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Evaluation of new semi-quantitative cryptococcal antigen Immy (immunochromatographic) SQ (semi-quantitative) and Biosynex tests in plasma for detection of subclinical cryptococcal meningitis in HIV positive patients with CD4 <100.

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DECLARATION

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

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ABSTRACT

Introduction: Blood cryptococcal antigen (CrAg) titres >160 are associated with concurrent subclinical cryptococcal meningitis (CM). When lumbar puncture (LP) is not immediately available in a CrAg screening programme, semi-quantitative CrAg assays may provide risk stratification for CM.

Materials and methods: Two semi-quantitative assays (SQ [Immuno-Mycologics, Norman, OK, USA] and CryptoPS [Biosynex, Strasbourg, France]) were evaluated against a qualitative lateral flow assay (LFA) using 194 plasma samples from a cohort of HIV-seropositive individuals with CD4 counts <100 cells/μL. We compared SQ and CryptoPS results to titres for LFA-positive samples. Among patients with LP, we examined the association between semi-quantitative CrAg results and CM. We used a Cox proportional hazards model to determine the association between SQ score and mortality.

Results: Of 194 participants, 60 (31%) had positive LFA results, of whom 41 (68%) had a titre of ≤160 and 19 (32%) a titre >160. Fifty individuals with antigenaemia had an LP; a clinically-useful SQ score that identified all ten cases of subclinical CM was ≥3 (100% sensitivity, 55% specificity). Patients with an SQ score of 3 or 4 also had a 2.2-fold increased adjusted hazards of 6-month mortality (95% CI, 0.79-6.34; p=0.13) versus those with score of <3. Nine of ten patients with subclinical CM had a strong-positive CryptoPS result versus 10/40 without subclinical CM (p<0.001).

Conclusions: Semi-quantitative assays offered a sensitive though not specific means of gauging the risk of concurrent CM in this patient population.

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DEDICATION

To my late grandmother Enid Kinase

TABLE OF CONTENT

ABBREVIATIONS AND ACRONYMS

CGB	Canavinine glycine bromothymol blue
CSF	Cerebrospinal fluid
CR	Computed tomography
C I	Confidence interval
CLAT	Cryptococcal latex agglutination test
CrAg	Cryptococcal antigen
CM	Cryptococcal meningitis
EIA	Enzyme immunoassay
EDTA	Ethylene diamine tetra acetic acid
GXM	Glucuronoxylomannan
HIV	Human immune deficiency virus
LFA	Lateral flow assay
LP	Lumbar puncture
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
OD	Optical density
PCR	Polymerase chain reaction
ROC	Receiver operating characteristic curve

SDA	Sabouraud dextrose agar
SQ	Semi-quantitative
TNF	Tumour necrosis factor
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Background

Cryptococcal meningitis (CM) is an opportunistic invasive fungal disease caused by *Cryptococcus* spp. and is most common in individuals with CD4 lymphocyte count <100 cells/µl (Park et al., 2009). CM is responsible for approximately 15% of HIV-related deaths globally, with most deaths occurring in sub-Saharan Africa (Rajasingham et al., 2017). CM was responsible for more than 60% laboratory-confirmed meningitis cases identified over a four-year period in Gauteng, South Africa (Britz et al., 2016).

The World Health Organization recommended cryptococcal antigen (CrAg) screening in individuals with a CD4 count <100 cell/µl followed by pre-emptive antifungal treatment of antigenaemia to reduce CM (World Health Organization, 2011). In 2016, National Health Laboratory Service (NHLS) implemented reflex CrAg testing of remnant blood specimens with a CD4 count <100 cells/µl (Govender, 2012, Govender and Glencross, 2018). CrAg screening is currently performed using a highly accurate and FDA-approved dipstick lateral flow assay (LFA) (Immuno-Mycologics, Norman, OK) (Govender et al., 2019). CrAg screening is progressing well so far, and 99% of blood samples eligible for CrAg testing are tested (Greene et al. 2019). CrAg-positive patients who are asymptomatic for CM are treated with fluconazole to prevent progression to CM. In addition to antifungal therapy, all CrAg-positive patients are now recommended to be investigated for subclinical CM by lumbar puncture (LP) since 2019 (Govender et al., 2019)

From February 2017 to September 2020, approximately 840 000 people were screened for CrAg and the prevalence of antigenaemia was 6.1% (Greene et al. 2019). In previous studies, antigenaemia was identified as a risk for developing CM and mortality (Ganiem et al., 2014, Letang et al., 2015). Therefore, there is an urgent need to evaluate antigen quantification for stratifying the risk of progressing to CM.

