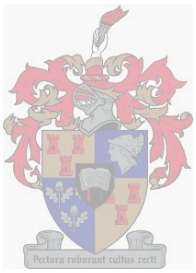
IMPROVING LABORATORY DIAGNOSTIC TECHNIQUES TO DETECT *M. TUBERCULOSIS* COMPLEX AND *C. NEOFORMANS* AS THE CAUSITIVE AGENTS OF CHRONIC MENINGITIS IN THE CEREBROSPINAL FLUID OF ADULT PATIENTS.

Yvonne Prince



Thesis presented in partial fulfillment of the requirements for the degree of Masters of Medical Sciences in Medical Microbiology at Stellenbosch University

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March 2010

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained is my own, original work, that I am the authorship owner therefore and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

INTRODUCTION

Mycobacterium tuberculosis (MTB) and *Cryptococcus neoformans* are the most common causes of chronic meningitis in South Africa. Conventional microbiology has limited utility in diagnosing these pathogens due to the paucibacillary nature of cerebrospinal fluid (CSF) and the diagnostic delay associated with culturing methods. This study aimed to evaluate the utility of an in-house polymerase chain reaction (PCR) method for the detection of the etiological agent of chronic meningitis.

METHODS

CSF samples (where volume exceeded 5ml) were submitted to the Medical Microbiology diagnostic laboratory of the Tygerberg Hospital from patients with suspected tuberculosis meningitis (TBM). Following routine bacteriology, the sample was used to inoculate two mycobacterial growth indicator tubes (MGIT A and B) and subsequently incubated in the BACTEC 960 automated system. MGIT A followed standard operating procedures and the time to culture positivity was noted.

Weekly aliquots (up to 6 weeks) were removed from MGIT B. These samples were boiled to inactivate the bacteria and then the DNA was extracted using the Promega Wizard SV Genomic DNA kit. The DNA was then speciated by PCR and high-resolution melting analysis (HRM) by using primers specific to either the RD9 region of MTB complex or primers specific to the partial internal transcribed spacer 1 (ITS1), 5.8S rRNA gene and partial ITS2 sequence of *C. neoformans*.

RESULTS

Routine CSF microscopy indicated that 14 of the 78 patients (17.9%) had typical CSF findings of TBM (lymphocytes predominant, increased protein levels and decreased glucose levels).

Ziehl-Neelsen (ZN) stains were positive for 12 (15.4%) samples, and MTB was cultured from 19 samples (24.4%). Our optimized PCR and HRM method was able to detect *M. tuberculosis* in 17 of the 19 culture positive specimens with a sensitivity of 89.5% and a specificity of 62.7%. The sensitivity of this method was higher than that of direct microscopy. In all of the PCR positive samples, the time to detection, compared to culture, could be shortened by 1 to 2 weeks.

Only one sample was positive for Cryptococcus culture and another sample was positive with a Cryptococcus latex test. PCR for Cryptococcus was positive in 2 cases (n=78), sensitivities and specificities could not be reported due to the low number of positive cases.

CONCLUSION

We demonstrated that a short culture period and the use of commercial DNA extraction kit on CSF samples increases the sensitivity of molecular tests to diagnose tuberculosis. Furthermore, the molecular techniques could significantly reduce the time to positivity of results, when compared to culture. Due to the low occurrence of Cryptococcus in the samples included in our study, we could not comment on the diagnostic utility of PCR in the diagnosis of Cryptococcal meningitis, when compared to the conventional methods.

ABSTRAK

INLEIDING

Mycobacterium tuberculosis (MTB) en *Cryptococcus neoformans* is die mees algemeenste oorsake van kroniese meningitis in Suid-Afrika. Routine mikroskopie dra beperkte waarde in die diagnose van hierdie patogene as gevolg van die klein hoeveelhede organismes wat in die SSV (serobrospinale vog) voorkom en die lang tyd wat dit benodig om hierdie organisms te kweek. Hierdie studie beoog om die diagnostiese waarde van ‘n polymerase ketting reaksie (PKR) metode wat intern ontwerp is te evalueer vir die identifikasie van patogene verantwoordelik vir kroniese meningitis.

METODES

SSV monsters (waarvan die volume 5ml oorskry) en waar daar ‘n kliniese vermoede van tuberkulose meningitis (TBM) was, is na die diagnostiese Mediese Mikrobiologie laboratorium van Tygerberg hospitaal gestuur vir roetine bakteriologiese ontleding. Die oorblywende monsters is gebruik om twee mikobakteriële groei-indikasiebuise (MGIT A en B) te innokuleer en hulle is geïnkubeer in ‘n BACTEC 960 geautomatiseerde sisteem. MGIT A is volgens roetine diagnostiese metodes geanaliseer en die tyd tot ‘n positiewe resultaat is aangeteken

Weeklikse monsters (tot en met week 6) is uit MGIT B verwyder en die monsters is gekook om sodoende die bakterië te inaktiveer. Die Promega Wizard SV Genomiese DNS ekstraksiemetode is gebruik om die DNS te versuiwer. Spesiëring van die DNS is deur middel van ‘n intern ontwerpte PKR en hoëresolusiesmeltingsmetode (HRS) gedoen met inleiers wat spesifiek is tot die RD9 gedeelte van die MTB kompleks en inleiers spesifiek tot die gedeeltelike interne getranskribeerde spasieerder 1 (ITS1), 5.8S rRNS geen en die gedeeltelike ITS2 DNS volgorde van *C. neoformans*.

RESULTATE

Roetine SSV mikroskopie het aangedui dat 14 uit 78 (17.9%) pasiënte tipiese SSV bevindings van TBM (oorwegend limfosiete, verhoogde proteïene en verlaagde glukose) gehad het. Ziehl-Neelsen (ZN) kleurings was positief vir 12 (15.4%) monsters, en MTB is gekweek in 19 (24.4%) van hierdie monsters. Ons geoptimaliseerde PKR en HRS metode het daarin geslaag om *M. tuberculosis* in 17 van die 19 kultuurpositiewe monsters aan te toon met ‘n sensitiviteit van 89.5% en ‘n spesifisitiet van 62.7%. Die sensitiwiteit van die direkte PKR was hoër in vergelyking met mikroskopie. In al die PKR positiewe monsters was die tyd tot aantoning, in vergelyking met kultuur, verkort met 1 tot 2 weke.

Slegs een monster het *C. neoformans* gekweek en ‘n ander monster was positief met die kriptokokkale latekstoets. PKR vir *C. neoformans* was positief in 2 gevalle (n=78). Die sensitiwiteit en spesifisiteit van die *C. neoformans* PKR kon nie bepaal word nie weens te min gevalle.

GEVOLGTREKKINGS

Ons het aangetoon dat ‘n verkorte inkubasieperiode en die gebruik van ‘n kommersiële DNS ekstraksiemetode op SSV monsters die sensitiwiteit van die molekulêre tegniek vir die diagnose van tuberkulose verhoog en dat hierdie metode die tyd na positiwiteit aansienlik verkort in vergelyking met kultuur. Weens die lae getalle van kriptokokkale meningitis in ons studie kon ons nie kommentaar lewer op die akkuraatheid van PKR in die diagnose van kriptokokkale meningitis, in vergelyking met meer konvensionele metodes, nie.

DEDICATION

To my departed **mother,** and **family,** for their love and support. My sister, **Henrietta Polman** for her endless support and love. And last but not least my **husband** and **daughters** for their patience and love.

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Finally, I would like to thank the **NHLS** for their funding of the project.

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LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
ART	Anti retroviral therapy
BLS	Biosafety level
BSC	Biological Safety cabinets
CFU	Colony forming units
CI	Confidence interval
CLAT	Cryptococcus latex antigen test
CM	Cryptococcal meningitis
CNS	Central Nervous systems
CO_2	Carbon dioxide concentration
CPT	Cotrimoxazole prevention therapy
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acids
DNTPs	deoxynucleotide triphoshate
GSH	Grootte Schuur Hospital
MGIT	Mycobacterium Growth Indicator tubes
MOTTS	Mycobacteria other than tuberculosis
NALC	Sodium Hydroxide-N-Acetyl-L-Cysteine
NaOH	Sodium hydroxide

NATs	Nucleic acid amplification test
NHLS	National Health Laboratory service
NICD	National Institute for Communal Diseases
NPV	Negative predictive value
NTM-	Nontuberculous mycobacteria
OADC	Oleic acid, albumin, dextrose, catalase
PANTA	Polymixin A, Amphotericin B, Naladixic Acid, Trimethoprim , Azocillin
PCR	Polymerase chain reaction
PDV	Positive predictive value
PPD	Purified protein derivative
rRNA	ribosomal Ribonucleic acid
RT-PCR	Reverse Transcription PCR
SOP	Standard Operating Procedures
Taq	Thermus aquaticus
TB	Tuberculosis
TBH	Tygerberg Hospital
TBM	Tuberculosis meningitis
TBM	Tuberculosis meningitis
WHO	World Health Organization
ZN	Ziehl-Neelsen

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CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 *Mycobacterium tuberculosis* and *Cryptococcus neoformans* as agents of chronic meningitis

Mycobacterium tuberculosis and *Cryptococcus neoformans* are the two most common causes of chronic meningitis encountered in the South African population (Karstaedt, Valtchanova et al. 1998). While the incidence rate of tuberculous meningitis (TBM) is gradually decreasing in most regions, it is still increasing in countries hardest hit by the HIV-epidemic, particularly in sub-Saharan Africa.

In the laboratory, it may be difficult to distinguish between these two pathogens because both may present with similar findings in the assessment of cell counts, protein and glucose levels in cerebrospinal fluid. The fast and accurate laboratory diagnosis of the cause of chronic meningitis is of the utmost importance in order to direct therapy, especially in immune compromised patients, where this type of infection is both common and life-threatening (Havlir and Barnes 1999).

Despite new diagnostic techniques and treatment regimens, mortality and morbidity associated with chronic meningitis remains high. Delay in diagnosis and treatment is directly related to poor patient outcome. As the number of mycobacteria in cerebrospinal fluid is extremely low and direct detection is therefore difficult and not sufficiently sensitive, the current gold standard for the diagnosis of tuberculosis remains the isolation of tubercle bacilli from cerebral spinal fluid (CSF) by culture (Thwaites, Chau et al. 2002). However, there is an urgent need for more

rapid diagnostic techniques as culture can take up to 8 weeks. Rapid methods for the diagnosis of Cryptococcus include Indian ink staining, detection of capsular antigen, and recently, polymerase chain reaction (PCR) (Bauters, Swinne et al. 2003).

1.2 Literature review of Mycobacterium tuberculosis

1.2.1 The organism

Tuberculosis is an ancient disease. It was endemic in animals in the Paleolithic period, long before it ever affected humans. The Mycobacteria, the causative agents of tuberculosis, are classified into 3 main groups: *M tuberculosis* complex, *M leprae* and Nontuberculous mycobacteria (NTM). The most important mycobacterium species that occur in humans belong to the *M. tuberculosis*-complex. These include *M. tuberculosis, M. bovis, M. bovis BCG*, *M africanum*, *M canetii,* and *M microtii*, all of which are capable of causing tuberculosis.

Mycobacteria are aerobic rods with gram-positive cell walls which are impermeable to the dyes used in Gram's method because of an abundance of the long chain of fatty acids. They are resistant to decolourisation and demonstrate acid fastness by mineral acid and alcohol (Ziehl-Neelsen stain).

Nontuberculous mycobacteria other than the *M. tuberculosis* complex occur widely in the environment. There are more than 110 species known, many of which can cause disease in humans under specific circumstances. These organisms are often referred to as NTM or mycobacteria other than tuberculosis (MOTTS). Runyon divided this group into rapid growers

(visible colonies form within 3-7 days on solid media) and slow growers (visible colonies form only after 2 weeks to 2 months incubation).

Mycobacterium leprae is the bacterium that causes leprosy (also known as Hansen's disease). The organism has never been successfully grown on artificial cell culture media. Instead it has been grown in mouse foot pads and more recently in nine-banded armadillos, because they are, like humans, susceptible to leprosy (Greenwood, Slack. et al. 2002, 210-213pg).

1.2.2 Epidemiology

Today, tuberculosis is still one of the leading infectious diseases in the world and is responsible for more than two million deaths and eight million new cases annually (Palomino 2005). The World Health Organization (WHO) estimated, for the year 2002, a global incidence of 8.8 million new cases, including 3.9-million smear positive subjects. Twenty-two high-burden countries concentrated 80% of these cases. The HIV pandemic worsens this situation, since it is estimated that 12 million patients are co-infected with HIV and *Mycobacterium tuberculosis* as of 2000, with the majority of cases living in sub- Saharan Africa and Southeast Asia. The risk of death in an HIV-infected patient with TB is twice that of an HIV-infected patient without TB (Palomino 2005).

An HIV- infected person is prone to infection due to virus replication that depletes the immune system and decreases CD4 counts. At a lower immunity even micro-organisms normally considered to be non-invasive or commensals become invasive pathogens with the capability to cause not only morbidity but also mortality.

1.2.3 Pathogenesis of tuberculosis

The most common route of infection of tuberculosis is by inhalation. Air borne droplets containing tubercle bacilli reach the terminal air spaces normally at the pleural and in the mid-lung zone (the lower parts of the upper lobes and the upper parts of the lower and middle lobes) where multiplication begins. The initial focus of infection is typically single, although multiple foci may be present in about one fourth of cases (Mandell 2005). The alveolar macrophages ingest the bacteria, and may be able to eliminate small numbers of bacilli. Bacterial multiplication destroys the macrophages. Blood-borne lymphocytes and monocytes are attracted to this focus and the infected macrophages are carried by the lymphatic system to regional lymph nodes. In the immune compromised host these cells are not effectively contained and may spread hematogenously throughout the body. Primary infection usually appears three to eight weeks after inhalation and in most cases in the immune competent host the infection is controlled, with the only evidence of infection being a positive skin test result (Mandell 2005).

1.2.4 Pathogenesis of tuberculosis meningitis

The development of TBM is a two- step process. *M. tuberculosis* enters the host by droplet inhalation, the initial point of infection being the alveolar macrophages. Localized infection escalates within the lungs, with dissemination to the regional lymph nodes to produce a primary complex. In persons who develop TBM, bacilli seed to the meninges or brain parenchyma, resulting in the formation of small or subendymal foci of metastatic caseous lesions (Ramachandran 2008). Immunocompetent individuals infected with *M. tuberculosis* have approximately a 10% lifetime risk of developing tuberculosis (Brock, Weldingh et al. 2004).

Many of the symptoms, signs and progression of TBM are the result of an immunological inflammatory reaction to the infection.

1.2.4.1 Clinical presentation

As the level of immune-suppression increases in HIV- infected patients, extra pulmonary tuberculosis and mycobacteremia become progressively common. When pyrexia occurs in HIV infected patients, diagnostic studies for extra pulmonary tuberculosis are often undertaken. HIV infected persons usually present with abdominal tuberculosis which is characterized by visceral lesions and intra abdominal lymphadenopathy with necrosis, whereas in HIV negative patients, ascites and omental thickening are observed.

The clinical presentation of tuberculous meningitis is similar in HIV- positive patients and in immuno-competent patients, except that an intracerebral mass lesion is more common in HIV positive patients. The radiographic findings in HIV positive patients with tuberculosis with a CD4 cell count of 200 or more is similar to those in HIV- negative patients with tuberculosis. Mediastinal adenopathy is common in both HIV-negative children with primary tuberculosis, and in HIV positive patients with a CD4 count of less than 200. Despite a normal chest radiograph an estimated 5 percent of HIV positive patients with pulmonary tuberculosis have a positive acid fast staining on sputum.

The degree of immuno- suppression is the most important predictor of survival in HIV infected patients with tuberculosis since low CD4 cell counts and opportunistic infections and negative skin test are all associated with increased mortality (Ramachandran 2008).

1.2.5 Clinical diagnosis of tuberculosis vs. tuberculous meningitis

1.2.5.1 Clinical diagnosis of TB

According to (Escalante 2009), there are four traditional steps in the diagnosis of TB disease**: Medical history, tuberculin skin test, chest x-rays, and bacteriological examination.**

The **medical history** includes asking patients whether they have been exposed to a person with TB; whether they are experiencing any symptoms of the disease; if they have had TB before; or other risk factors for developing TB.

The symptoms for pulmonary TB include:

- Coughing
- Pain in chest when coughing or breathing
- Coughing up blood

The general symptoms of TB (pulmonary or extra pulmonary) include:

- Weight loss
- Fatigue
- Malaise
- Fever
- Night sweats

The symptoms of extra pulmonary TB depend on the part of the body that is affected by the disease.

Patients with symptoms of TB may be given a **tuberculin skin test**. They should be evaluated for TB, regardless of their skin test results.

The **chest x-ray** is used to rule out the possibility of pulmonary TB in a person who has a positive reaction to the tuberculin skin test, and check for lung abnormalities in people who have symptoms of TB disease. However, the results cannot confirm that a person has TB.

The fourth step towards definite diagnosis is a **bacteriological examination**. A sputum specimen is obtained from a patient suspected of having pulmonary TB disease. The specimen is examined under a microscope for the presence of acid- fast bacilli (AFB). When AFB are seen, they are counted. Patients with positive smears are considered infectious. The specimen is then cultured, or cultured to determine whether it is indeed *M. tuberculosis*. A positive culture for *M. tuberculosis* confirms the diagnosis of TB.

