	3	100%	1	0	+	-	+	-	1	0
308	1	1	1	3	100%	1	0	+	-	+	-	1	0
309	1	1	1	3	100%	1	0	+	-	+	-	1	0
310	1	1	1	3	100%	1	0	+	-	+	-	1	0
311	1	1	1	3	100%	1	0	+	-	+	-	1	0

312	1	1	1	3	100%	1	0	+	-	+	-	1	0
313	1	1	1	3	100%	1	0	+	-	+	-	1	0
314	1	1	1	3	100%	1	0	+	-	+	-	1	0
315	1	1	1	3	100%	1	0	+	-	+	-	1	0
316	1	1	1	3	100%	1	0	+	-	+	-	1	0
317	1	1	1	3	100%	1	0	+	-	+	-	1	0
318	1	1	1	3	100%	1	0	+	-	+	-	1	0
319	1	1	1	3	100%	1	0	+	-	+	-	1	0
320	1	1	1	3	100%	1	0	+	-	+	-	1	0
321	1	1	1	3	100%	1	0	+	-	+	-	1	0
322	1	1	1	3	100%	1	0	+	-	+	-	1	0
323	1	1	1	3	100%	1	0	+	-	+	-	1	0
324	1	1	1	3	100%	1	0	+	-	+	-	1	0
325	1	1	1	3	100%	1	0	+	-	+	-	1	0
326	1	1	1	3	100%	1	0	+	-	+	-	1	0
327	1	1	1	3	100%	1	0	+	-	+	-	1	0
328	1	1	1	3	100%	1	0	+	-	+	-	1	0
329	1	1	1	3	100%	1	0	+	-	+	-	1	0
330	1	1	1	3	100%	1	0	+	-	+	-	1	0
331	1	1	1	3	100%	1	0	+	-	+	-	1	0
332	1	1	1	3	100%	1	0	+	-	+	-	1	0
333	1	1	1	3	100%	1	0	+	-	+	-	1	0
334	1	1	1	3	100%	1	0	+	-	+	-	1	0

335	1	1	1	3	100%	1	0	+	-	+	-	1	0
336	1	1	1	3	100%	1	0	+	-	+	-	1	0
337	1	1	1	3	100%	1	0	+	-	+	-	1	0
338	1	1	1	3	100%	1	0	+	-	+	-	1	0
339	1	1	1	3	100%	1	0	+	-	+	-	1	0
340	1	1	1	3	100%	1	0	+	-	+	-	1	0
341	1	1	1	3	100%	1	0	+	-	+	-	1	0
342	1	1	1	3	100%	1	0	+	-	+	-	1	0
343	1	1	1	3	100%	1	0	+	-	+	-	1	0
344	1	1	1	3	100%	1	0	+	-	+	-	1	0
345	1	1	1	3	100%	1	0	+	-	+	-	1	0
346	1	1	1	3	100%	1	0	+	-	+	-	1	0
347	1	1	1	3	100%	1	0	+	-	+	-	1	0
348	1	1	1	3	100%	1	0	+	-	+	-	1	0
349	1	1	1	3	100%	1	0	+	-	+	-	1	0
350	1	1	1	3	100%	1	0	+	-	+	-	1	0
351	1	1	1	3	100%	1	0	+	-	+	-	1	0
352	1	1	1	3	100%	1	0	+	-	+	-	1	0
353	1	1	1	3	100%	1	0	+	-	+	-	1	0
354	1	1	1	3	100%	1	0	+	-	+	-	1	0
355	1	1	1	3	100%	1	0	+	-	+	-	1	0
356	1	1	1	3	100%	1	0	+	-	+	-	1	0
357	1	1	1	3	100%	1	0	+	-	+	-	1	0

358	1	1	1	3	100%	1	0	+	-	+	-	1	0
359	1	1	1	3	100%	1	0	+	-	+	-	1	0
360	1	1	1	3	100%	1	0	+	-	+	-	1	0
361	1	1	1	3	100%	1	0	+	-	+	-	1	0
362	1	1	1	3	100%	1	0	+	-	+	-	1	0
363	1	1	1	3	100%	1	0	+	-	+	-	1	0
364	1	1	1	3	100%	1	0	+	-	+	-	1	0
365	1	1	1	3	100%	1	0	+	-	+	-	1	0
366	1	1	1	3	100%	1	0	+	-	+	-	1	0
367	1	1	1	3	100%	1	0	+	-	+	-	1	0
368	1	1	1	3	100%	1	0	+	-	+	-	1	0
369	1	1	1	3	100%	1	0	+	-	+	-	1	0
370	1	1	1	3	100%	1	0	+	-	+	-	1	0
371	1	1	1	3	100%	1	0	+	-	+	-	1	0
372	1	1	1	3	100%	1	0	+	-	+	-	1	0
373	1	1	1	3	100%	1	0	+	-	+	-	1	0
374	1	1	1	3	100%	1	0	+	-	+	-	1	0
375	1	1	1	3	100%	1	0	+	-	+	-	1	0
376	1	1	1	3	100%	1	0	+	-	+	-	1	0
377	1	1	1	3	100%	1	0	+	-	+	-	1	0
378	1	1	1	3	100%	1	0	+	-	+	-	1	0
379	1	1	1	3	100%	1	0	+	-	+	-	1	0
380	1	1	1	3	100%	1	0	+	-	+	-	1	0

381	1	1	1	3	100%	1	0	+	-	+	-	1	0
382	1	1	1	3	100%	1	0	+	-	+	-	1	0
383	1	1	1	3	100%	1	0	+	-	+	-	1	0
384	1	1	1	3	1005	1	0	-	+	+	-	0	1
	372	384	384			372	12						
	384	384	384			384	384	384		384	384	384	384
	HAIN	GE	DM		Average agreement	3 agree	2 agree						
	97%	100.0%	100.0%		99%	97%	3%						