CrAg can be semi-quantified using CrAg assays such as Immy LFA, cryptococcal latex agglutination test (CLAT) and enzyme immunoassays (EIA), all of which require serial dilutions. Although blood CrAg LFA titres can predict the risk of developing CM, the cost and time taken to perform serial dilutions makes this method unsuitable for routine laboratory testing in South Africa. Given these conditions, a more reasonable approach would be evaluating single-step semi-

quantitative CrAg assays for blood CrAg quantification. Since higher titres were found to predict CM, investigations could be done to establish a specific threshold in semi-quantitative assays. CrAg results within the identified threshold could be flagged as critical to alert clinicians to investigate CM. Additionally; semi-quantitative assays can assist clinicians to determine the probability of developing CM in patients with antigenaemia who are declining an LP. Although LP refusal has not been reported in South Africa, it is common in low resource settings (Thakur et al., 2015).

In this study, two semi-quantitative CrAg assays, Immy SQ assay (Immy, Norman, OK, USA) and Biosynex CryptoPS (Biosynex, Strasbourg, France) were investigated for accuracy to quantify antigen in remnant plasma samples from CrAg-screened adults with a CD4 count of <100.

1.2 Study objectives

1. To determine specificity and sensitivity of the SQ and CryptoPS assays in detecting CrAg in plasma compared to the standard LFA and enzyme immunoassay

2. To compare CrAg positive SQ scores and CryptoPS semi-quantitative results to LFA titres ≤160 and > 160.

3. To determine if SQ scores and CryptoPS semi-quantitative results can distinguish between patients with subclinical CM and patients without subclinical CM, and the association with mortality.

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CHAPTER 2: LITERATURE REVIEW

2.1 *Cryptococcus* species, history and classification

Cryptococcus is an encapsulated yeast that causes cryptococcosis, and was discovered from fermented peach juice in Italy in 1894, and later identified as a human pathogen on tibia lesions (Busse, 1894, Sanfelice, 1894). *Cryptococcus* spp. was initially classified under the genus *Saccharomyces* but was re-assigned to a new genus *Cryptococcus* due to the inability to ferment sugars (Busse, 1895). In 1951, *Cryptococcus* spp. was first identified in the environment from soil contaminated with pigeon droppings. Years later, *Cryptococcus* spp. was again detected from another environmental source, Eucalyptus trees in Australia (Ellis and Pfeiffer, 1990, Emmons, 1951)

Cryptococcus spp. grows in the lab as a unicellular oval-round yeast surrounded by a polysaccharide capsule (Benham, 1956). The capsule has been studied extensively to understand its role, and based on its structure and antigen agglutination reactions, *Cryptococcus* spp. was categorised into 5 serotypes (Evans, 1950, Wilson et al., 1968). Strains with serotypes A, D and AD hybrid were allocated to *Cryptococcus neoformans* and B and C strains to *Cryptococcus gattii*. Furthermore, through multi-locus sequence typing, *C. neoformans* and *C. gattii* are divided into 9 molecular subtypes. Molecular types VNI, VNII, VNB, VNIII and VNIV fall under *C. neoformans* and VGI, VGII, VGIII and VGIV belong to *C. gattii* (Meyer et al., 2010, Hagen, 2015).

2.2 Environmental niches

Currently, there are more than 37 *Cryptococcus* species distributed in the environment worldwide, and among these, *C. neoformans* and *C. gattii* are the main cause of cryptococcal disease in humans. Environmental sources of *C. neoformans* include soil, pigeon droppings and decaying wood (Emmons, 1955, Mitchell et al., 2010, Vanhove, 2017). *C. neoformans* serotype A molecular type VNI is the most common strain distributed in the environment worldwide (Mitchell et al., 2010). For many years, the environmental niche for *C. gattii* was known to be tropical and subtropical trees, until years later when the fir and oak trees in the Vancouver Island were identified as another source (Ellis and Pfeiffer, 1990, Kidd et al., 2004).