1.2.5.2 The diagnosis of TBM

The clinical diagnosis of TBM is complicated as it causes various clinical manifestations, which overlap with those of other chronic diseases of the central nervous system (CNS) such as viral and pyogenic meningitis (Kashyap, Kainthla et al. 2006). Because untreated TBM is almost always fatal, it is essential that diagnostic testing is sensitive enough to pick up the low number of organisms present in the CSF. Diagnosis must also be rapid, because poor outcome is strongly associated with delayed treatment (Thwaites 2006). Features that have been found to be predictive in the diagnosis of tuberculosis meningitis are age, length of history, blood white cell count, total CSF white cell count and CSF neutrophil percentage. The diagnosis of meningitis should also be considered when the patient presents with headache, pyrexia, nausea and vomiting, neck stiffness, confusion, seizures, abnormal behavior, new onset of

psychiatric symptoms, neurological signs and unexplained blindness or coma (Thwaites, Chau et al. 2002).

1.2.6 Microbiological and immunological diagnostic methods

1.2.6.1 Direct examination for acid fast bacilli

Ever since Robert Koch first saw the tubercle bacilli in 1882, the search for acid-alcohol fast bacilli in clinical specimens has remained the cornerstone of diagnosis. Although the conventional technique of direct smear examination with Ziehl-Neelsen (ZN) staining is cheap and easy to perform, its low sensitivity is a major drawback (Thwaites 2006). Greater than 10^4 bacilli per ml are required for reliable detection (Palomino 2005). Therefore the demonstration of AFB in CSF requires diligence and time if excellent results are to be achieved (Thwaites 2006). As far as the diagnosis of tuberculous meningitis is concerned, the sensitivity of a direct smear depends on the volume of CSF: 10-20 ml is considered optimal (Thwaites 2006). Factors such as specimen type, staining method, and culture method can influence the sensitivity of an acid- fast smear. In general the overall sensitivity of an acid fast smear ranges from 20% to 80%. Increasing the volume of CSF taken, centrifuging the CSF and careful examination of a smear made from the deposit, increases the chance of detecting acid-fast bacilli (Katti 2004). Due to the low bacillary load in CSF, the practice is to do cultures rather than to do smears as experience has shown that performing a smear may result in the loss of bacilli required for subsequent culture.

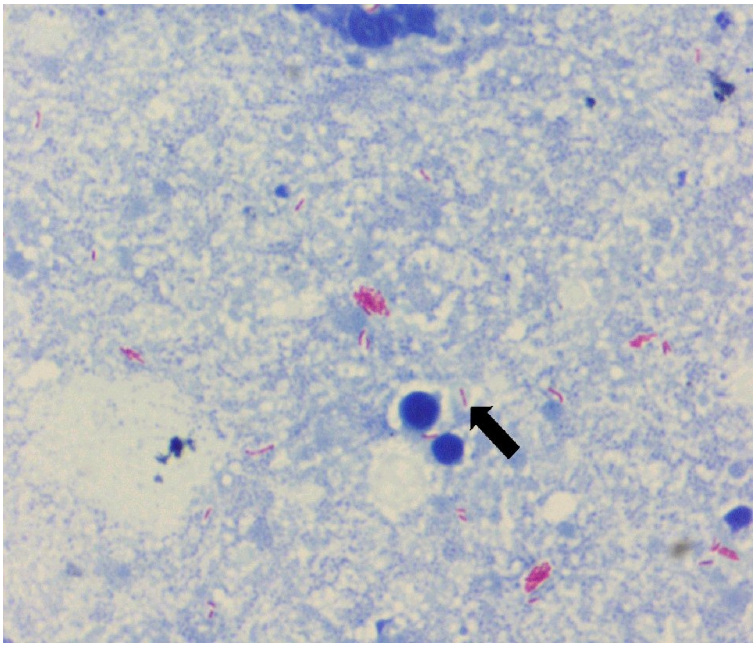

Figure 1.1 **Ziehl-Neelsen** stain displaying the red acid-fast bacilli, against a blue background using a methylene blue counter stain (magnification, 100X). (Photograph taken at Tygerberg Hospital).

An alternative staining technique is the fluorescence Auramine-O stain. An Auramine-O smear can be examined under a lower magnification than the ZN stain but a special fluorescence microscope is needed, as well as a well-trained technologist (Lafras, 1995).

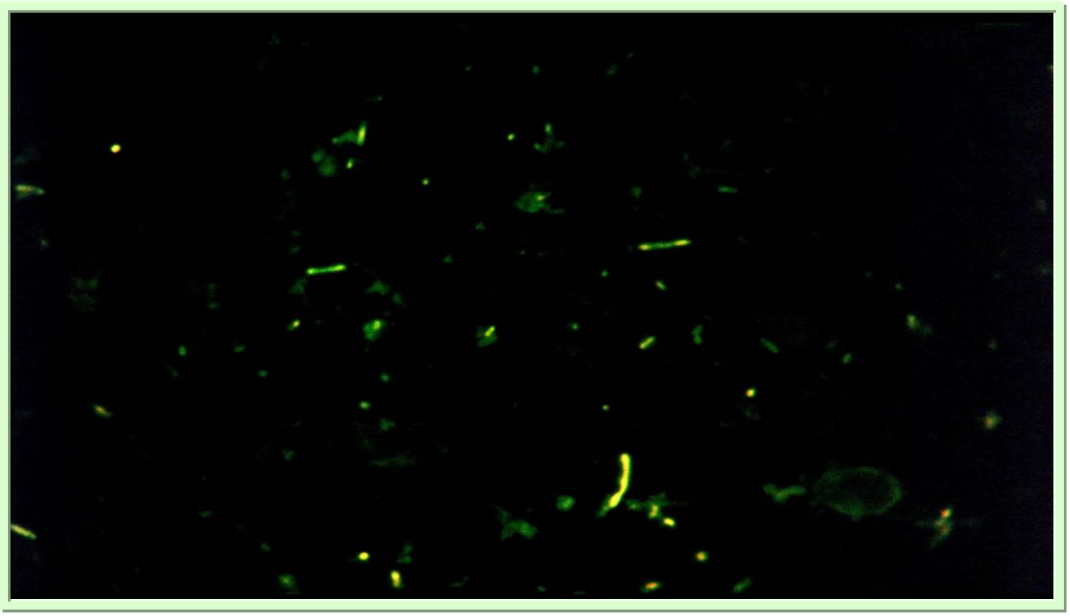

Figure 1.2 Auramine-O fluorescent stain prepared from a paediatric sputum sample displaying bright yellow fluorescent acid-fast bacilli against a dark background. (Magnification 40X).

The use of Light- Emitting Diode Fluorescence (LED) Microscopy was recently introduced. In addition to an increase in the sensitivity, it also facilitates a quicker scanning of each field because of increased visibility of the mycobacteria, and the decreased magnification used during fluorescence microscopy as compared to the light microscope. It is reported to be quicker to read a smear with fluorescence microscopy (1.4 minutes compared to 3.6 minutes with light microscopy), reflecting a time saving of 61% with a fluorescence microscope (Marais, Brittle et al. 2008).

1.2.6.2 CSF culture for Tuberculosis

Although culture of CSF for *M. tuberculosis* is the diagnostic "gold standard" for TBM, it may take up to 42 days to yield results. The factors that govern sensitivity are the same as for direct microscopy. Culture of tuberculosis can be accomplished in different ways, using different media types. *M. tuberculosis* can be cultured on solid media in which case the isolation of the organism from clinical specimens can take up to 6-8 weeks. In contrast, culture from liquid medium leads to a more rapid detection of the organism (1-2 weeks), and as few as 10 viable organisms can be detected (Greenwood, Slack. et al. 2002, 210-213pg)

Currently the BACTEC MGIT (Mycobacterium Growth Index Tube) 960 automated system is used by most microbiology laboratories to optimize the culture of mycobacteria. This system is based on a glass tube containing modified Middlebrook 7H9 broth together with a fluorescence quenching based oxygen sensor embedded at the bottom of the tube. When inoculated with a sample containing mycobacteria, consumption of the dissolved oxygen by growing bacteria produces fluorescence, which is detected by a UV light (Palomino 2005).

However, recovery of mycobacterium from the MGIT system depends on the number of organisms present in the sample (10-100 viable organisms is required), sample collection technique and processing of the (De Wit, Steyn et al. 1190).

1.2.6.3 Immune based methods

Several immune-based diagnostic tests are commercially available. QuantiFERON-TB Gold assay (Cellestis Limited, Carnegie, Victoria, Australia) detects the in vitro cell mediated immune

response to *M. tuberculosis* infection by measuring the release of interferon gamma assay (IFN-γ) in whole blood incubated with *M. tuberculosis* purified protein derivative (PPD). An enzyme-linked immuno sorbent assay (ELISA) detects the amount of INF-γ produced. Despite the long history of serological test and persistent attempts to improve them, no assay is currently accurate to replace microscopy and culture (Pai, Kalantri et al. 2006).

The T-SPOT (Oxford Immuno tec) is an in vitro diagnostic assay measuring T-cell reaction to *M. tuberculosis*-specific antigens (Taggart, Hill et al. 2004).

These assays have been recommended by the Centers of Disease Control to be used in the detection of TB in children, but little evidence has been provided to support these assays because the pediatric studies that have been performed were small and inconsistent and inadequate to clinically recommend these assays at this time. These assays fail to make the crucial distinction between latent tuberculosis infection and active disease (Marais and Pai 2007).

1.2.7 Molecular methods

1.2.7.1 Polymerase chain reaction (PCR)

Polymerase chain reaction **(**PCR) is an method first conceived in the 1980's by Kary Mullis (Wikipedia 2008). Today, PCR is a technique widely used in molecular biology. It derives it name from one of its key components, a DNA polymerase used to amplify a piece of DNA by in vitro enzymatic replication.

PCR make use of two 15-30 base oligonucleotides primers that are complementary to nucleic acid sequences that flank the region of interest in the target DNA. These primers are included in the reaction mixture containing the target nucleic acid, a heat stable DNA polymerase, a defined solution of salts often containing magnesium, such as MgCl and $MgSO_4$, and excess amounts of each of the four deoxynucleotiside triphoshate (dNTPs). The total reaction mix is placed in a thermocycler. The enzyme derived from the hemophilic bacterium *Thermus aquaticus* "Taq" remains stable under high temperatures, even after the DNA denatures. The mixture is subjected to repeated cycles of defined temperature change, these thermal changes facilitates the denaturing (94-97℃) of the template DNA, annealing, and the extension (72℃) of the primers so that the target DNA sequences is replicated.

Denaturation causes the double stranded helix to separate by disrupting the hydrogen bonds between complementary bases of DNA strands, resulting in 2 single stranded DNA templates. During the annealing phase, the forward and reverse primers anneal to their complementary sequence on the template DNA. The primers are elongated by the DNA polymerase using the dNTPs, and the time needed for elongation is dependant on the target sequence and sequence length. During the next heating cycle, the stands separate and the original DNA and the newly DNA strand serves as a template for another round of DNA replication, thus the number of

target DNA strands double with each cycle. Amplification is logarithmic leading to the easy detection of the amplicon.

PCR is now a well-developed technique and is most widely used as an alternative rapid diagnostic technique. Although PCR was previously found to be superior to the currently available techniques for the diagnosis of TBM in terms of sensitivity, specificity and rapidity, one of its limitations is that PCR only indicates the presence of DNA, and not viable bacteria (Desai 2002; Katti 2004).

PCR methods

A variety of PCR methods have been developed for the detection of specific sequences of *M. tuberculosis* and other mycobacteria. These PCR assays may target either DNA or rRNA.

- **Reverse Transcription PCR** (RT-PCR) is a method used to amplify, isolate or identify a known sequence from cellular or tissue RNA. The reaction is preceded by a reaction using reverse transcriptase to convert RNA to DNA. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites and, when the genomic DNA sequences of a gene is known, to map the location of exons and introns in the gene.

- **Quantitative real- time PCR (**also known as real time PCR). This uses fluorescent dyes such as Syber Green, or flourophore- containing DNA probes, such as Tag man, to measure the amount of amplified product in 'real time', i.e. as the reaction occurs, and not as a single measurement of an end-product.

- **Multiplex-PCR**: multiple primers sets are used within a single PCR mix to produce amplicons of varying sizes specific to different DNA sequences. This can be used to detect, for example, more than one pathogen present in a single specimen.

- **Nested PCR**: increases the specificity of DNA amplification by reducing background due to non specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non- specifically amplified DNA fragments. The products are then used in a second PCR with a set of primers whose binding sites are complementary to sequence within the amplicon. Nested PCR is often more successful specifically amplifying long DNA fragments than conventional PCR but requires more detailed knowledge of the target sequences.

Contamination of PCR tests is a problem but can be reduced with appropriate laboratory design, strict discipline about collection and processing of specimens, handling of reagents and use of certain blocking reagents (Katoch 2004).

1.2.7.2 Commercially available kits

Nucleic acid amplification test (NAATs) also known as 'direct amplification test' is designed to amplify nucleic acid regions specific to the *Mycobacterium tuberculosis* complex. The specificity and sensitivity of NAATs is lower and highly variable across studies (Pai, Kalantri et al. 2006). The sensitivity of NAATs has shown to be higher in smear positive specimens than in smear negative specimens. (Pai, Kalantri et al. 2006).

1.2.7.3 DNA Probe assays

Several investigators have developed DNA probes for the detection of *M. tuberculosis* and one of these DNA probe tests that are commercially available is the Gen- Probe assay (San Diego, Calif.) that is used for the detection of *M. tuberculosis complex*. However, the sensitivity of these tests on direct specimens are similar to that of the ZN stain (De Wit, Steyn et al. 1990). The lack of sensitivity of DNA probes was the motivation, in part, for the development of the PCR.

Line probe assays are a family of novel DNA strips tests that uses PCR and reverse hybridization methods for the rapid detection of mutations associated with drug resistance (Karstaedt, Valtchanova et al. 1998). Commercial line probes assays include the Geno type MTBDR assay (Hain life-science GmbH, Nehren, Germany) (Pai, Kalantri et al. 2006). In general, line probe assays are expensive and require sophisticated laboratory infrastructures (Pai, Kalantri et al. 2006).

1.3 LITERATURE REVIEW OF CRYPTOCOCCUS NEOFORMANS

1.3.1 The organism

Cryptococcus neoformans is an encapsulated yeast that is present in the environment worldwide and can cause disease in both immunecompetent and immune compromised hosts. Patients with a T- cell deficiency are most susceptible. *C. neoformans* var. *neoformans* is the strain that causes most cryptococcal infections in humans. The basidiospores (sexual spores) are approximately 1.8µm to 3µm in diameter and result from crosses of the α-end, and a-

mating types on appropriate medium. *C. neoformans* has a number of virulence factors that can contribute to progression of the disease. These virulence factors include the capsule, cryptococcal products, melanin production, mannitol production, and proteases, amongst others. The polysaccharide capsule and the soluble extra cellular constituents of *C. neoformans* are the dominant virulence factors. The capsules of *C. neoformans* are primarily composed of high molecular weight polysaccharides that have a backbone of α 1, 3- D mannopyranose units with single residues of β- D- glucoronopyranosyl attached. This polysaccharide is referred to as glucuronxylomannan and has four serotypes: A and D produced by *C. neoformans* var. *neoformans*, and B and C produced by *C. neoformans* var. *gattii.* It has been indicated that the virulence factor of *C. neoformans* lies in its capsule where as a capsular mutants are typically avirulent. *C. neoformans* var.*gattii* has never been cultured from bird droppings but has been cultured from vegetation around and associated with the river red trees (*Eucalyptus camaldulensis*), and forest red gum trees (*E. teriticornis*) in Australia and in other parts of the world (Kent, Buchanan et al. 1998).

1.3.2 Epidemiology

C. neoformans is not part of the normal microbial flora of humans. Their main habitats include debris around pigeon roosts and soil contaminated with decaying pigeon or chicken droppings. The organism causes disease in immune competent, as well as immune compromised, hosts. The yeast enters the host by the respiratory route in the form of dehydrated haploid yeast or as basidiospores. After some time in the lungs, the organism spreads to extra pulmonary tissues, and, since it has a predilection for the brain, infected persons usually contract meningoencephalitis (Kent, Buchanan et al. 1998). The vast majority of patients with symptomatic disseminated cryptococcosis have an identified underlying immune compromised condition. The most common underlying conditions worldwide include the acquired immunodeficiency syndrome (AIDS), prolonged treatment with corticosteriods, and organ transplantation. In less well-developed countries with major epidemics of HIV, such as sub-

Saharan Africa, cryptococcal disease appears to reach very high prevalence (Bicanic and Harrison 2004).

C. neoformans is the leading cause of meningitis in central and southern Africa, accounting for 26.5% of cases in a series from Malawi, 31% in a series from the Central African Republic and 45% from Zimbabwe (Jarvis and Harrison 2007). Access to antifungal therapy is limited in developing countries. Whereas in sub- Saharan Africa reported mortality rates with antifungal treatment are very high, median survival time of 19 days in a Zambian study and 64% in-hospital mortality in a South African study (Jarvis and Harrison 2007) have been reported.

1.3.3 Pathogenesis

The primary route of infection, as well as the pathogenesis of cryptococcal disease, has certain similarities with tuberculosis.

Invasive fungal infections have become major causes of morbidity and mortality among immune compromised patients including individuals with AIDS. The pathogenesis of cryptococosis is determined by three factors: (1) the status of the host's defenses, (2) the virulence of the strain of *C. neoformans*, and (3) the size of the inoculum. In the alveoli, the yeast makes contact with the alveolar macrophages, which recruit other inflammatory cells through cytokines and a proper Th1 response with granulomatous inflammation is elicited. The infection can follow one of three pathways:

1. In an immune suppressed host, the yeast continues to proliferate and disseminate, causing clinical disease.
2. The effective immune response completely eliminates the yeast from the host.