2.3 Pathogenesis

Cryptococcal infection to humans occurs by inhalation of basidiospores or dried yeast cells from the environment into the lungs (Wozniak et al., 2012). Infection can also occur during traumatic injury of tissue with an object contaminated with *Cryptococcus* (Christianson et al., 2003). Clinical manifestation of cryptococcosis is determined by the host's immune status. The infection can be asymptomatic for many years until reactivation occurs due to immunosuppression, or can be disseminated to other organs including the central nervous system (Wozniak et al., 2012).

When the spores reach the lungs, the host immune response is triggered and alveolar macrophages are activated to remove the pathogen through phagocytosis (Chang et al., 2004). Tumour necrosis factor (TNF), interferon-γ and interleukin-2 are also activated to form granulomatous inflammation. In addition, pulmonary surfactant proteins in the lungs bind on *Cryptococcus* spp. to opsonise uptake by phagocytes. After phagocytosis, *Cryptococcus* cells are capable of surviving inside macrophages. In most cases, infection to immunocompetent hosts can be cleared by macrophages, whereas in immunocompromised individuals, the yeast cells proliferate in the lungs and dissemination to the brain occurs resulting in meningoencephalitis (Chang et al., 2004).

2.4 Virulence factors

Cryptococcus produces several virulence factors for surviving within the host, and for evading the host immune system (Coelho et al., 2014). Among these, *Cryptococcus* polysaccharide capsule and melanin pigment are the main virulence factors (Jacobson et al., 1982, Kwon-Chung and Rhodes, 1986). The main function of the capsule is to protect *Cryptococcus* cells against phagocytosis and dehydration (Nosanchuk and Casadevall, 2006, Rosas and Casadevall, 1997). The capsule also inhibits agglutination of *Cryptococcus* cells by surfactant proteins. In order to fully understant the role of a capsule as a virulence factor, capsule genes were studied, and four genes, *CAP59, CAP64, CAP60* and *CAP10* were associated with capsule formation (Chang and Kwon-Chung, 1994, Chang et al., 1996, Chang and Kwon-Chung, 1998, Chang and Kwon-Chung, 1999). It was noted that, inactivating these *CAP* genes in *C. neoformans* strains resulted in loss of capsule and virulence. Furthermore, when avirulent acapsular mutant *C. neoformans* strains were transformed to restore the capsule through complementation, the strains became virulent to mice (Tefsen et al., 2014).

Similar to the capsule, melanin also plays an important role in protecting *Cryptococcu*s, because the pigment shields the cells from being damaged by free radicals, heat and ionisation radiation (Coelho, et al., 2014). Melanin synthesis requires the presence of *LAC1* gene encoding laccase enzymes (Pukkila-Worley, 2005). Another function of laccase enzymes is to facilitate dissemination of *Cryptococcus* to the brain (Noverr et al., 2004). The effect of melanin in *Cryptococcus* virulence was investigated and strains lacking melanin were observed to be less virulent than those that were melanised (Kwon-Chung, et al., 1982).

2.5 Epidemiology

Prior to the HIV pandemic in the 1970s, common risk factors for cryptococcosis included immunosuppressive treatment, haematological malignancies and organ transplantation. In the 1980s, the incidence of cryptococcosis increased and 80% of the cases were associated with HIV/AIDS (Hajjeh et al., 1999, Perfect, 2000). A CD4+ T lymphocyte count of <100 cell/µl was identified as a major risk factor for cryptococcosis. Although the incidence of HIV-related cryptococcosis has decreased in countries with access to antiretroviral therapy (ART), it remains high in low resource settings such as sub-Saharan Africa (Bratton et al., 2012). As a result, globally, more than 200 000 cases of HIV-associated cryptococcal infection are reported every year (Rajasingham et al., 2017). Cryptococcosis in immunocompromised individuals is mainly caused by *C. neoformans* serotypes A and D (Meyer et al., 2010). Serotype A accounts for approximately 95% of CM cases worldwide, while serotype D cases are estimated to be around 5% and are common in Europe. *C. gattii* is associated with cryptococcosis in both immunocompromised and immunocompetent individuals (Ellis and Pfeiffer, 1990)