3. The yeast produces a small lung/lymph-node complex and remains dormant in the tissues. The infection is clinically asymptomatic until loss of local immunity occurs, for example corticosteriod use or progression of an HIV infection. The yeast may then begin to replicate in the pulmonary lymph-node complex and eventually disseminate to the organs outside the lung.

The two most common sites of infection with encapsulated yeast are the lung and central nervous system (CNS) (Mandell 2005, pg 2999-3002).

1.3.4 Clinical presentation

The main clinical feature of AIDS is the occurrence of opportunistic infections in individuals that are known to be immune deficient. Patients with T cell defects and patients subjected to chemotherapy are also susceptible to opportunistic pathogens. In central nervous system invasion by *C. neoformans*, symptoms are common to those seen in other forms of chronic meningitis. Lymphadenopathy, night sweats, fever and weight loss have also been noted (Rosen 1985).

1.3.5 Clinical diagnosis

The clinical diagnosis of cryptococcal meningitis is similar to the clinical diagnosis of other forms of chronic meningitis, and relies on the laboratory for the diagnosis of a specific ethological agent.

1.3.6 Microbiological diagnostic methods

There are four approaches to the laboratory diagnosis of fungal disease:

- Direct microscopy
- Culture of organism
- Serological testing
- DNA probe test

1.3.6.1 Direct detection

Microscopy can be performed quickly, but accuracy depends on the experience of the microbiologist and quality of equipment (Levins 2006).

Samples sent to the laboratory for direct microscopy include: sputum, lung biopsy and skin scrapings. Rapid methods include Indian ink stain technique and examination by light microscopy, which is a time-honored approach for the detection of the encapsulated yeast cells of *C. neoformans*. Some diagnostically important findings including the *C. neoformans* capsule are seen by the India ink preparation (Levins 2006).

However, these test are not as sensitive as the Cryptococcal latex test, and the specificity is also limited because leucocytes can mimic the encapsulated yeasts (Levins 2006).

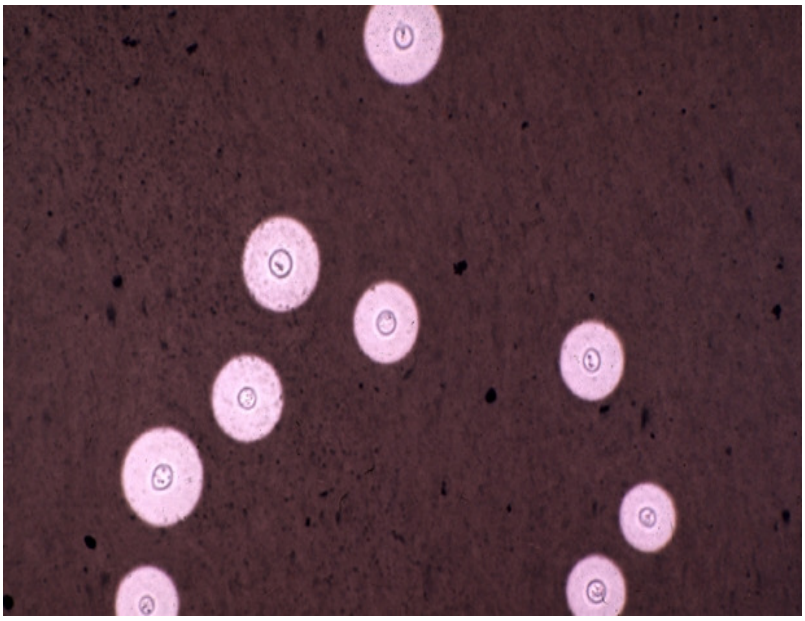

Figure 1.3 India Ink Stain illustrating Encapsulated yeast (photograph taken at Tygerberg NHLS laboratories).

Detection of the polysaccharide antigen of *C. neoformans* by antibody–coated latex particles agglutination has become an important adjunct in the diagnosis of Cryptococcus. The antigen test is fast and rapid, but false-positive reactions and false negative latex reactions can occur, due to factors like the presence of rheumatoid factors in the specimens, which may lead to false positive reactions, and the prozone phenomenon which leads to false negative result (Currie, Freundlich et al. 1993). Other factors that may lead to false-positive Cryptococcal Antigen Agglutination include disinfectants and soaps used to clean the glass slide on which the test is performed (Blevins, Fenn et al. 1995). Capsule deficient *C. neoformans* may also present difficulties in the direct detection of this pathogen because the latex agglutination test for cryptococcal antigen detects solute capsular polysaccharide antigens with the antigens fixed to the latex particles (Sugiura, Homma et al. 2005).

1.3.6.2 CSF Culture methods for Cryptococcus

Diagnosis is confirmed by culture of the organism, which is considered to be the gold standard of laboratory diagnosis. Culture can be time consuming because the yeast can take between 5 to 10 days to grow and the sensitivity of culture depends on the organism load present in the sample. The total organism load in a specimen may be determined by the volume of the specimen taken and the number of organisms/ml. The number of organisms/ml can be influenced by the viability of the immune system, and by treatment.

The ability of *Cryptococcus spp.* to produce melanin in media containing phenol compound is widely used for identification of the organism. Melanin production is evaluated on the basis of colony pigmentation (Wang, Aisen et al. 1995).

An agar medium containing inositol, urea and Caffeic acid for selective growth of *C. neoformans* has been developed (Difco). This shows dark brown pigment after 36 hours incubation. The media itself often manifests a light to medium brown color after colonies become pigmented, but as it was observed that *Rodotorula* species also change the color of the media, it has been recommended that the yeast-like organism isolated on this media should be identified by standard identification systems. This can be time consuming and expensive (Healy, Dillavou et al. 1977).

C. neoformans can also be recovered from Modified Dubos Liquid TBC Media (Pfizer) used to culture *Mycobacteria spp.* Specimens like bone marrow, cerebrospinal fluid; pleural fluid and lung biopsies do not need to be subjected to decontamination but may be inoculated directly into this media. Laboratories should remain aware of the potential recovery of unsuspected pathogenic yeasts or fungi (Damsker and Bottone 1975).

An urgent need to develop a quicker and more sensitive method for detection of *C. neoformans* still remains (Cohen 1984).

1.3.6.3 Commercial available identification kits

Available tests includes the RapID Yeast Plus system that gives same day identification of clinical yeasts but the performance depends on the inoculum's density and isolate source (Freydiere, Guinet et al. 2001).

The API 20C AUX system is also considered as a reference method, but newer methods such as Auxocolor and Fungi chrome are as accurate and more convenient (Freydiere, Guinet et al. 2001).

Automated system include the VITEK Yeast Biochemical Card (BioMérieux, South Africa) which correctly identify >93% of common yeasts as well as the ID 32 C strips.

Spectroscopic methods such as Fourier transformed- infrared spectroscopy offer potential advantages for the future (Freydiere, Guinet et al. 2001).

1.3.6.4 Serologically based methods

Clinical studies have indicated that the enzyme immunoassay (ELISA) is a useful supplementary tool for measuring IgG- specific antibodies in Cryptococcosis. The literature indicates the present methods for detection of Cryptococcus antibodies are lacking in specificity

and/or sensitivity. Kaufman discussed and reviewed the methods which have been developed to date (Kaufman and Blumer 1968). The tube agglutination test will detect antibodies in only 30% of Cryptococcus patients. The Indirect fluorescent antibody (IFA) technique will detect approximately 38 % of Cryptococcus cases but quantification is difficult and subjective. Therefore the recommendation is to use both assays which only detect about 50% of cases of cryptococcosis as many of the specimens will give false negative results (Kaufman and Blumer 1968).

1.3.7 Molecular methods

Conventional methods to identify fungi have relied on isolation and culture of environmental organisms and laboratory identification by biochemical test and morphology. Although these methods are still of fundamental importance there is an increasing move towards molecular diagnostic tools like PCR in all fields (Atkins and Clark 2004).

1.3.7.1 Polymerase chain reaction (PCR)

PCR has become a fundamental part of molecular fungal diagnostics since its introduction in the mid 1980's. PCR involves the enzymatic exponential amplification of a specific target region using short primers, leading to detectable amounts of amplified DNA from a single or a few sequences (as described in 1.2.7). Conventional PCR is not quantitative but qualitative and has been used to detect and identify fungi from a range of environmental samples and is the core of fungal molecular diagnostic (Atkins and Clark 2004).

- **Reverse transcription PCR (rtPCR)**

Reverse transcription PCR is often used in the identification of fungi. It uses the enzyme reverse transcriptase to convert RNA to CDNA before PCR amplification therefore the detection of several fungal elements can be detected if highly specific primers are designed to anneal at the same temperature and the PCR products are different sizes to allow discrimination (Amjad, Kfoury et al. 2004).

- **Quantitative PCR**

PCR is a well suited to detect small amounts of target DNA, but without quantifying them, therefore decisions have to be made whether treatment should be commenced prior to confirmation with culture. Quantification is done visually on a gel; when the ratio of both target signals are the same then the level of unknown fungal DNA matches the quantity of the DNA added, the amount of fungus in the sample can be quantified. This method was successfully described in quantifying the fungus *Pochinia chlamydo* spores from soil (Atkins and Clark 2004).

- **Multiplex PCR**

Multiplex PCR was used to amplify the internal transcribed spacer 1 (ITS1) region between the 18S and 5.8S rRNA genes and a specific DNA fragment within the region of ITS2 region (Chang, Leaw et al. 2001).

1.3.7.2 DNA Probing

The use of fluorescent probes has allowed direct *in situ* analysis of organisms, even those that are not able to be cultured. This technique has been referred to as fluorescent *in situ*

hybridization (FISH) and has been used in the study of fungal interaction (Atkins and Clark 2004).

This technology uses probes with similar sequences to the target fungi DNA. These DNA probes have been used for the detection of specific sequences in DNA and RNA samples in several studies. The DNA probes are either labeled with radio- active isotope or a chemiluminescence's reporter. Once the probes are bound to the DNA sample, which is immobilized on a nitocellulor or nylon membrane, the probe is detected by exposure to X- ray film (Atkins and Clark 2004).

- **Hyper branched rolling circle amplification Probes**

Targeting species- specific single nucleotide polymorphisms at the end of internal transcribed spacers (ITs) of the RNA gene locus were developed to speciate Cryptococcus species complex which is a rapid and sensitive method (Kaocharoen, Wang et al. 2008).

1.3.7.3 Micro array technology

Micro array technology was first introduced by Schena et al. in 1995 (Atkins and Clark 2004). This technology made it possible to measure the expression levels simultaneously of all or most genes in a given genome using DNA probes as the target. However, with the arrival of short DNA fragments arrays, they can be custom made to identify the presence and the quantity, in theory of specific fungal species using ITS or ribosomal RNA information (Atkins and Clark 2004).

- **Reverse Line Blot Hybridization Assay**

Reverse Line Blot Hybridization Assay is an assay designed to identify medical important fungi based on internal transcribed spacer (ITS1) and ITS2 region. (Zeng, Kong et al. 2007)

CHAPTER 2

2 AIM OF STUDY

The aim of this study was to improve our current laboratory techniques for the diagnosis of the two most important infectious agents causing chronic meningitis in our patient population, namely *Mycobacteria* species and *C. neoformans*.

2.1 OBJECTIVES FOR THE STUDY

The objectives of this study were therefore:

1. To compare the sensitivity and specificity of laboratory investigations including microscopy and in-house PCR against the 'gold standard' of culture for the diagnosis of Mycobacteria and Cryptococcus as the causative agents of chronic meningitis in HIV positive and negative adult patients presenting at Tygerberg Hospital, Western Cape, South Africa.

2. To evaluate the cost-efficiency of alternative tests such as real time PCR and alternative culture methods to the conventional tests currently used in the NHLS laboratories.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Study settings and patient population

This study was a prospective hospital-based analysis of cerebrospinal fluid (CSF) samples referred to the National Health Laboratory Service (NHLS) at Tygerberg Hospital in the Western Cape, South Africa which occurred over a period of 16 month, for the routine diagnosis of chronic meningitis. These same samples were also used to evaluate the utility of an in-house PCR for the detection of *M. tuberculosis* and Cryptococcus. The samples were obtained from adult patients from the emergency admission ward (F1), clinics and other departments located in Tygerberg Hospital. We included samples from adult patients with suspected chronic meningitis as indicated by the treating clinician on the requisition form. This study formed part of a collaboration between the Divisions of Medical Microbiology (NHLS) and the Division of Molecular Biology and Human Genetics at the University of Stellenbosch. Bacteriological processing of the samples was done in the Microbiology laboratory, Tygerberg hospital (NHLS) and the PCR analysis was done in the Division of Molecular Biology and Human Genetics at the University of Stellenbosch.

3.2 Ethical approval

Ethical approval for this study, as well as annual extensions, was granted by the Institutional Review Board, Faculty of Health Sciences, University of Stellenbosch, Western Cape. Project number: N07/02/040 (Appendix A).

3.3 Data collection of patient demographics

All selected samples were routinely processed according to standard operating procedures (SOPs) which included macroscopic evaluation, cell count, microscopy and culture. Data collected from the laboratory work sheet included the age and sex of the patient, macroscopic appearance of the sample, volume, chemistry, cell count, Ziehl-Neelsen stain, results of the Cryptococcus latex test if performed, India ink stain, bacteriology and MTB culture results.

3.4 Sample processing methods

3.4.1 Safety Considerations

This laboratory based study was carried out in the Biosafety Level 2 (BLS2) Mycobacteriology laboratory of the National Health Laboratory Services (NHLS), situated at Tygerberg Hospital, and in the Biosafety level 3 (BSL3) laboratory at the Division of Molecular Biology and Human Genetics, University of Stellenbosch, South Africa. All positive cultures were confirmed with a Ziehl-Neelsen (ZN) stain where after positive cultures were submitted to the NHLS Microbiology laboratories at Grootte Schuur hospital, Cape Town, South Africa, for identification and drug susceptibility testing according to routine practice at that time. Safety precautions were strictly followed when all samples were processed; all samples were inoculated and processed in class ll biological safety cabinets (BSC) under sterile conditions. The BSC working area was disinfected with 2 % Hycolin (William Pearson chemicals, Coventry, UK) before and after processing all clinical samples. Protective clothing was worn (disposable laboratory coats and double sterile latex gloves) while handling the specimens. To avoid cross contamination only one tube was opened at any given time and all materials that were to be used were arranged

correctly within the safety cabinet so as to avoid any interference with the air flow. All procedures were conducted by qualified and experienced technologists.

3.4.2 Preparation of sputum samples for diagnosis of MTB by culture

A higher volume of sputum samples were received by the diagnostic laboratory, and as sputum samples have a higher positivity rate for TB, ten sputum samples (5 known positive samples and 5 known negative samples) were used to optimize the PCR method used.

Sputum samples are mucoid and usually contaminated by normal nasopharyngeal flora, therefore a **digestion** (liquefication) and a **decontamination** step (selective elimination of bacteria other than mycobacteria) is required (Figure 1). The Sodium Hydroxide-N-Acetyl-L-Cysteine (NAOH-NALC) (Merck, Darmstadt, Germany) procedure was used to digest and break up the mucus in samples so as to free the trapped bacilli and to kill normal flora present in the sample. The Sodium Citrate procedure is the standard decontamination procedure used in our laboratory in conjunction with the BACTEC 960 automated culture system (Becton Dickinson, Maryland, USA)). Briefly, NACL (5g/L) was added to a 1:1 solution of 4% Sodium Hydroxide and 2.9% Sodium citrate (2.9%). This decontaminating solution was then added to an equal volume of sputum in a sterile 25 ml capped centrifugation tube shaken for approximately 2 minutes and incubated at 37° C for 20 minutes. Thereafter phosphate buffer (NHLS, South Africa) with a pH 6.8 was added to neutralize the decontamination reaction. Sample containers were filled with equal volumes of phosphate buffer and sputum plus decontamination fluid to a total volume of 25ml to balance the tubes for centrifugation. The tubes were then centrifuged at 3000 rpm for 20 minutes to concentrate the sputum samples. The supernatant was subsequently discarded and the pellet resuspended in 1 ml of phosphate buffer.

One drop of resuspended pellet was placed on a clean slide, heat fixed on a hot tray (Lasec, South Africa) and left to air dry for subsequent Ziehl-Neelsen staining (Diagnostic Media Products, NHLS, South Africa). These slides were then viewed under an 100x magnification using an oil immersion microscopic lens for at least 20 minutes and positive slides were reported according to the Centre of Disease Control and Prevention (CDC) as: 1+ (1-9 AFB / 100 fields), 2+ (1-9 AFB /10 fields), 3+ (1-9 AFB / single field) , 4+ (> 9 / single field). Null (0) acid fast bacilli were reported as negative.

Two BD BBL Mycobacterium Growth Indicator tubes (MGIT) containing 7ml of modified Middlebrook 7H9 liquid broth and 0.8 ml Middlebrook oleic acid, albumin, dextrose and catalase (OADC) enrichment medium (Beck ton Dickinson) were used as culture media.

According to the SOPs, a mixture of antimicrobials containing Polymixin B (10,000 units), Amphotericin B (1000 μg), Naladixic acid (4000 μg), Trimethoprim(1000 μg) and Azlocillin (1000 μg) (PANTA, Becton Dickinson), was added to samples at this point, in order to prevent the overgrowth of other bacteria. Thereafter a half a milliliter of the resuspended pellet was added to two PANTA enriched (0.8 µl per tube) MGIT tubes, respectively and annotated as MGIT A (processed according to standard laboratory procedures as a gold standard) and MGIT B (the seed sample culture for PCR).