2.6 Clinical manifestations

2.6.1 Cryptococcal antigenaemia

Cryptococcus polysaccharide capsule contains glucuronoxylomannan (GXM), a polymer that is released into the bloodstream during cryptococcal disease (Zaragoza et al., 2009). GXM capsular antigen can be detected in body fluids through antigen testing, and is identified in blood weeks to months before onset of CM (Ganiem et al., 2014, Jarvis et al., 2009). Globally, the prevalence of cryptococcal antigenaemia is around 6%, and similar findings were reported in South Africa where prevalence was estimated to be 6.1% from 2017-2020 (Rajasingham et al., 2017). Cryptococcal

antigenaemia is strongly associated with CM and mortality, and early detection followed by fluconazole treatment reduces mortality (Liechty et al., 2007).

2.6.2 Pulmonary cryptococcosis

Pulmonary cryptococcosis in immunocompetent individuals may be asymptomatic or have mild symptoms (Salyer et al., 1974). In advanced HIV disease, the infection may present as severe progressive pneumonia. Pulmonary nodules can be visualised using a computed tomography (CT) scan and chest x-ray (CXR)

2.6.3 Cutaneous cryptococcosis

Cutaneous cryptococcosis can be classified as primary or secondary, and is currently ranked as the third common clinical manifestation of cryptococcosis. Primary cutaneous cryptococcosis occurs after direct inoculation with a contaminated object, and usually presents as a single lesion at the site of injury (Christianson et al., 2003). Some of the risk factors for primary cutaneous cryptococcosis include solid organ transplantation and immunosuppression.

Secondary cutaneous cryptococcosis results from disseminated cryptococcosis, and usually presents as multiple lesions. Most cases of secondary cutaneous are caused by *C. neoformans* (Salyer et al., 1974, Wang et al., 2015). Since cutaneous cryptococcosis lesions cannot be easily differentiated from lesions caused by other infections, diagnosis is through skin biopsy with culture and histopathology.

2.6.4 Central nervous system (CNS) cryptococcosis

HIV-related CM is the most frequent clinical presentation of CNS cryptococcosis. CM occurs when *Cryptococcus* yeast cells disseminate from the lungs to the brain and spinal cord and following reactivation of latent infection. Symptoms may develop over several weeks and may include headache, nausea, altered mental status and raised intracranial pressure (Chang et al., 2004, Jarvis and Harrison, 2007). In severely immunocompromised individuals, the signs and symptoms could present within a shorter period. These patients may also have higher CrAg titres and higher intracranial pressures. Raised intracranial pressure affects 75% of patients with CM and occurs when there is a blockage of cerebrospinal fluid (CSF) outflow through the arachnoid villi resulting in building up of CSF pressure (Graybill et al., 2000)

2.7 Laboratory diagnosis of CM

Cryptococcus spp. is identified in clinical specimens using microscopy, culture, CrAg detection and polymerase chain reaction (PCR).

2.7.1 Microscopy

India ink stain is a well-known microscopic method for identification of *Cryptococcus* species in CSF. India ink stains the background leaving the *Cryptococcus* cells unstained while demonstrating the capsule as a zone of clearance (halo) around the cells to be visualised using a light microscope (Coovadia et al., 2015). Although India ink microscopy is able to provide results rapidly, sensitivity is around 86% compared to culture (Boulware et al., 2014).

2.7.2 Culture

Culturing CSF, blood, sputum, urine and other specimen types on Sabouraud dextrose agar (SDA) can primarily isolate *Cryptococcus* spp. On SDA, *Cryptococcus* spp. grows as creamy white colonies, which is not specific for genus identification as other yeasts such as *Candida* spp. also grow similar colonies (De Pauw et al., 2008). To differentiate *Cryptococcus s*pecies from other yeasts, the creamy white yeast-like colonies can be cultured on Niger-seed agar, where both *C. neoformans and C. gattii* produce phenoloxidase enzyme to synthesise melanin resulting in brown-pigmented colonies (Kwon-Chung et al., 1982). Since Niger-seed agar cannot differentiate between *C. neoformans* and *C. gattii*, both species can be sub-cultured on canavinine glycine bromothymol blue (CGB) agar for identification. On CGB agar, *C. gattii* will grow and turn the agar blue, and *C. neoformans* will not grow or cause colour change (Klein et al., 2009).