The two MGIT tube samples (MGIT A and B) were incubated in a BACTEC 960 automated machine. MGIT A was removed when it flagged positive. The time to culture positivity was noted and these positive samples were once again subjected to ZN staining to confirm acid fastness before speciation. Cultures with no growth were reported as negative after 42 days of incubation. Daily aliquots (time 0, day 1 to 14) of 200 µl culture were removed from MGIT B with sterile, disposable 3 ml graduated plastic pipettes (Lasec, South Africa) from the bottom of each MGIT B tube. These aliquots were subsequently boiled in a 100℃ heating block for 30

min to kill and lyse the bacterial cells in order to extract DNA (see Figure 1). The extracted DNA was then stored at – 20ºC.

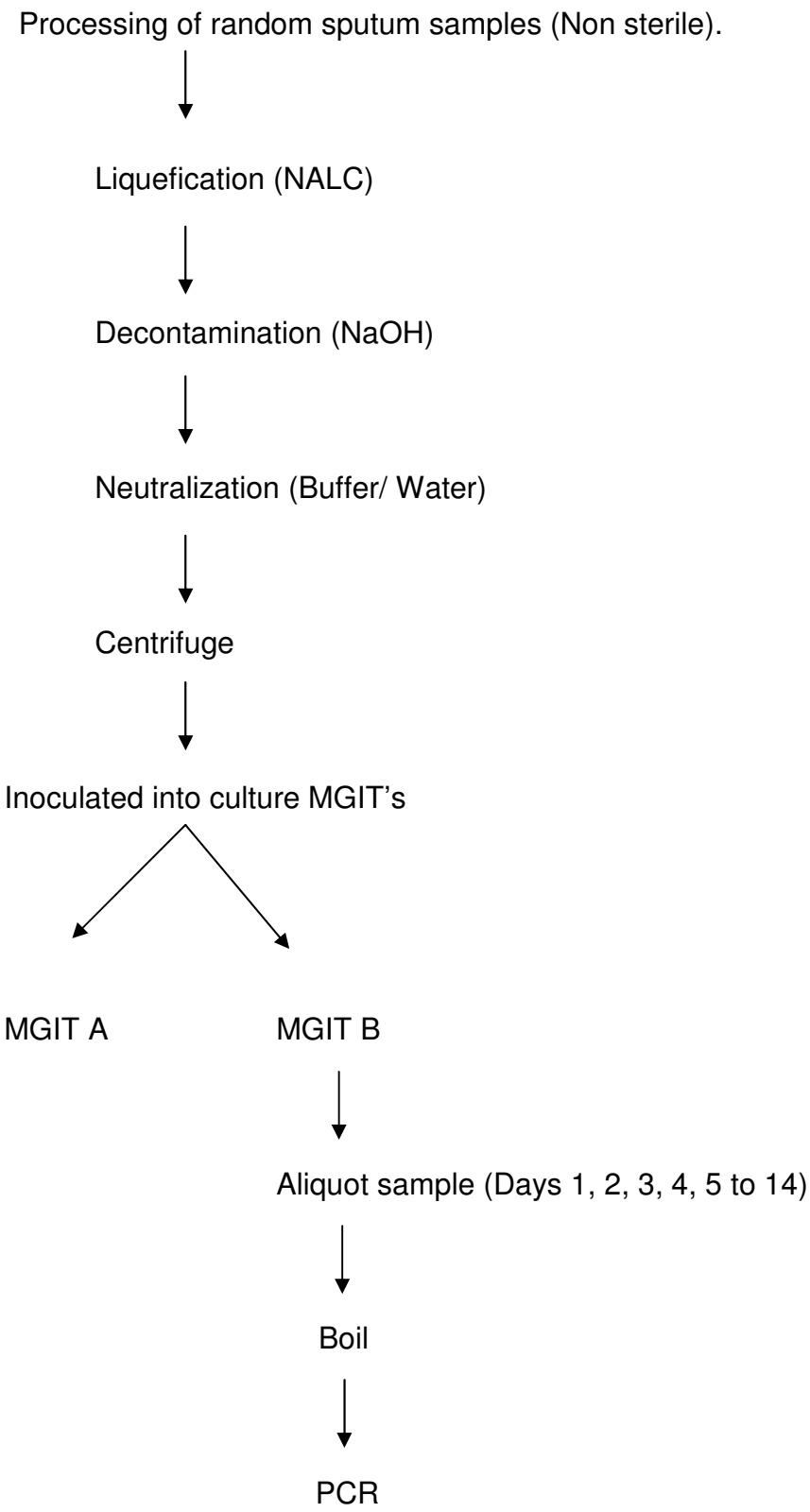

Figure 3.1 Diagrammatic representation of processing of sputum samples.

3.5 Development and standardization of PCR method using reference stains of *M. tuberculosis* and *C. neoformans*

3.5.1 Preparation of MTB reference strain

A known reference strain (H37Rv) was cultured in a MGIT tube containing PANTA. It was incubated in the BACTEC 9260 automated machine until it flagged positive. The time to positivity was recorded and a serial dilution of the culture was made and plated onto 7H11 solid medium containing OADC (NHLS, Green point, South Africa), for determination of the number of colony forming units (CFU) by count. The positive culture was stored at -80℃ (to ensure longer viability) to be used again as stock culture. Stock cultures consisted of 800 µl H37Rv strain and 200 µl of Glycerol broth.

Nine hundred micro liters of Tween 80 normal saline (Difco Laboratories) was transferred to labeled sterile tubes for serial dilutions of the reference strain culture. The positive MGIT tube containing the H37Rv strain was mixed well (by vortex) and a 100 µl aliquot was transferred into the first tube where after the sample was mixed again before plating 100 µl thereof onto the 7H11 solid medium plates. Using a sterile plastic loop the aliquots were streaked out to enable single colony formation. By using a sterile tip (to avoid carrying over of previous dilutions) 100 µl of tube 1 (1 in 10 dilution) was pipette into tube 2 (1 in 100 dilution), mixed well and plated out onto 7H11 plates. The above procedure was repeated until tube 5 (1 in 100 000 dilution) was reached. All plates were incubated for up to 3 weeks in a 5% oxygen Scientific Series 9000 incubator (Lasec, South Africa) and visually evaluated after 7 days and then every 4 days. Colony counts were done after 2 weeks of culture.

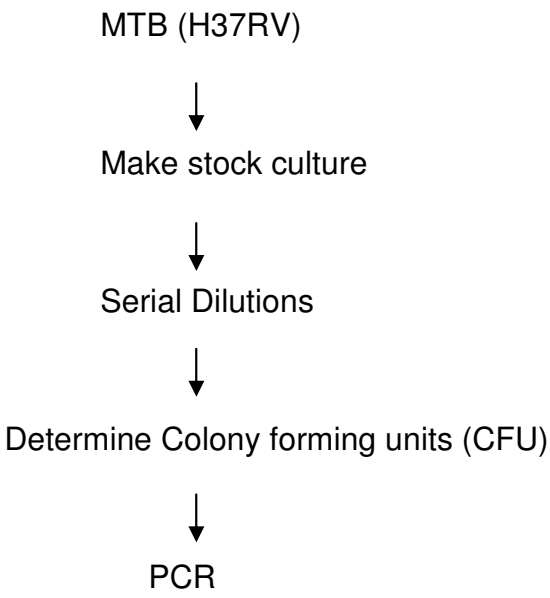

Figure 3.2 Development and standardization of PCR method using MTB reference strain.

3.5.2 Preparation of *C. neoformans* ATCC 66031 reference strain

The ATCC 66031 strains of *C. neoformans var neoformans* and *C. neoformans var gatti* (no ATTC available) were used as positive controls during PCR. These are the two most prevalent infectious strains causing chronic cryptococcal meningitis. Both strains were ordered and received on micro beads from the National Institute for Communal Diseases (NICD). Subsequently, a single bead was removed using a sterile needle, transferred into 1 ml of sterile nutrient broth, (NHLS Green Point, South Africa) and then incubated until a optical density of a 0.5 McFarland (10^8 organisms/ml) was obtained. Subsequently, a 100 µl aliquot was plated onto Sabourauds Dextrose (SAB) culture plates (NHLS, Green Point) and incubated for 24-48 hrs at 35℃ in a 5% to 10% Carbon dioxide (CO_2) concentration. Plates were then visually evaluated to determine the viability of the organism, as judged by colony formation. If growth was successful, a 0.5 McFarland turbidity standard was made and the suspension left at room temperature for 30 min to allow the organism to reach its exponential growth phase. Stock cultures consisted of 900µl nutrient broth and 100µl of the respective reference strains.

Nine hundred micro liters of nutrient broth was pipetted and transferred into labeled sterile tubes for double dilutions. The reference tube containing *C. neoformans* reference strain was mixed well (vortexed) and a 100 µl of aliquot was transferred into the first tube where after the sample was mixed again before plating 100 µl onto the plates. Using a sterile plastic loop the aliquots was streaked for single colonies. By using a sterile tip (to avoid carrying over of previous dilutions) 100 µl of tube -1 was pipette into tube -2 (mixed well) and was plated out onto 7h11 plates. The remainder of the suspensions were clearly marked and stored at –80℃ prior to DNA extraction/concentration, to be used as a positive control for PCR. The above procedure was repeated until tube -5 was reached. All plates were incubated for 24-48 hours in a CO_2 incubator, and visually evaluated after 24 and 48 hours. Counts were performed after 48 hours.

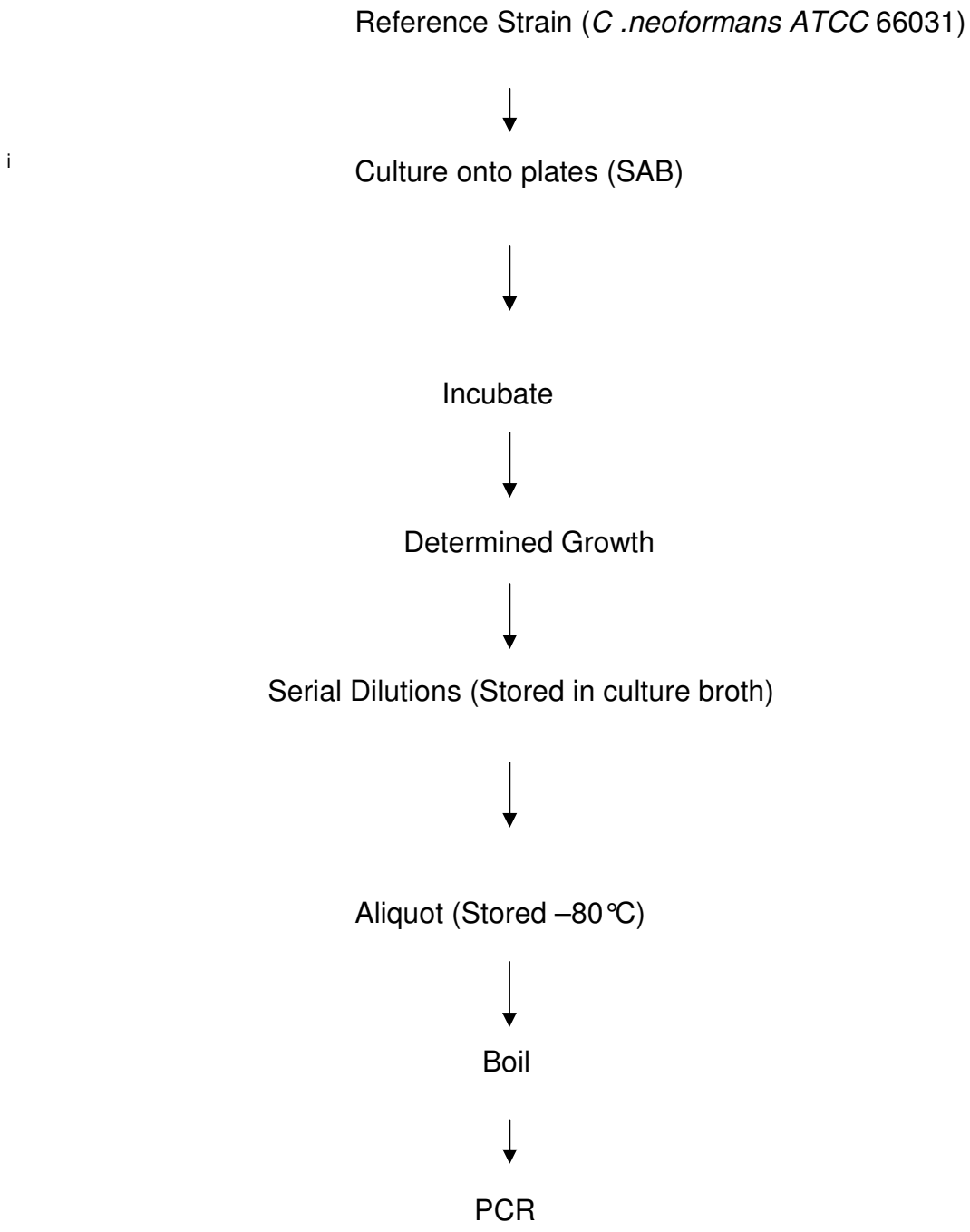

Figure 3.3 Development and standardization of PCR method using *C. neoformans var neoformans* and *var gattii* reference strains.

3.6 Preparation of CSF sample by routine Bacteriology culture and MTB culture

3.6.1 CSF sample for routine bacteriology culture

Cerebrospinal fluids included in this study were collected via lumbar puncture by qualified clinicians. The normal practice is to take three separate samples in three containers for analysis of chemistry and microbiology. These include a sodium fluoride tube for glucose testing, an anticoagulant free tube for CSF protein determination and a sterile tube for microbiology. Each tube should contain approximately 1-5 ml fluid. These tubes are sent to the laboratory *directly* after the procedure so as to minimize the effect of cell lysis, which can start soon after specimen collection (Betty A). In the microbiology laboratory the samples were first sent to the routine laboratory for microscopy and bacteriological culture. In the routine laboratory the appearance of the CSF sample was noted. Routine examination of CSF samples included the following tests: direct cell count, Gram stain, methylene blue stain (if necessary), India ink (for identification of Cryptococcus), bacterial culture, as well as protein and glucose determination. The latter two tests are done in the chemistry laboratory according to the NHLS SOP's. All CSF results were reviewed by a pathologist or registrar who decided whether further testing was required. Further tests may include MTB culture, Cryptococcus culture on media to specifically support fungal growth, Cryptococcus Latex antigen test, and serology for syphilis. These additional tests were only done on specimens if requested by a clinician.

Macroscopic evaluation of CSF samples

The volume of fluid received was noted and the macroscopic appearance of the CSF sample was reported as clear and colorless, clear, turbid or yellow.

Cell count

A cell count was done using a Fuchs- Rosenthal counting chamber and a cover slip was placed on it. Ten micro liters of crystal violet was pipette into a clean tube and with a sterile Gilson pipette 100 µl of CSF was added and mixed well. The counting chamber was filled using a capillary tube. The area to be viewed was selected using a 10x objective focus, the cells counted using the 40x objective. The white cells and red blood cells were counted in 5.5 big or 88 small blocks. If more than 100 red blood cells per small block were noted, one small block was counted and multiplied by 88.

If excess red blood cells were observed that might obscure white blood cells, the cell count was not done. Instead the quantity and type of white blood cells seen on the Gram stain or Methylene blue stain was commented on.

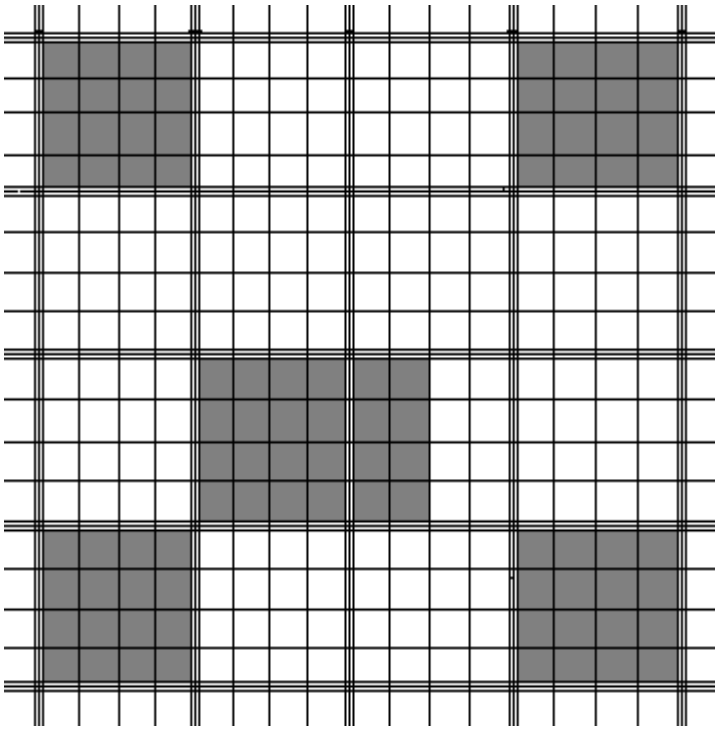

Figure 3.4 Representation of a **Fuchs- Rosenthal counting chamber**. Shaded areas represent the blocks counted.