Although culture is considered the gold standard for CM diagnosis, *Cryptococcus* spp. can take days to weeks to grow on culture, especially from patients on antifungal treatment, and sensitivity depends on the volume of CSF specimen cultured (Tanner et al., 1994b). The effect of specimen volume on culture sensitivity was observed in a cohort of 832 HIV-infected persons investigated for CM in Uganda and South Africa, increasing the CSF specimen volume from 10µl to 100µl resulted in increased sensitivity from 82.4% to 94.2% (Brouwer et al., 2004)

2.7.3 Cryptococcal antigen detection

Detection of CrAg in whole blood, serum, plasma and CSF indicate cryptococcal disease. The presence of CrAg in specimens can be determined qualitatively and semi-quantitatively using three

methods, cryptococcal latex agglutination test (CLAT), lateral flow assays (LFA) and enzyme immunoassays (EIA). The principle of CrAg testing is based on *C. neoformans* and *C. gattii* antibodies coated or embedded in the assay for targeting the polysaccharide antigen in the specimen. During CrAg testing, an antigen-antibody reaction occurs, and the presence of CrAg in the specimen is detected when the antigen in the specimen binds to the antibody in the provided reagent. Depending on the CrAg assay used for testing, the results are interpreted by observing agglutination, visible lines, colour change, or spectrophotometer readings.

Latex agglutination: CrAg testing using CLAT is not simple, particularly in low resource settings as electricity is required, refrigeration of reagents and pre-treatment of specimens. CLAT can detect CrAg qualitatively and semi-quantitatively. However, for semi-quantitative CrAg testing, the specimen should be diluted in serial dilutions of 1:2 to determine titres. CLAT's sensitivity compared to the EIA is around 93% with specificity > 98% in serum and CSF (Tanner et al., 1994a, Wu and Koo, 1983). Although CLAT has been used for CrAg testing for many years, it is now less common due to lower sensitivity compared to the Immy LFA (Boulware et al., 2014). In addition, CLAT sometimes misidentifies specimens with a high antigen load as false-negative (prozone-effect). This can be resolved by performing dilutions (Stamm and Polt, 1980). False negative results can also occur when testing specimens with low fungal burden (Bloomfield et al., 1963).

Enzyme immunoassays: The EIA provides automation and objective interpretation of CrAg results as spectrophotometer readings can be used as a tiebreaker to resolve discordant results between other CrAg assays (Binnicker et al., 2012, Jarvis et al., 2020). Two commercially-available EIAs, Premier Meridian and the Immy Alpha were compared with CLAT using serum. Both EIAs were highly specific in detecting CrAg, but sensitivity was very low (55.6%) for the Premier EIA while the Alpha was 100% sensitive (Binnicker, 2012). One of the possible reasons for the low sensitivity of the Premier EIA could be that it uses polyclonal antibodies to detect CrAg, and polyclonal antibody-based assays are not able to detect all *C. neoformans* serotypes, in particular serotype C (Percival, 2011). This is confirmed in the package insert of the Premier EIA which states that this assay is specific to the *C. neoformans* antigen (serotypes A, D and AD) (Immy, Alpha, Bioscience, Premier).

Immy LFA is an immunochromatographic device for rapid detection of CrAg in body fluids. The Immy LFA was introduced over a decade ago and is highly sensitive (>99%) and very specific (>99%) in detecting CrAg in whole blood, serum, plasma and CSF compared to other CrAg assays (Boulware et al., 2014). The CrAg LFA offers a rapid turnaround time for results as specimen pre-treating is not required prior to testing. Furthermore, LFA testing is less laborious and requires no laboratory infrastructure, making this assay suitable for point of care testing (Wake et al., 2018b). For semi-quantitative CrAg LFA testing, titres are determined by performing specimen serial dilutions of 1:5. The LFA is simple and easy to use as a CrAg screening test more specifically in reflex CrAg screening of individuals with advanced HIV disease (Govender and Glencross, 2018).