Centrifugation of CSF sample

If more than 1ml of CSF was received the remainder of the CSF was centrifuged for 10 minutes at 2000-3000 rpm. The macroscopic appearance of the supernatant was reported as colorless, yellow, orange or brown. The supernatant was aspirated with a sterile pipette into a sterile tube, leaving approximately 0.5 to 1.0 ml of fluid in the specimen tube. The supernatant was retained for additional week, in case more investigations were requested. The sediment was mixed to resuspend the pellet. If less than 1 ml of CSF was received, we did not centrifuge the sample but we rather mixed it gently by swirling.

Inoculation of media for routine bacterial culture

A sterile pipette was used to inoculate Blood agar and Chocolate agar with 1 or 2 drops of the sediment. The plates were then streaked and incubated at 35°C in a 5-10% CO_2 incubator for 18-24 hours. All media was incubated for 48 hours before reported as 'no growth'. If the India ink or Cryptococcus latex (CLAT) was positive, an additional Sabourauds dextrose agar (SAB) plate was inoculated and the plate was incubated for 5 days at 37°C. Positive growth was followed up with identification and drug sensitivity testing, according to SOPs.

Microscopy

Three smears were prepared from the sediment: one Gram stain, one methylene blue, and one wet preparation for India ink stain. All positive microscopy results were reported to the clinician immediately by telephone. If organisms were seen on the Gram stain and there was sufficient CSF left, direct drug sensitivity testing was performed according to the standard laboratory protocol (SOP).

3.6.2 CSF samples for MTB culture

In the TB laboratory the remainder of the CSF sample was split into two MGIT tubes annotated MGIT A (processed according to standard laboratory procedures as a gold standard) and MGIT B (the study sample that acted as the seed culture for PCR). Five hundred micro liters (500 µl)

of CSF was added to the individual tubes, together with 800µl PANTA. The remainder of the sample was stored at -20°C for further investigation.

The two MGIT tubes (MGIT A and B) were incubated in a BACTEC 960 automated machine. MGIT A was removed as soon as it flagged positive. The time to culture positivity was noted and ZN staining was performed to confirm acid fastness of the culture before speciation by PCR. Cultures with no growth were reported as negative only after 42 days of incubation. Weekly aliquots (time day 1, week 7, 14, 21, 28, 35 and 42) of 200 µl culture were removed from MGIT B with sterile, disposable 3 ml graduated plastic pipettes (Lasec, South Africa) taken from the bottom of the tube. These aliquots were subsequently boiled in a 100℃ heating block for 30 min to kill and lyse the bacterial cells and for subsequent DNA extraction (see figure 1). The extracted DNA was then stored at – 20ºC.

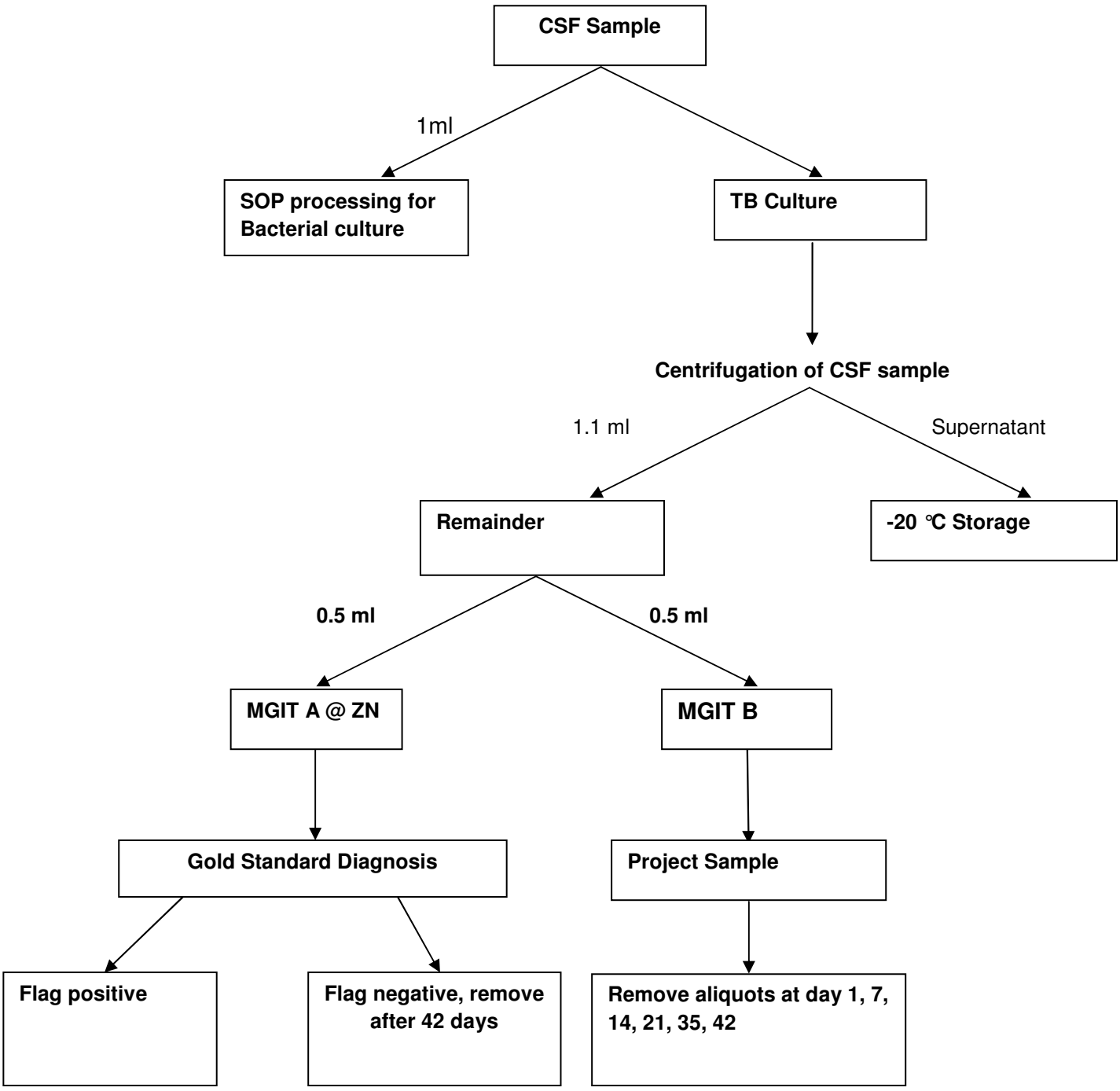

Figure 3.5 Flow-diagram describing the processing of the CSF samples included in this study.

3.7 Molecular Methods

Different DNA extraction, DNA concentration methods, and testing for the presence of possible inhibitors were performed on subsets of samples in order to optimize the methods.

3.7.1 DNA extraction

To reduce the cost and ensure a rapid diagnostic procedure, it would be ideal to avoid having a DNA extraction technique. However, when working directly on specimens like sputum, many PCR inhibitors are present, including neutrophil protease, elastase and cathepsin G. An improvement would be to dilute these inhibitors as is undertaken with culture. However, short term cultures are hampered by the lack of mycobacterial DNA as the mycobacterium has not yet had enough time to grow. This problem can be overcome by concentrating the DNA prior to boiling by (a) centrifugation and subsequent resuspension of the pellet in a smaller volume of media, or (b) to use a commercialized kit following boiling of the specimen, which will trap the DNA released from cells, and then to elute this DNA in a smaller volume than initially used. As the latter method will add cost to the diagnostic procedure, it is preferable to concentrate the DNA prior to boiling. However, in this study, only the specimens collected at time naught (time 0) were not boiled and could therefore be processed by method (a), the remaining specimens (those cultured for different time periods), were boiled and thus needed a subsequent DNA extraction/concentration procedure by commercial kit.

The Wizard® SV Genomic Purification System (Promega, USA; Appendix B) was used according to manufacturer's instructions to purify these specimens. Briefly, specimens were vortexed and a ratio of sample to Lysis Buffer of 1:2.5 added (300 µl culture: 750 µl Lysis buffer). The lysates were then transferred to minicolumn assemblies and centrifuged at 11000 g for 3 min. The flow-through was subsequently discarded and the sample washed four times with 650 µl of the Wash Buffer (centrifuging for 1 min at 13000g and discarding the flow-

through in between washes). The minicolumns were then transferred to sterile Eppendorf tubes and the DNA eluted with 15 µl ddH_2O (heated to 65°C). These samples were then ready for analysis by PCR.

3.7.2 PCR optimization and amplification

3.7.2.1 Primer design

Correct primer design is essential to ensure that PCR diagnostics are specific and sensitive. Cross-reactivity of primers to other organisms must be avoided so as to ensure amplification of the target. One of the aims of this study was to differentiate between *M. tuberculosis* (causing TBM) and *C. neoformans* (causing cryptococcal meningitis (CM), however *M. tuberculosis* forms part of the *M. tuberculosis* complex (including *M. africanum, M. bovis*, *M. bovis BCG* vaccine strain*, M. microti and M. canetti*), which all have common ancestry and therefore share very similar DNA sequences. It is essential to be able to differentiate whether TBM is caused by *M. tuberculosis* or one of the other members of the complex so as to adjust the patients' treatment accordingly. Numerous deletions and insertions have occurred over time in members of the MTC, which includes the Region of Deletion 9 (RD9), present in *M. tuberculosis* and *M. canetti*, but absent from all other members of the complex. Human disease by *M. canetti* is very rare and therefore this RD is an ideal target for differentiating MTB from other members of MTC. Many current diagnostic tests focus on the common insertion element *IS6110*, which is found in all members of the *M.tuberculosis* complex; thus it offers no use in speciation.

Primers for detecting *M. tuberculosis* were designed according to the RD9 region of the standard laboratory reference strain H37Rv, the sequence of which was obtained from the NCBI website (accession number NC 000962) and subjected to primer design using DNAMan

primer design software. Primer selection criteria included that the primers must have an annealing temperature of 62℃, GC% content of between 40 and 60 and end on a C/G base on the 3' end. Selected primer sequences were analysed for hairpin, homo- and hetero-dimer formation using the IDT® (Integrated DNA Technologies, Inc, Coralville, IO, USA) online. OligoAnalyser followed by a NCBI Basic Local Alignment Tool (BLAST) search to ensure the primers were specific to mycobacteria only. All oligonucleotide primers were synthesised by IDT® in the 100nMolar range. The primers (table 1) were designed to flank the RD9 as well as have an internal reverse primer within the RD9. Priming with the outer primers produce a 108 bp fragment indicating that the specimen belong to members of the *M. tuberculosis* complex other than *M. tuberculosis*, however if priming occurred with the internal primer, to produce a product of 235 bp, the presence of *M. tuberculosis* was diagnosed. (See Appendix C)

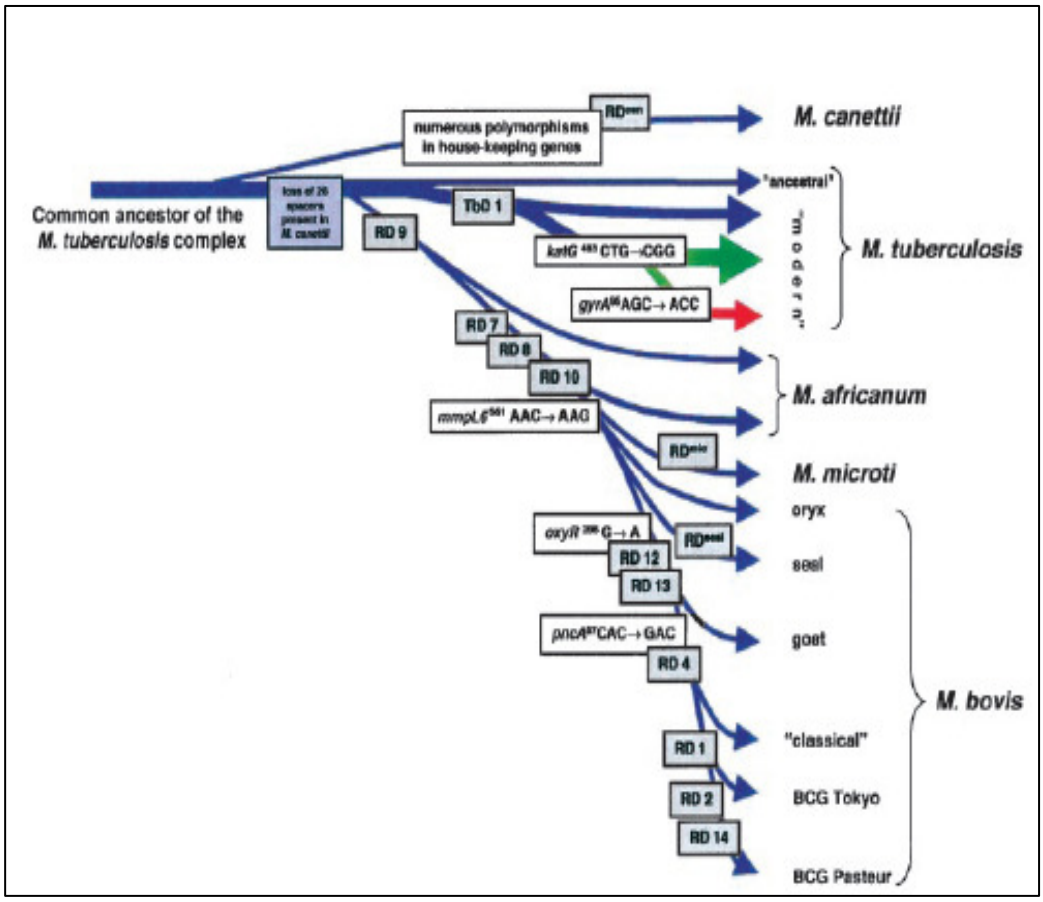

Figure 3.6 An evolutionary tree showing that the Region of Deletion 9 (RD9) is present in *M.tuberculosis* and *M.canettii*, however absent from all other members of the *M.tuberculosis* complex (Brosch, Gordon et al. 2002).

Primers specific to both *C. neoformans var. neoformans* and *C. neoformans var. gattii* were designed based on the sequence encoding the partial internal transcribed spacer 1 (ITS1) 5.8S rRNA gene and partial ITS2 obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/; accession number EU402440) as described in the paragraph above (Table 3.1).

Table 3.1 Primer sequences used in the study

Locus	Primer Name	Primers (5' to 3')	Organism specific to
RD9	**RD9Fs**	CAA GTT GCC GTT TCG AGC C	*M. tuberculosis* complex
RD9	**RD9int**	CAA TGT TTG TTG CGC TGC	*M. tuberculosis*
RD9	**RD9FR**	GCT ACC CTC GAC CAA GTG-TT	*M. tuberculosis* complex
5.8s rRNA	**CNrD-F**	CCT GTT GGA CTT GGA TTT GG	*C. neoformans**
5.8s rRNA	**CNrD-R**	CGA AAC TTA TTA CGC CGG G	*C. neoformans**

**C. neoformans var. neoformans and var. gattii*

3.7.2.2 PCR amplification

PCR amplification for the detection of *M. tuberculosis* DNA and *C. neoformans* DNA was optimized using the H37RV and ATCC *C. neoformans* laboratory strains, respectively. Briefly, in a reaction mixture containing 25 ng DNA template, 5 µl Q-Buffer, 2.5 µl 10 x Buffer, 2 µl 25 mM $MgCl_2$, 4 µl 10 mM dNTPs, 1 µl of each primer (50 pmol/µl) (for detection of *M. tuberculosis* RD9 Fs1 5'-CAA GTT GCC GTT TCG AGC C-3', RD9FR 5'-GCT ACC CTC GAC CAA GTG TT-3' and RD9INT 5'-CAA TGT TTG TTG CGC TGC-3' and for detection of *C. neoformans* CNrD-F-5' CCT GTT GGA CTT GGA TTT GG 3', CNrD-R- 5' CGA AAC TTA TTA CGC CGG G 3'), 1 µl (1/100 dilution) SYTO 9 fluorescent dye (Molecular Probes, USA), 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) were mixed and made up to 25 µl with dH_2O. Amplification was initiated by incubation at 95°C for 15 minutes, followed by 45 cycles at 94°C for 45 seconds, 62°C for 45 seconds, and 72°C for 45 seconds. After the last cycle, the samples were incubated at 72°C for 10 minutes. Amplification was confirmed by high resolution melt analysis (see below).

PCR analysis of the concentrated sputum and CSF specimens was exactly the same; however, since the concentration of the CSF specimens was unknown, 2µl of specimen was added to the PCR reaction. Every PCR run included a positive control (laboratory H37Rv or ATCC *C. neoformans* strain) as well as a contamination control (no DNA present). Results were only included if these controls were normal.

NOTE: To minimize the risk of laboratory cross contamination during PCR the following steps were adhered to:

- The PCR reaction mixture, addition of the DNA and the actual PCR amplification was conducted in physically separate rooms,
- PCR workstations were cleaned with 10% Sodium Hypo chloride and 70% ethanol prior to use and treated with UV light to remove any contaminants,
- Good laboratory practice was adhered to, including the use of laboratory coats and regular changing of latex gloves

3.7.2.3 Speciation using High Resolution Melting Analysis

The resulting amplification products were subjected to high resolution thermal melt analysis in a Rotorgene 6000 (Corbett, Australia). The thermal denaturation profiles were measured over the temperature range from 80°C to 95°C and fluorometric readings were taken every 0.1°C. Rotorgene software was used to calculate the derivative of the intensity of fluorescence at different temperatures (dF/dT), thereby generating a plot where the derivative peaks represent the melting temperature of the PCR products. The infectious agent was identified by the software according to the presence of a peak located within defined temperature bins. *M. tuberculosis* products melted at 90.5℃, *M. tuberculosis* complex at 86℃ and *C. neoformans* at 84.5℃.