Dynamiker and StrongStep LFAs: Other available LFAs include Dynamiker (Dynamiker Biotechnology, China) and StrongStep (Liming Bio, Nanjing, China). Dynamiker was compared to the Immy LFA using plasma, serum and CSF, and the sensitivity was >96% but specificity was lower (<91%) (Kwizera et al., 2017). StrongStep was also compared to the Immy LFA using CSF and plasma. The accuracy in CSF was better with 100% sensitivity and 98% specificity, and in plasma, sensitivity was 98% with low specificity of 90% (Mpoza et al., 2018).

Immy SQ is a single-step semi-quantitative dipstick assay for detecting CrAg in whole blood, plasma, serum and CSF. The SQ quantifies CrAg in specimens by grading antigen in scores of 1 to 5. A few studies evaluated the SQ to determine accuracy against the standard LFA, and to assess if the SQ scores are equivalent to LFA titres. In three separate studies that evaluated the SQ using CSF, plasma and serum, sensitivities for detecting CrAg were 100%, 98% and 98% with specificities of 100%, 93%, 98% respectively (Jarvis et al., 2020, Tadeo et al., 2021) (Skipper, 2020). Semi-quantitatively, CSF samples with SQ scores >3 were positive on culture, and SQ demonstrated the ability to detect high dose hook effect from a CSF sample with LFA CrAg-negative result with a titre of 1,310,000 and culture-positive. In plasma, SQ scores of 3 corresponded with LFA titres of 160, whereas serum SQ scores of 3 had a median LFA titre of 320. The association between SQ scores and mortality was investigated in CSF and plasma, and mortality at 14 days was 5% in patients with CSF SQ scores from 1 to 3, and 21% in patients with higher CSF SQ scores of 4 and 5. In plasma, increasing SQ scores were associated with 10-week mortality.

Biosynex CryptoPS lateral flow assay is for semi-quantitative determination and titration of the *Cryptococcus* spp. antigen in serum, plasma, whole blood and CSF. CryptoPS can detect all serotypes for *C. neoformans* and *C. gattii.* CryptoPS has two test bands, the T1 band for indicating qualitative CrAg results, and the T2 band for detecting higher CrAg titres >160. The CryptoPS assay was compared to the standard LFA for accuracy, and to investigate a relationship between semi-quantitative results and LFA titres (Skipper et al., 2020, Tenforde et al., 2020). CryptoPS had poor accuracy in detecting CrAg qualitatively, with a tendency to detect CrAg in Immy LFA CrAg-negative samples. Based on two evaluations in whole blood and serum, sensitivity in blood was 61% and 88% in serum with similar specificities around 95%. For semi-quantitative results, the median titre for CryptoPS T2 positive was 40 and 320, and in whole blood, the median CrAg titre for CryptoPS T2 results was 2560 (IQR, 1:280 to 1: 10240).

2.8 Laboratory-based reflex CrAg screening in South Africa

Previously, CrAg testing was only performed when requested by a clinician in the South African public sector. Following WHO recommendations to CrAg screen individuals with a CD4 count of <100 cells/µl, a non-clinician initiated laboratory-based CrAg screening programme was developed in 2012. A pilot was conducted for over 3 years where remnant blood samples with a CD4 counts results <100 cells/µl were reflexively tested for CrAg using the LFA. At the end of the pilot period, an analysis was performed to determine the benefits of the screening programme, and reflex CrAg screening was found to be more cost effective, and saved more lives compared to clinician-initiated CrAg testing (Larson et al., 2016). Based on these developments, CrAg screening was included in the ART guidelines in 2015, and finally implemented nationwide in 2016. Reflex CrAg testing is now performed routinely at 47 NHLS CD4 laboratories.

Antigenaemia in asymptomatic individuals is pre-emptively treated with fluconazole to reduce CM. However, even after receiving fluconazole therapy, patients with antigenaemia remain at high risk of mortality, suggesting that the treatment is not completely effective (Wake et al., 2020).

CM is treated using three antifungals, amphotericin B, fluconazole and flucytosine (Loyse et al., 2013, Sarosi et al., 1969). Although flucytosine is currently not registered in SA or in other African countries, when combined with other antifungal agents, the treatment is effective in reducing mortality (Molloy et al., 2018). CM treatment is divided into 3 phases, induction, consolidation

and maintenance. The induction phase takes two weeks: in week 1 patients are treated with amphotericin B and 4 doses of flucytosine daily, and fluconazole in week 2. Consolidation phase is 8 weeks of fluconazole, followed by maintenance phase with fluconazole for 10-12 months or until the CD4 count is >200 cells/µl and the viral load is suppressed. Raised intracranial pressure is relieved using therapeutic LPs and combination therapy of amphotericin B with either fluconazole or flucytosine (Govender et al., 2019)

Although a CD4 count of <100 cells/µl was identified as a cut-off for reflex CrAg screening, it is not including other patients with antigenaemia who have CD4 counts falling between 100 and 200 cells/µl. In a review of 21 studies, the prevalence of cryptococcal antigenaemia was 2% in patients with a CD4 count from 101-200 cells/µl (Ford et al., 2018). Also, in a randomised clinical trial conducted in Zambia and Tanzania, antigenaemia was detected from outpatients with a CD4 count <200 cells/µl who were screened for CrAg and treated with fluconazole, mortality was reduced by 28% (Mfinanga et al., 2015, Hurt, 2021). Based on the above facts, South African HIV Clinicians Society (SAHCS) guidelines now recommend CrAg screening in individuals with a CD4 count <200 cells/µl (Govender, 2019). In addition, all patients with antigenaemia are recommended to have an LP to investigate subclinical CM.

2.9 Association between CrAg titres, CM and mortality

Serial dilution CrAg titres are generally performed to investigate the high-dose hook effect or prozone-effect, a phenomenon where specimens test false negative for CrAg due to the high antigen load (Lourens et al., 2014). CrAg titres are also used to semi-quantify CrAg in specimens through identifying a titre as the highest dilution that yields a positive result. Some of the challenges for determining CrAg titres include using more reagent to perform serial dilutions, and testing requires expertise and additional resources. Performing serial dilutions is labour-intensive even with easy to use assays such as the CrAg LFA, and implementation for routine CrAg testing would be inconvenient.

High CrAg titres are associated with developing of CM and death in patients with antigenaemia (Jarvis et al., 2009). Individuals with blood CrAg titres <80 are less likely to develop CM (Beyene et al., 2017). Higher blood CrAg titres are associated with high fungal burden, a risk of CM and death (Jarvis, 2009, Letang et al., 2015). Individuals with CD4 <100 cells/µl, with CrAg titres

>160 are more likely to have disseminated cryptococcosis, and CNS involvement should be assumed in those with titres >640 even without confirmatory CSF results (Letang et al., 2015, Wake et al., 2018a). Blood CrAg titres >1280 are highly associated with CNS involvement. Although titres are not routinely performed in South Africa, patients with blood CrAg titres >160 are recommended to be monitored for signs and symptoms of CM.

In the next chapter, research results for this study are presented in the form of a published article in the Medical Mycology journal.

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CHAPTER 3: RESEARCH RESULTS
Manuscript

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Association of semi-quantitative cryptococcal antigen results in plasma with subclinical cryptococcal meningitis and mortality among patients with advanced HIV disease

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Association of semi-quantitative cryptococcal antigen results in plasma with subclinical cryptococcal meningitis and mortality among patients with advanced HIV disease

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Abstract

Introduction: Blood cryptococcal antigen (CrAg) titres >160 are associated with concurrent subclinical cryptococcal meningitis (CM). Semi-quantitative CrAg assays may provide a simple and rapid alternative to screen for CM.

Materials and methods: Two semi-quantitative assays (SQ [Immuno-mycologics, Norman, OK, USA] and CryptoPS [Biosynex, Strasbourg, France]) were evaluated against a qualitative lateral flow assay (LFA) using 194 plasma samples from a cohort of HIV-seropositive individuals with CD4 counts <100 cells/μL. We compared SQ and CryptoPS results to titres for LFA-positive samples. Among patients with lumbar puncture, we examined the association between semi-quantitative CrAg results and CM. We used a Cox proportional hazards model to determine the association between SQ score and mortality.