3.7.2.4 DNA extracted by boiling and centrifugation

Sixty five samples were subjected to boiling, centrifugation and resuspension of the pellet at the day of sample collection according to method described in section 3.6.2.

3.7.2.5 Spike test

The Spike test was done on DNA samples which failed to amplify to determine if the absence of amplification was due to the presence of inhibitors in the sample. Two micro liters of a MTB positive sample (positive by culture and PCR) and 2 μl of a MTB positive sample (positive by culture but not PCR) were added to the reaction mixture and subsequently amplified by PCR.

3.8 Statistical analysis

For the comparison of PCR method with routine culture phenotyping the statistical analysis was done with the program Statistica and we calculated the sensitivity and specificity of PCR amplification at a confidence interval (CI) OF 95 %. The negative predictive value (NPV) and the positive predictive value (PDV) of all results were also calculated.

CHAPTER 4

4 RESULTS

4.1 Demographic information of the patient population

Cerebro-spinal fluid specimens from a total of 78 adult patients were collected over a period of 16 months and included in this study, according to the criteria set out in Chapter 3.1. The demographical data of the patients from whom the CSF samples were taken, was recorded and analysed. The principle findings were the following:

Ages ranged between 14 and 76, with a mean of 37 years. Two patients (2.6%) were under the age of fifteen. Of the whole study population, 42 were female (54%) and 36 were male (46%).The female patients were between the ages of 14-75 years, and the males were 14-63 years. There was no statistical significant difference between the age and sex distribution of the study population. Details of the treatment regimens of patients, clinical response and HIV status were not included in this study.

4.2 Results of PCR optimization of sputum sample

To optimize our in-house PCR method, 10 sputum samples selected from routine samples known to be either positive or negative for MTB, were prepared as described in section 3.4.2. PCR were performed on aliquots of duplicate cultures (MGIT B) on day 1, 14 and the results of MTB culture and PCR are indicated in Table 4.1.

Table 4.1 Culture, ZN, and PCR results performed on random culture selected sputum samples.

Sputum sample number(n=10)	Enumeration of bacilli observed in ZN stain*	Day to culture positivity		Day of PCR positivity sample B
		Sample A	Sample B	
1	4+	9	10	5
2	1+	7	7	5
3	2+	4	10	1
4	3+	6	7	1
5	3+	3	10	1
6	Not observed	Negative	Negative	No amplification
7	Not observed	Negative	Negative	No amplification
8	Not Observed	Negative	Negative	No amplification
9	Not observed	Negative	Negative	No amplification
10	Not observed	Negative	Negative	No amplification

Sample A was the gold standard MGIT that was not manipulated, the original sample.

Sample B was the duplicate MGIT that was manipulated daily for PCR amplification

1+ =1-9 AFB/100 field seen

2+ = 1-9 AFB/10 field seen

3+ = 1-9 AFB/single field

4+ = >9 AFB/ single field seen

Of the 10 samples, 5 were culture and ZN negative, and 5 were culture and ZN positive. The average time taken for the MGIT B sample to flag MTB positive was 9 days. PCR could correctly identify the MTB positive and negative samples in those cultured, with in the first two PCR analyses.

4.3 Calculation of the sensitivity of the in- house PCR for MTB by using H37Rv as a reference strain

Serial dilutions of H37Rv in a broth culture were made (1:10 to 1:100 000) and colony forming units were calculated in order to determine the lowest colony count that could be detected by our in-house PCR (Table 4.3).

The formula in Table 4.2 was used to calculate the number of molecules (representing organisms) present in our PCR optimization methods by using the Avogadro constant.

Table 4.2 Formula of Avogadro constant.

Avogrado Constant	**Formula**	**Values of N_A[1]**	**Units molecules/ mole**
The **Avogadro** constant (symbols: *L, NA*) is the number of ''elementary entities'' (usually atoms or molecules) in one mole, that is (from the definition of the mole), the number of atoms in exactly 12 grams of carbon-12. It is was originally called Avogadro number	***(Conc. / Mw) × Avogrado Constant*** ***Mw = (# bp in genome × 700)***	**N_A 6.022 x 10^{23}**	**mol^{-1}**

= number; bp = base pairs; Mw = molecular weight; Conc. = concentration

The results of the PCR amplification of these serial dilutions is presented in Table 4.3

Table 4.3 Culture and PCR results of optimization of our in-house PCR (boiling method) using H37Rv as a reference strain.

CSF sample dilution	Colony forming units (CFU) /ml	PCR	Number of organisms in PCR run calculated
Tube 0	Confluent growth	Amplified	2912
Tube 1 (1:10)	Confluent growth	Amplified	291
Tube 2 (1:100)	1000 CFU	Amplified	29
Tube3 (1:1 000)	500 CFU	Amplified	3
Tube4 (1:10 000)	100 CFU	Amplified	3
Tube5 (1:100 000)	0 CFU	No Amplification	0

PCR amplification was successful up to a dilution of 1:10 000 which corresponded to 100 CFU (number of organism counted) as stated in Table 4.3. No amplification was evident in the sample that failed to grow on the 7H11 plates. The maximum dilution at which amplification was evident corresponded to 2912 ng (number of organisms calculated during PCR run) of the H37Rv DNA. According to the formula (Table 4.2), the PCR was able to detect a number of 3 MTB organisms in a reaction stated in table above.

4.4 Optimization of study CSF sample by using *Cryptococcus neoformans* ATCC 66031 as reference strain for culture and PCR

Serial dilutions of Cryptococcus were made in liquid broth (1:10 to 1:100 000) and colony forming units were determined, in order to determine the sensitivity of the in-house PCR performed (Table 4.4).

Table 4.4 Culture, PCR results of optimizing our in-house PCR (boiling method) using *Cryptococcus neoformans* ATCC 66031as reference strain.

CSF sample dulutions	Colony forming units (CFU) /ml	PCR	Number of organisms in PCR run calculated
Tube 0	Confluent	Amplification	630
Tube 1 (1:10)	500 CFU	Amplification	63
Tube 2 (1:100)	250 CFU	Amplification	6
Tube3 (1:1 000)	101 CFU	Amplification	0-6
Tube4 (1:10 000)	5 CFU	Amplification	0
Tube5 (1:100 000)	0 CFU	Amplification	0

PCR amplification was successful up to a dilution of 1:100 000, and no growth was evident from the 1: 100 000 dilution. This maximum dilution at which amplification was evident corresponded to 630ng (organisms calculated in PCR run) of Cryptococcus DNA. According to the formula (Table 4.2), the PCR was able to detect a number of 6 cryptococcus organisms/yeasts in a reaction, as indicated in Table 4.4.

4.5 Results of the routine laboratory processing of the CSF samples included in this study

4.5.1 Macroscopic evaluation of CSF results

In the routine laboratory, the practice is to record the CSF appearance as *Clear and Colorless, Clear, Turbid,* or *Yellow*. Sixty one samples (79%) were recorded as clear and colorless, one sample (1%) was clear, eleven samples (14%) were turbid, and five samples (6%) were recorded as yellow (Figure 4.1).

The average volume of CSF samples examined was 6.2 ml (range from 5.0 to 12 ml).

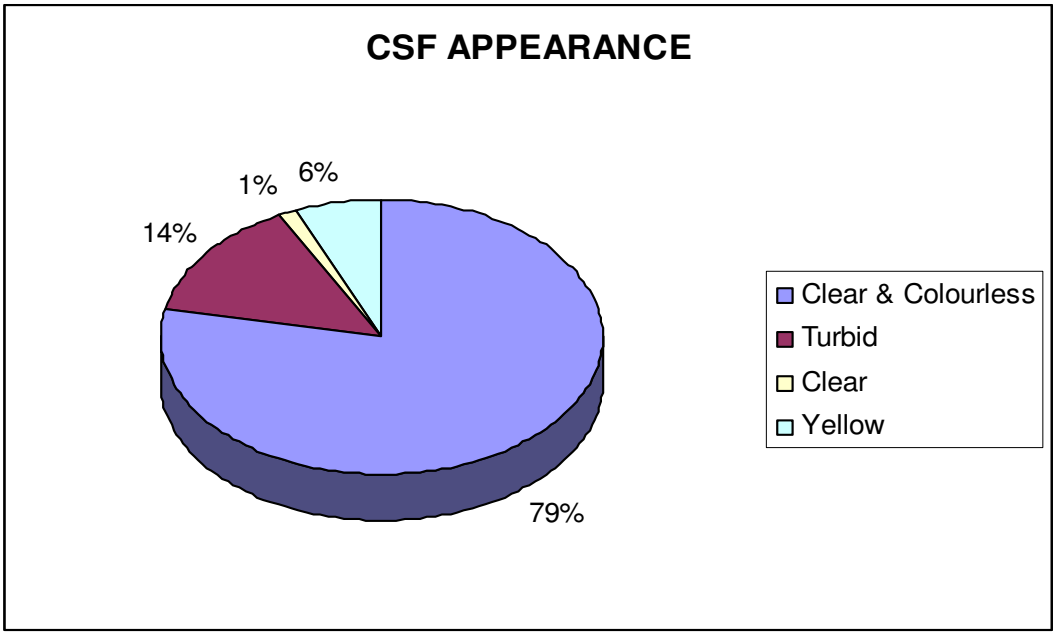

Figure 4.1 Graph showing the percentages of the CSF appearance, as macroscopically evaluated.

4.5.2 Cell count and biochemistry

Cell counts were done on all (n=78) samples of which twenty four samples had a normal cell count (Normal range 0-5 lymphocytes per milliliter), and fifty four showed an increase in lymphocytes (6-1110 lymphocytes per ml).

The routine biochemical analysis performed on the CSF samples included in the study was recorded, and these are described in Table 3.5. The averages protein content was 1.6 gram / liter and average glucose content was 2.6 mmol / liter.

Table 4.5 Results of routine laboratory investigations performed on the CSF samples

Test performed	**Range of results**	**Mean**	**Normal values**
Volume of CSF samples (ml)	5-12	6.2	N/A
Lymphocytes (cell / ml)	0- 1110	100.15	0-5
Protein (g / l)	0.11- 5.00	1.6	0.15-0.45
Glucose (mmol / l)	0.30- 6.70l	2.6	2.2-3.9l

N / A = not applicable

Analysis of the subgroups of patients that subsequently proved to be culture positive for tuberculosis indicated that out of 14 samples (n=78) with had CSF findings suggestive of TBM (lymphocytes predominant, increased protein levels and decreased glucose levels), four of these were negative on TB culture. Of the TB culture positive (n =19) samples, only 10 cases (53 %) showed a raised CSF, lymphocyte count (more than 5 lymphocytes), elevated protein and decreased glucose, in keeping with the 'text book' findings of tuberculous meningitis. A

normal CSF analysis was recorded in 47 % (n=9) of MTB culture positive samples. The remaining CSF sample population was either normal, or had a raised lymphocyte count and normal protein or vice versa.

The CSF analysis of the one sample that was positive for cryptococcal meningitis displayed typical changes with elevated lymphocytes, elevated proteins (0.82 g / l), and decreased glucose levels (1.3 mmol / l).

4.5.3 Microscopy for Bacteriology and MTB

4.5.3.1 Bacteriology microscopy

The gram stain showed a yeast-like organism present in one sample (1.3%) of which the India ink was also positive, which was suggestive of *Cryptococcus neoformans*. The other seventy seven samples (98.7%) showed no organism present.

4.5.3.2 MTB microscopy

In our collection of clinical samples, ZN stain positive and MTB culture positive was 12 (63%) and 7 (36%) were ZN negative but MTB culture positive (Table 4.5.1.

Table 4.5 1 2 by 2 Table of the observed Frequencies of culture results vs Zn results

	Culture Positive	Culture Negative
Zn Positive	12	0
Zn Negative	7	59
Total	19	59

For ZN's performed on CSF samples in our study , a sensitivity of 89 % was obtained with a confidence interval (CI) of 95 %, a lower CI of 41.5 % and an upper CI limit 84 %. A specificity of 100 % was obtained with a 95 % CI, a lower CI limit of 100% and an upper CI Limit 100%. The negative predictive value (NPV) was 0.89 % with a positive predictive value (PPV) of 100%.

4.5.4 Routine Bacteriology culture and MTB culture

4.5.4.1 Bacteriology culture

In this study seventy eight samples (n=78) were cultured on routine media according to laboratory SOP, and in only one sample (n=1) growth was obtained, and identified as *Cryptococcus neoformans*. No other pathogens were isolated. Time to positivity for Cryptococcus culture was 2 days. Another sample was positive by Cryptococcus agglutination latex test (Remel Diagnostic test, Bio- web).

4.5.4.2 MTB culture

The number of CSF samples that yielded a positive culture for MTB culture (gold standard) were 19 (n=19), and 59 (n=59) were culture negative as seen in Figure 3.5.3. Time to positivity by culture ranged between 7 and 28 days.

The culture results of the CSF samples included in our study we summarized in Table 4.5.2

Table 4.5.2 Microbiological analysis of the CSF samples included in this study

	Total	**Percentage (n=78)**
MTB culture positive	19	24%
MTB culture negative	59	76%
Cryptococcus latex positive	1	1.3 %
Cryptococcus culture positive	1	1.3%
Cryptococcus culture negative	77	99%

4.6 Optimization of molecular methods

Different methods of DNA extraction and purification were attempted, in order to optimize specimen handling and molecular methods.

4.6.1 DNA extracted by boiling and centrifugation

Sixty five samples were subjected to boiling, centrifugation and resuspension of the pellet at the day of sample collection. Eleven of these samples were MTB culture positive, but only 3 samples were amplified by PCR. All three of these samples had typical CSF findings of TBM, and therefore possibly high organism loads.

4.6.2 Spike test

To exclude the presence of inhibitors in the original samples, a spike test was conducted according to the method described in Chapter 3.7.2.5. Of the 8 samples that were tested no DNA amplification were observed, indicating the presence of inhibitors. A DNA Purification (clean up) was therefore done as a next step.

4.6.3 DNA purification

All seventy eight samples of all aliquots were subjected to the WIZARD SV Genomic Purification kit after aliquots were removed during the culture method (as described in method section 3.7.2). Of the seventy eight samples tested, 17 of the19 samples that were MTB culture positive were now amplifiable by PCR.

4.6.4 PCR done on cultures at different incubation times

PCR were done on day of collection (time naught), and on weekly aliquots of the parallel culture (MGIT B) were subjected to PCR (day 1, 7, 14, 21, 28, 35 and 42).

4.7 Results of molecular methods to detect MTB

The RD9 PCR detected *M. tuberculosis* in 17 of the 19 positive samples as cultured by the BACTEC 960 automated system, after the improvements to the method described above. However, 22 of the 59 samples that were negative by culture amplified by PCR.

It is not excluded that the PCR positive but culture negative results obtained during our PCR analysis could be the result of carry over contamination by previously amplified nucleic acids, from clinical specimens containing large numbers of organisms, since all these samples were numbers that that followed right after each other. Alternatively, false culture negative results could be due to low number of organism present, because of their paucibacillary nature. Two of these PCR positive but culture negative samples had an atypical CSF finding (raised lymphocytes, decreased glucose, and raised proteins), three samples had cell- and protein

levels within normal range, and seventeen samples had either an raised lymphocyte count, or protein count, and normal glucose count or vice versa.

These results are demonstrated in Table 4.6. Included in these results is one sample that was identified as MTB complex by PCR.

Our PCR results correlated well with our *M. tuberculosis* culture results: eighteen samples were identified as *M. tuberculosis* and one sample was identified as *MTB* complex with our PCR analysis. Of the positive cultures, eighteen were identified as *M. tuberculosis* and one sample was identified as MTB complex.

Table 4.6 Results of TB culture compared to in-house PCR (RD9 primers).

		PHENOTYPE	
		TB Positive	**TB Negative**
GENOTYPE	**TB Positive**	17	22
	TB Negative	2	37

The sensitivity of our in-house PCR was therefore calculated to be 89.5 %, with a 95% Confidence Interval (CI) and a lower CI 75.7% and an Upper CI limit 103.3%. A specificity of 63 % with a 95% CI and a lower CI limit 50.4% and an upper CI limit 75% was obtained. The NPV was 0.949 and the PPV of 0.5.

4.8 Time to Positivity of PCR and MTB culture.

The time to positivity of conventional culture was compared to PCR as performed on aliquots of the parallel culture (MIGIT B) in weekly time intervals. These results are illustrated in Figure 4.2.

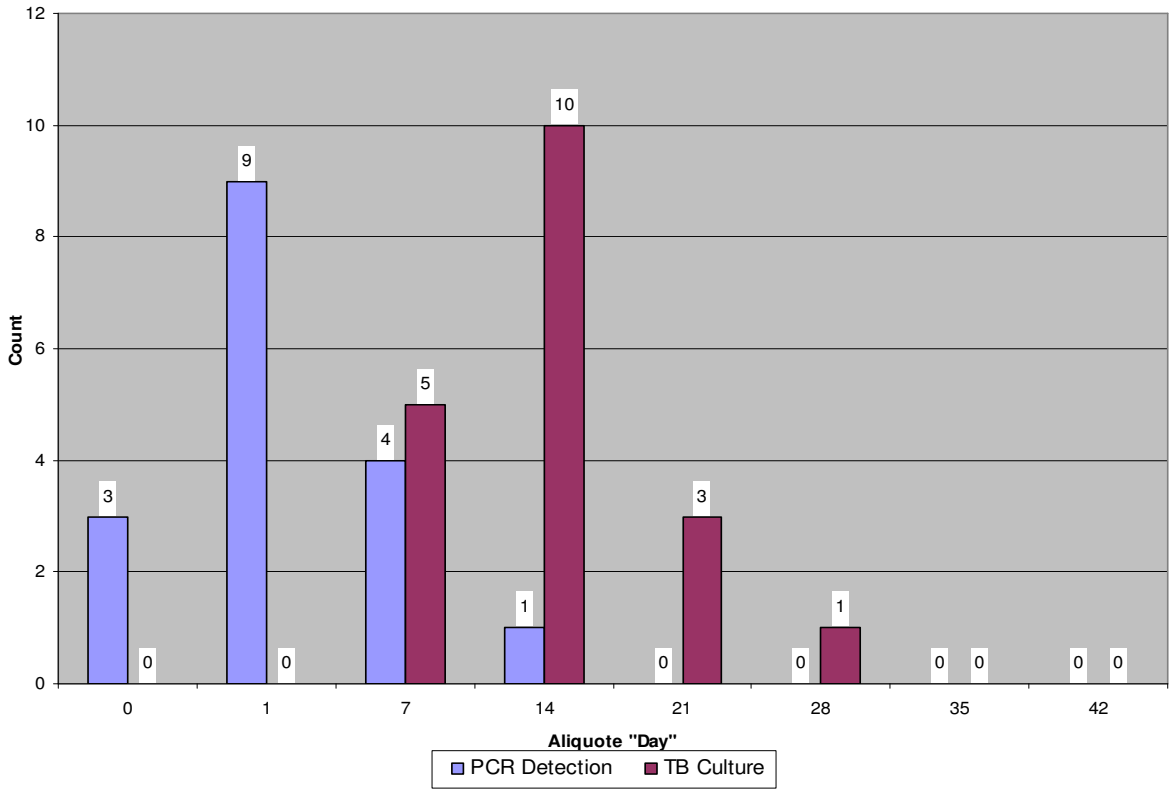

Figure 4.2 Graph illustrating Time to Positivity of PCR vs MTB culture.

4.8.1 PCR results

In the graph shown in figure 4.2, three samples were positive at Crude time naught, nine samples were positive at day 1, four samples were positive at day 7, and one sample was positive at day 14.

4.8.2 MTB culture results

Figure 4.2 illustrates that ten samples were positive at day 14, three samples were positive at day 21, and one sample was positive at day 28.

4.9 CSF analysis of Cryptococcus by culture and PCR

The samples in our study had a very low yield of crytococcus by all diagnostic methods. The detection of cryptococcal meningitis by culture and PCR is illustrated in Table 4.4. Only one sample (n=1) of the seventy eight (n=78) was positive for Cryptococcus culture and the time to culture positivity was 2 days. Cryptococcus latex was not performed on this sample as the India ink test was positive. This sample was also positive for *M. tuberculosis* by PCR, and MTB culture.

This sample, as well as two other samples (culture negative) tested positive by PCR for *C. neoformans*. The one sample that tested positive by PCR, had an elevated lymphocytes count (26 lymphocytes) and its protein levels was also elevated (0.82 g / l) and a decreased glucose level (1.3 mmol / l) which is a typical finding in cryptococcal meningitis.

The other sample that was positive by PCR was cryptococcal culture negative, India ink was negative, and cryptococcal latex was not performed. The CSF findings, the lymphocyte count was normal (3 lymphocytes), the protein levels were elevated (0.49 g / l) and glucose level were in the normal range. The sensitivity and specificity of the PCR compared to routine phenotyping could not be calculated due to the low number of cryptococcus positive samples.

Table 4.7 Cryptococcal culture compared to PCR (CNrD primers).

		PHENOTYPE	
		Cryptococcus Positive	**Cryptococcus Negative**
GENOTYPE	**Cryptococcus Positive**	1	1
	Cryptococcus Negative	0	76

CHAPTER 5

5 DISCUSSION

This study evaluated the use of an in house PCR method to improve the laboratory diagnostic technique for the diagnosis of TBM and CM in adult patients with chronic meningitis as compared with the routine practice of ZN staining and culture. Our method resulted in an improved time to positivity when we compared it to culture. We also evaluated different DNA extraction methods regarding their time and cost efficacy, and the influence of DNA clean up kits on the positivity rates of PCR on CSF.

5.1 Patient demographics of study population

A prospective, laboratory-based study was conducted on the cerebro-spinal fluid submitted from adult patient admitted to Tygerberg hospital with suspected TBM. Cerebro-spinal fluid specimens from a total of 78 cases were included. The average age of the patients from whom the specimens were analysed was 37 years. The gender distribution was equal with 54 % females (n=42), and 46 % males (n=36). Of those that were MTB positive 63 % (n=12) of the patients were females, and 37 % (n=7) were males. This supported a previous study which showed that TBM was more common in females in a rural valley in Kashmir. (Wani, Hussain et al. 2008). The one patient that was positive for CM was male, but due to the low occurrence of Cryptococcus in our sample series we cannot comment on this finding.

5.2 The optimization of in house PCR method using sputum samples for MTB culture

The results obtained during the optimization of the in house PCR of sputum samples showed that the PCR method was sensitive and specific, for the diagnosis of TBM in clinical samples and therefore appropriate to be applied to other samples suitable for PCR, including CSF samples. The in house PCR was also able to detect CN in CSF samples.

Table 4.1 illustrates that the number of acid fast bacilli observed by ZN stain did not necessarily determine a shorter time to culture positivity, and therefore the ZN enumeration may not be an accurate indication of the number of organisms in the specimen. One possible explanation is the presence of non viable bacteria present in the specimen that will still be detected by the ZN stain. Compared to the MTB positive culture samples, the time to PCR positivity was significantly shorter ($p<0.0001$). This improvement in turnaround time is significant for patient care, as identification of an etiological agent can assist early targeted therapy.

5.3 The calculation of the sensitivity of our in-house PCR using H37Rv as reference strain

We used a reference strain of MTB to determine the sensitivity of our PCR. We were able to demonstrate that the in - house PCR method was able to detect the equivalent of 3 mycobacteria in an in vitro testing. This is comparable with the BACTEC MIGIT 960 automated system where it has been described to detect as few as 1 to 10 viable organisms from a processed sample in less than 49 days.

5.4 Calculation of the sensitivity of our in-house PCR for cryptococcal meningitis, using ATCC 66031 *Cryptococcus neoformans* as reference strain

During the optimization of the PCR method, we were able to demonstrate that our in-house PCR method was able to amplify cryptococcal DNA in a dilution where no growth was obtained by culture. Negative controls included during our PCR analysis showed no amplification. This illustrated that PCR has the potential to be more sensitive than culture.

5.5 Results of the routine laboratory processing of the CSF samples included in this study

5.5.1 Macroscopic evaluation of the CSF

The macroscopic appearance of the CSF is an important observation as it may give an indication of disease. The volume of the fluid received, the chemistry and CSF macroscopic appearance were recorded on all the samples. It is well known that the diagnosis of TBM and CM by culture is dependent on the volume of CSF submitted to the laboratory (Thwaites, Chau et al. 2002). We could not include samples in the study that had a volume lower than 5 ml, as we had to ensure that the routine laboratory had an optimal amount of fluid to process for routine diagnostic procedures. The average volume of the samples included in the study was 6.2 ml (range from 5 to 12 ml).

Differences in the appearance of the CSF were not very helpful in predicting the samples that tested positive for MTB. In total, only four of the samples (5%) which were MTB culture positive had a macroscopic appearance suggestive of tuberculosis.

5.5.2 CSF cell count and biochemistry

We found that CSF cell count and chemistry was predictive of a positive MTB culture in only 53% of the culture positive samples included in our study. This indicates that, in our population, cell count and chemistry offers little diagnostic prediction in adult patients with tuberculous meningitis. This is in keeping with the findings of Anderson et al., that clinical feature and changes in cell count, protein and glucose in the CSF are not reliable in distinguishing one form of CM from another.

Our sample size was too small to comment on the value of CSF cell count and chemistry in the diagnosis of cryptococcal meningitis. However, there is enough evidence in the literature that points to the fact that this has limited value, especially in immune compromised patients (Karstaedt, Valtchanova et al. 1998).

5.5.3 Microscopy for Bacteriology

The number of cryptococcal cases in our study was too small to comment on the value of microscopy on the diagnosis of chronic meningitis.

5.5.4 MTB microscopy

ZN stains remain popular in the diagnosis of tuberculosis as they are fast, cheap, and relatively easy to perform. On CSF, however, the value of ZN staining is controversial as the yield is notoriously low (Kivihya-Ndugga et al., 2004). Nevertheless, the microscopic examination of CSF is important for the definitive early diagnosis of TBM, although the sensitivity is known to be low and it is very time consuming. A study by Thwaites et al. has shown that by increasing the volume of the CSF and examining the ZN slide for at least 20 minutes, the yield can be increased. In our study, ten of the twelve ZN positive slides had an average volume of between 5 and 12ml (refer to Table 4.5, Chapter 4). Many of the samples included in our study had a low volume (less than 10 ml), which may have compromised the value of the ZN stain in identifying positive samples. However, statistically there was no significance in the average volumes between the two groups of ZN positive and ZN negative samples. The sensitivity and specificity for ZN smears was 89% and 100%, and Positive Predictive Value (PPV) and Negative Predictive Values (NPV) of 100%, and 89%. Our ZN sensitivity and specificity compares well to a study performed by Caws et al. (2007) who found a sensitivity of 52.6% and 100% specificity.

Of the patients with ZN negative but MTB culture positive results, the average volume was 7 ml, slightly higher than the average of the whole sample set. Our study confirms that ZN stains are often negative in CSF samples of patients with TBM meningitis (NPV of 89%), and in many laboratories this step is omitted because of this low yield.

The higher average volume of ZN positive CSF samples in our study confirms the notion that low volumes of sample may contain less bacilli, and decreases the probability of the ZN stain to yield a positive result, as described in the literature. (Thwaites, Chau et al. 2002) The recommendation to clinicians to submit an adequate volume (10 – 20 ml) of CSF to the laboratory should increase the yield of this quick and relatively cheap investigation.

5.5.5 Bacteriology culture

For *Cryptococcus neoformans,* the initial Gram stain may show yeast like bodies, allowing presumptive diagnosis which is sensitive in 90 % of cases. Usually, such diagnoses are subsequently confirmed within 2 to 5 days by culture. Only one sample in our study was positive for cryptococcus on culture. This sample was positive on India ink stain, suggesting CM. The time to culture positivity was 2 days and cryptococcus latex was not done on this sample.

5.5.6 MTB culture

Culture methods for the diagnosis of MTB are sensitive but require 10 to 100 viable organisms per sample, and are time consuming. Culture techniques also require viable organisms, and this is a problem in partially treated patients. The time to culture positivity may take up to 8 weeks, depending on the type of specimen and the initial organism load. (De Wit, Steyn et al. 1990) In our study, we had a culture positivity rate of 24.4%. Caws et al. evaluated different culture techniques and found a sensitivity of 70.2% (Caws, Dang et al. 2007).

5.6 PCR results for *Cryptococcus neoformans*

By PCR analysis, amplification was evident in 2 of the 78 samples included in our study. This indicates a promising advantage of PCR above conventional methods, but our positivity rate for Cryptococcus was too low in this study sample (2.6%) to reach any conclusions, and should be investigated further, using a larger number of samples and a prospective study in order to better correlate results to the clinical presentation and response of the patient to treatment.

5.6.1 PCR results for MTB

Although every possible precaution was taken to avoid contamination, we did not have the resources to characterize gene products in order to exclude possible carry-over of amplicons as a possible cause for false positive PCR reactions (see section 3.7.2.2 of methods section). Positive controls included were amplifiable Our sensitivity (89%) and specificity (63%) exceeds that cited in the literature (Bergmann and Woods 1996). In a study performed by Guy E Thwaites et al, PCR as compared to other methods had a sensitivity of 79.4% and a specificity 99.6% (Thwaites, Caws et al. 2004).

5.7 DNA extraction methods

In a subset of specimens, we utilized DNA concentration. We found that by using a DNA clean up kit our results were more sensitive than other DNA methods described in our method sections.

One novel aspect of our study is the pre-culturing of specimens before submitting to PCR testing. This is particularly of potential value in paucibacillary samples like CSF. Using a combination of experimental methods, our average time to positivity was 1.9 days, showing that the main benefit of the in house PCR method was an improvement in turnaround time.

CHAPTER 6

6 CONCLUSION

It is of utmost importance to make a definite diagnosis of the etiological agent of chronic meningitis as soon as possible, in order to institute correct treatment. Ideally the diagnosis should be within hours of presentation. Microscopy for acid-fast bacilli is cheap and remains the most rapid diagnostic test, although the sensitivity depends on the volume of CSF examined. Although we made every effort during the course of our study to alert clinicians of the value of adequate volumes of CSF, our results reflect that volumes generally were still low.

Culture remains the gold standard for diagnosis of TBM, but the extended incubation required for isolation of *M. tuberculosis,* result in delayed diagnosis and treatment of patients, which may result in increased morbidity and mortality in these patients.

At present, molecular techniques (also known as Nucleic Acid Amplification Tests or NAATs) to detect *M. tuberculosis* from sputum specimens are well developed, and accepted for routine use by the American Food and Drug Association (FDA). The Amplified *Mycobacterium tuberculosis Direct* (MTD) test, (Gen-Probe), and the Amplicor *M. tuberculosis* test (Roche Diagnostics) were approved by the FDA for use on respiratory tract samples that tested positive for AFB on smears. In 1999, an enhanced test was approved for AFB negative smears and instituted in many laboratories (Dorman 2009). Molecular tests on other specimens such as CSF are increasingly being offered, and are said to be ‘coming of age’ (Dorman 2009). However, false negative results may be obtained from samples containing low numbers of *M. tuberculosis* or substances inhibiting the assay. Therefore, regardless of NAATs results, culturing of samples still remains the gold standard.

Because of the high incidence of chronic meningitis in our patient population, and because the present conventional methods mentioned are less sensitive as well as time consuming, we developed an in house PCR diagnostic method amplifying the RD9 region of *M. tuberculosis* complex with speciation based on melting point analysis, and primers specific to both *C. neoformans var. neoformans* and *C. neoformans var. gattii* were designed based on the sequence encoding the partial internal transcribed spacer 1 (ITS1) 5.8S rRNA gene and partial ITS2, for the detection of *C. neoformans,* in order to diagnose these two common etiological agents of chronic meningitis. We optimized and evaluated these tests on CSF samples obtained from adult patients admitted to Tygerberg Hospital, over a 16 month period.

The initial PCR positivity for TBM in this study was less sensitive than conventional culture, but after DNA clean up we were able to increase our sensitivity significantly. We also demonstrated that both initial incubation and DNA clean up of CSF samples increases the sensitivity of molecular tests to diagnose tuberculosis. However, a low specificity occurred in our study. This could be due to possible laboratory contamination, or to the use of culture as the gold standard of diagnosis. As this was a laboratory based study, we were not able to evaluate our results against clinical parameters. Arguably, patient outcome and response to therapy may be a better 'gold standard' against which to measure the sensitivity and specificity of our molecular methods.

Due to the low frequency of cryptococcus in the samples included in our study, we cannot comment on the diagnostic value of PCR in the diagnosis of cryptococcal meningitis when compared to more conventional methods.

The cost-efficiency of our in-house PCR should be considered taking into account the benefit of improved patient outcome brought about by diagnosing *M. tuberculosis* or cryptococcus as ethological agents of chronic meningitis within the shortest possible timeframe. In view of the significant decrease in turnaround time to positivity it can be considered to be of value. The

results of this study lead us to conclude that a cost – efficient way to implement molecular diagnostics of chronic meningitis into the routine laboratory, would be to perform PCR on filtrated CSF specimen at time naught, and, if negative, again on an aliquot of culture after 7 and 14 days of incubation.

In summary: to determine the potential value of our in house PCR method for the diagnosis of patients with chronic meningitis in CSF samples, a larger study is needed to be evaluated on a greater sample set, in order to determine the impact of these new tests on treatment and patient outcome. The inclusion of primers to screen for common genes of resistance against anti-tuberculosis drugs such as rifampicin and isoniazid in the same PCR reaction can also be investigated in further studies.

The following algorithm for application of NAATs detection of TBM has been concluded:

- Volume of CSF should be more than 10 ml
- Centrifugation to ensure concentration
- The use of 50μl aliquots for ZN and Auramine stain
- 50μl aliquots for DNA purification by SV genomic purification kit
- Remaining concentrated pellet inoculated into MGIT tube for 1 week incubation and removal of aliquots
- PCR analysis

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APPENDICES

8.1 APPENDIX A

8.2 APPENDIX B

8.3 APPENDIX C

8.4 APPENDIX D

APPENDIX A

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
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25 August 2008

Prof E Wasserman
Division of Medical Microbiology
Dept of Pathology

Dear Prof Wasserman

RESEARCH PROJECT: **"IMPROVING LABORATORY DIAGNOSTIC TECHNIQUES TO DETECT M.TUBERCULOSIS COMPLEX AND C.NEOFORMANS AS THE CAUSATIVE AGENTS OF CHRONIC MENINGITIS IN THE CEREBROSPINAL FLUID OF ADULT PATIENTS"**

PROJECT NUMBER : N07/02/040

At a meeting of the Committee for Human Research that was held on 6 August 2008 the progress report for the abovementioned project has been approved and the study has been granted an extension **for a period of one year from this date**.

Please remember to submit progress reports in good time for annual renewal in the standard CHR format.

For standard CHR forms and documents please visit:
www.sun.ac.za/knowledgepartner/committees_CHR.htm

Kind regards

pp
Prof PJT de Villiers
Chairperson: Committee for Human Research
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: mertrude@sun.ac.za

Approval Date: 6 August 2008 **Expiry Date**: 6 August 2009

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APPENDIX B

Wizard® SV Genomic DNA Purification System

1. Add 315ml of 95% EtOH to Wash Buffer prior to use
2. Heat 250µl ddH_2O/sample to 65°C
3. Ratio 1:2.5 for medium to Lysis buffer (300µl sample + 750µl Lysis buffer)

4. Transfer Lysate to minicolumn assembly
5. Centrifuge@ 13 000g for 3min, discard flowthrough
6. Add 650µl Wash Buffer
7. Centrifuge@ 13 000g for 1min, discard flowthrough
8. Repeat step 6 and 7 three times for a total of four washes
9. After last wash, centrifuge @ 13 000g for 2min to dry the binding matrix
10. Transfer column to new 1.5ml Eppi
11. Add 15µl heated ddH_2O and leave for 1 min
12. Centrifuge @ 13 000g for 1min

APPENDIX C

H37RV Sequence (accession number: NC_000962)

LOCUS NC_000962 4411532 bp DNA circular BCT 17-JAN-2006

DEFINITION Mycobacterium tuberculosis H37Rv, complete genome.

2329801 catacacgtt ccagcccatc cgggcgcacg ccaacgtcac cgcggacacg tgcggcaaca

2329861 cggtcacgtt gtcgtggccg aacagccgga tcagggtgga gccgatacca tgcaacaacg

2329921 ggtcgccgct ggcaacc**acg tgtaggtcag ccccatccgg tgacaggcct tgcaccgcgg**

5'-CAA

2329981 gcagcatcgg cgtcggccac tcccagcgct cggcggtgac ggtatcgtcg agcagggcaa

GTTGCCGTTT CGAGCC-3'(RD9 Fs)

2330041 gttgccgttt cgagccgtaa attactgtgg cc ***ctgcgcaa ttcggagcga gaatgctcgg

2330101 agagaccggt catgccgtcg gcgccgatcc cgacaacgat gatcatcggc gccgctctcc

2330161 cccgcaagcg ggcggtaccc ccaccgcatc gctgcgctct gcatcgtcgc ggatcatcgc

2330221 ggcatcctgc gccagacgaa ccggggaagc aaccgcagcg caacaaacat tggccgcagc

3'-CGTCGC GTTGTTTGTA AC-5' (RD9 int)

2330281 gcccacggaa tccacaccac gcgcttaccg ttgaccagcg cacgcgcggt cgcggcggcc

2330341 acccgctccg gggtgaccga caggggtgcg ggcgtcatgc cctcggtcat gcgcccgatg

2330401 acgaatcccg gccgcgcgat cagtaaccgc accccggtgc cgtgcaacgc atcggccagg

2330461 ccgctggcga agccgtccag gccggctttg gccgatccgt agacatagtt ggcgcggcgc

2330521 acccgaatcc cggcgaccga ggagaacacc accagcgatc cccgtccggc ggtgcgcatc

2330581 gccgctgcca gatgagtcag caggctgacc tgggcgacgt agtcggtgtg cacgatggcc

2330641 accgcgtgcg ccgcgtctgt ctcggcgcgg gcctggtcgc cgagtatccc gaaggccagc

2330701 accgcggtgc cgatggggcc gtgctcggca acgagcgaag cgaccaacgg gccgtgtgcg

2330761 gccaggtcgt cggcgtcgaa ctcccgggtg tgcaccgcta tagcgccagc tgcgcggagt

2330821 gcggcggcct ggtcggcgag ttgatcggcg ttccgcgcgg ccagcaccat cgtcgccccg

2330881 gcagccaggc gtcgcgcgag ttcgccgccg atctggctgc ggccgccgaa aattactacc

2330941 ggagcagcgc ccgtgtcgtc cacggctgcg attattgcct gcgctagcgt gagtggcgat

2331001 ggtcaacacc actacgcggc ttagtgacga cgcgctggcg tttctttccg aacgccatct

2331061 ggccatgctg accacgctgc gggcggacaa ctcgccgcac gtggtggcgg taggtttcac

2331121 cttcgacccc aagactcaca tcgcgcgggt catcaccacc ggcggctccc aaaaggccgt

2331181 caatgccgac cgcagtgggc ttgccgtgct cagccaggtc gacggcgcgc gctggctctc

2331241 actggagggt agggcggcgg tgaacagcga catcgacgcc gtgcgcgacg ccgagctgcg

2331301 ctacgcgcag cgctatcgca ccccgcgtcc caatccacgc cgagtggtca tcgaggtcca

2331361 gattgagcgc gtgctgggat ccgcggatct gctcgaccgg gcctgacaac cgaggtcatg

2331421 gcggcagtag gtaatgcacc caggcgccac cggcgggccc ggccacggcg tgcagacggg

2331481 cgttctgatt gcccgttcgg ggcagggtaa agtccgcgcc gatggctgtg caggctaggg

2331541 cagccccggc gaagaccacg ggtgccggcg tcacggtcca cctgcctgcc gcgtcccgac

2331601 aggccgcagg gtgtgggtca ccgcacgatg cggcgaccca gcggccatcc gcgccctgca

2331661 gggcgcatgc tccggcaccg gcacgcggtt cgtccggtgc ccagctccac aacgacgcct

2331721 gaatgcggcc gtcttcgggg agcagctgat cgaagccgaa cagattgacc ccgcaatcgg

2331781 tcatcgccgg caccttcggc ggggtaagcg cctgcggatt ggccggtgga cgggtcgggt

2331841 tggccaacgc cgtggccagc gtggagtcct cgtaatagcg gaccagtcgc caagcgtaga

2331901 caccgcggcc ataggtggca tcgcaggccg ggtatggccg gtagccggag ttcgagccgc

2331961 tttccagctc aacgccgctc cagtcgaaga cggcggccga ccaacctggc gcacaagacc

2332021 cgacgagcac ggctcgtgcg ccggatgcgc ggatttcctc ccgcgacacg tcgagtggaa

2332081 gcgggacaca gccgttggtg g*****cacgccggg ccgggttggg acggtagata aggcttgttc**

2332141 cgtccgcacg ccgcaacact tggtcgaggg tagccaccac cgactcatac gccgacgcgt

3’-TTGTGA ACCAGCTCCC ATCG -5’ (RD9 FR)

2332201 tcttcagctg gtcctccagg tagagcagga tgacctcctc ggtatgcccg ggtgcgttca

2332261 accagttggc gatctgcggc agcactgtgg ccagcagagg ttcgacggtg cagcctaggt

2332321 tcgcgttctt cggtcccagc ccgtgacaca cggtgacgcc gggggcgccg tggccctcga

2332381 ggcggggcaa gtagtgcagg tctagctcga gcgcgcggac gtcgatgtcg agctgttggg

2332441 ccaacgacag ctgctggttt gagtctgcgt gcgagaccgt gaacgaatcg ctgaggctgt

2332501 tgaacgagtt gtgcgtgccg agccactgag tttcccgcag cggcaccggg tcttgcaacg

2332561 catcctggaa ccgcgcggtg cgatgcaccc aagactgtag gtaggcatca cgcgcggcct

2332621 gggtcacccg gtgcgcgagc ggaagcacgc accgcgcatc gggcacaccg acgcggcgac

2332681 actccgcagc gaccgcgtcg gcgaacttgc cgagcgccac gcaggggatc gcaaccgggc

APPENDIX D

Sample Number (n=78)r	Gender	Age	Volume ml	Appearance	Biochemistry			ZN results	Culture A		CRYPTOCOCCUS		India results	RD9 PCR	CRYPTO PCR
					Protein g/l	Glucose mmol/l	Lymphs /cu mm		Result	Positivity day	culture day	latex			
1	M	14	10	CC	0.28	4.2H	53	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
2	F	31	5	CC	1.26H	2.3	21	NEG	NEG	NEG	NEG	NEG	NEG	P 28	
3	F	36	5	CC	3.23H	2.3	209	POS	POS	14	NEG	ND	NEG	1	
4	M	37	5	CC	0.43	3.7	1	NEG	NEG	NEG	NEG	ND	NEG	NEG	
5	F	72	7	T	0.62H	4.9H	77	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
6	F	33	10	CC	0.41	2.4	31	POS	POS	28	NEG	ND	NEG	1	
7	F	33	10	T	0.28	2.8	23	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
8	F	32	5	CC	2.77H	1.3L	192	POS	POS	7	NEG	NEG	NEG	1	
9	F	23	10	CC	1.76H	2.2L	4	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
10	F	54	5	CC	1.12H	ND	5	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
11	F	20	5	CC	3.52H	2.6	161	NEG	NEG	NEG	NEG	NEG	NEG	P 42	
12	M	48	5	CC	0.36	4.0H	1	NEG	NEG	NEG	NEG	NEG	NEG	P 42	
13	M	27	10	CC	2.37H	3.6	49	NEG	NEG	NEG	NEG	ND	NEG	NEG	
14	F	28	10	T	0.27	3	48	NEG	NEG	NEG	NEG	NEG	NEG	NEG	

Sample Number (n=78)r	Gender	Age	Volume ml	Appearance	Biochemistry			ZN results	Culture A		CRYPTOCOCCUS		India results	RD9 PCR	CRYPTO PCR
					Protein g/l	Glucose mmol/l	Lymphs /cu mm		Result	Positivity day	culture day	latex			
15	M	37	10	YELL	1.11H	0.7L	22	POS	POS	7	NEG	NEG	NEG	1	
16	F	25	5	CC	0.46H	3	19	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
17	M	47	5	CC	1.41	3.7	15	NEG	NEG	NEG	NEG	ND	NEG	P 1	
18	M	31	10	CC	0.42	1.3L	4	NEG	NEG	NEG	NEG	ND	NEG	NEG	
19	M	49	5	CC	0.86H	3.3	51	NEG	NEG	NEG	NEG	ND	NEG	P 1	
20	M	29	5	T	2.75H	0.9L	1110	NEG	NEG	NEG	NEG	ND	NEG	NEG	
21	F	20	5	CC	0.77H	2.6	110	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
22	M	40	5	T	ND	1.3L	185	POS	POS	21	NEG	ND	NEG	1	
23	F	39	5	C	5.00H	2.1L	803	NEG	POS	14	NEG	NEG	NEG	1	
24	M	35	5	T	1.45	2.6	110	NEG	NEG	NEG	NEG	ND	NEG	NEG	
25	M	22	5	CC	0.49H	3.1	3	NEG	NEG	NEG	NEG	ND	NEG	NEG	CRYPTO
26	F	22	5	CC	0.24	2.8	6	NEG	NEG	NEG	NEG	ND	NEG	NEG	
27	M	27	10	CC	2.00H	2.1L	110	POS	POS	7	NEG	NEG	NEG	1	
28	M	32	5	CC	0.21	3.6	0	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
29	M	42	5	CC	ND	2.6	69	NEG	NEG	NEG	NEG	NEG	NEG		
30	F	41	10	T	3.22H	0.8L	347	POS	POS	14	NEG	NEG	NEG	0	

Sample Number (n=78)r	Gender	Age	Volume ml	Appearance	Biochemistry			ZN results	Culture A		CRYPTOCOCCUS		India results	RD9 PCR	CRYPTO PCR
					Protein g/l	Glucose mmol/l	Lymphs /cu mm		Result	Positivity day	culture day	latex			
31	F	14	5	CC	0.66H	1.0L	258	POS	POS	14	NEG	ND	NEG	7	
32	M	46	5	CC	0.61H	4.5H	0	NEG	NEG	NEG	NEG	ND	NEG	NEG	
33	M	53	5	Y	4.22H	0.9L	62	NEG	NEG	NEG	NEG	POS	NEG	P 21	
34	M	63	6	CC	0.41	4.7H	1	NEG	NEG	NEG	NEG	ND	NEG	P 42	
35	F	47	5	CC	0.22	3.2	1	NEG	NEG	NEG	NEG	ND	NEG	NEG	
36	M	17	5	CC	2.37H	1.66	301	NEG	NEG	NEG	NEG	ND	NEG	NEG	
37	M	34	10	T	1.99H	3.1	8	NEG	NEG	NEG	NEG	ND	NEG	NEG	
38	F	74	5	CC	1.52H	2.28H	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
39	F	18	10	CC	1.21H	2.4	193	NEG	POS	14	NEG	ND	NEG	7	
40	M	52	5	Y	5	2.1	130	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
41	M	17	10	CC	0.53	2.8	153	NEG	NEG	NEG	NEG	ND	NEG	NEG	
42	F	61	10	T	1.69H	2.6	34	POS	POS	14	NEG	ND	NEG	NEG	
43	F	22	8	CC	1.41H	1.3L	20	POS	POS	7	NEG	ND	NEG	1	
44	F	40	7	Y	2.73H	2.7	46	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
45	M	33	5	CC	0.57H	3.1	16	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
46	M	24	12	CC	0.59	2.1	42	NEG	NEG	NEG	NEG	ND	NEG	P 28	

Sample Number (n=78)r	Gender	Age	Volume ml	Appearance	Biochemistry			ZN results	Culture A		CRYPTOCOCCUS		India results	RD9 PCR	CRYPTO PCR
					Protein g/l	Glucose mmol/l	Lymphs /cu mm		Result	Positivity day	culture day	latex			
47	F	17	5	CC	0.17	3.7	0	NEG	NEG	NEG	NEG	ND	NEG	NEG	
48	M	28	5	T	1.93H	4.2	605	NEG	NEG	NEG	NEG	NEG	NEG	P 28	
49	F	57	8	CC	0.28	3.5	2	NEG	NEG	NEG	NEG	ND	NEG	NEG	
50	F	31	10	CC	2.47	1.1L	359	NEG	POS	14	NEG	ND	NEG	1	
51	F	26	5	T	4.67H	2.4	77	NEG	NEG	NEG	NEG	NEG	NEG	P 1	
52	F	19	5	CC	0.36	2.8	0	NEG	NEG	NEG	NEG	ND	NEG	P 28	
53	F	44	10	CC	0.82H	ND	0	NEG	NEG	NEG	NEG	NEG	NEG	P 14	
54	F	26	5	CC	3.2	1.25	86	NEG	NEG	NEG	NEG	ND	NEG	P 28	
55	F	39	5	CC	4.57H	2.3	18	NEG	NEG	NEG	NEG	NEG	NEG	P 35	
56	F	36	5	CC	2.33H	0.8L	4	NEG	NEG	NEG	NEG	ND	NEG	P 28	
57	M	34	5	CC	0.38	3.3	8	NEG	NEG	NEG	NEG	ND	NEG	P 14	
58	F	42	10	CC	0.68H	1.9L	0	NEG	NEG	NEG	NEG	ND	NEG	P 1	
59	F	23	5	CC	1.70H	0.5L	60	POS	POS	14	NEG	NEG	NEG	0	
60	F	42	5	CC	0.29	3	3	NEG	NEG	NEG	NEG	NEG	NEG	P 1	
61	F	40	5	CC	0.53H	2.7	0	NEG	NEG	NEG	NEG	ND	NEG	P 1	
62	F	34	6	CC	0.52H	4.3H	5	NEG	NEG	NEG	NEG	ND	NEG	NEG	

Sample Number (n=78)r	Gender	Age	Volume ml	Appearance	Biochemistry			ZN results	Culture A		CRYPTOCOCCUS		India results	RD9 PCR	CRYPTO PCR
					Protein g/l	Glucose mmol/l	Lymphs /cu mm		Result	Positivity day	culture day	latex			
63	F	21	7	CC	5.0H	ND	6	NEG	POS	14	NEG	ND	NEG	14	
64	M	24	6	CC	0.65H	0.3L	13	NEG	NEG	NEG	NEG	ND	NEG		
65	M	36	6	CC	2.39H	0.4L	49	POS	POS	7	NEG	NEG	NEG	0	
66	M	56	7	CC	4.6H	2.4	61	NEG	POS	14	NEG	ND	NEG	7	
67	M	35	5	CC	1.21H	3.9	33	NEG	NEG	NEG	NEG	NEG	NEG	NEG	
68	M	27	5	CC	0.82H	1.3L	26	NEG	POS	21	POS	ND	POS	7	CRYP 7
69	M	33	5	Y	5.0H	0.3L	95	NEG	NEG	NEG	NEG	POS	NEG	NEG	
70	M	64	5	CC	0.11L	6.7H	0	NEG	NEG	NEG	NEG	ND	NEG	NEG	
71	M	44	10	CC	0.82H	ND	0	NEG	NEG	NEG	NEG	ND	NEG	NEG	
72	F	37	7	CC	0.67H	2.3	20	NEG	NEG	NEG	NEG	ND	NEG	NEG	
73	M	39	9	CC	0.88H	ND	157	NEG	NEG	NEG	NEG	ND	NEG	NEG	
74	F	43	5	CC	0.89H	1.8L	30	NEG	NEG	NEG	NEG	ND	NEG	NEG	
75	F	73	5	CC	5.0H	0.3L	980	NEG	NEG	NEG	NEG	NEG	NEG	P 14	
76	F	75	5	CC	0.75H	6.7	5	NEG	NEG	NEG	NEG	ND	NEG	NEG	
77	M	23	5	CC	0.28	1.5L	0	NEG	POS	21	NEG	NEG	NEG	NEG	
78	F	36	5	CC	1.05	5.5	0	NEG	NEG	NEG	NEG	NEG	NEG	28 BCG	