Results: Of 194 participants, 60 (31%) had positive LFA results, of whom 41 (68%) had a titre of ≤160 and 19 (32%) a titre >160. Fifty individuals with antigenaemia had a lumbar puncture; 10 with subclinical CM had SQ scores of 3 or 4. The optimal cut-off SQ score for predicting CM was ≥4 (93% specificity, 50% sensitivity, area under receiver operating characteristic curve 0.83). Patients with an SQ score of 3 or 4 had a 2.2-fold increased adjusted hazards of 6-month mortality (95% CI, 0.79-6.34; p=0.13) versus those with score of <3. Nine of ten patients with subclinical CM had a strong-positive CryptoPS result versus 10/40 without subclinical CM (p<0.001).

Conclusions: Semi-quantitative assays offered a simple though not completely accurate means of gauging the risk of concurrent CM and subsequent mortality in this patient population.

Lay summary: We evaluated two simple laboratory tests that can quantify the amount of cryptococcal antigen in plasma of patients with advanced HIV disease and could thus gauge the risk of concurrent meningitis and subsequent mortality. In our cohort, neither test clearly predicted meningitis or mortality.

3.1 Introduction

Cryptococcal meningitis (CM) accounts for an estimated 15% of AIDS-related deaths in sub-Saharan Africa [1]. A lateral flow assay (LFA) (Immuno-Mycologics Inc. [IMMY], Norman, OK, USA) can detect cryptococcal antigen (CrAg) in plasma, serum, whole blood and cerebrospinal fluid (CSF) very accurately [2-3]. Reflex laboratory CrAg screening using this LFA to test remnant blood samples with a CD4+ T-cell count of <100 cells/µL has been routinely performed across a national network of CD4 laboratories in South Africa since 2016 [4-5]. CrAg-positive patients are recommended to be investigated for CM since up to 40% may have subclinical CM [5]. Pre-emptive fluconazole therapy is offered to asymptomatic patients with no microbiological evidence of CM [6-7]. Despite pre-emptive fluconazole, CrAg-positive patients have a 2- to 3-fold increased risk of mortality compared to CrAg-negative patients with similar CD4 counts, suggesting that current treatment regimens may be inadequate [8-9]. Furthermore, lumbar punctures are not always offered to CrAg-positive patients, and when offered, are often declined [5,8,10]. A CrAg LFA titre in blood can stratify the risk of subclinical CM prior to lumbar puncture. A blood titre of >160 had 88.2% sensitivity and 82.1% specificity to predict concurrent subclinical CM in a cohort of CrAg-screened adults with a CD4 count of <100 cells/µL [11]. However, measuring CrAg titres using serial dilutions of CrAg-positive blood samples is costly and challenging to implement in high-volume pathology laboratories. Approximately 10 LFA strips were required for serial dilutions and an experienced technician took approximately 20 minutes to obtain a single CrAg titre result (unpublished data, National Institute for Communicable Diseases). Semi-quantitative CrAg assays could provide a simple alternative, assisting clinicians to gauge the probability of subclinical CM among people with antigenaemia prior to lumbar puncture. We evaluated the accuracy of the SQ (Immy) and CryptoPS assays (Biosynex, Strasbourg, France) compared to the currently-used LFA

in a cohort of CrAg-screened HIV-seropositive patients with a CD4 count of <100 cells/µL. We also determined the association of SQ assay results with subclinical CM and mortality.

3.2 Materials and methods

A prospective cohort study was conducted at Helen Joseph and Tambo Memorial hospitals in Johannesburg, South Africa from June 2015 through to October 2017 [9,11]. HIV-seropositive adults aged ≥18 years with a CD4 count of <100 cells/µL and no symptoms or signs of CM, who were identified during routine reflex laboratory CrAg screening, were recruited. For each CrAg-positive participant, two CrAg-negative participants were concurrently enrolled with a similar CD4 count. Lumbar punctures were performed for a subset of asymptomatic CrAg-positive patients to investigate for subclinical CM at enrolment and all participants were followed up for 6 months. CrAg LFA titres were determined using ethylene diamine tetra acetic acid (EDTA)-plasma samples stored at -70^{o}C. Concurrent subclinical CM was defined as a positive CSF India ink microscopy, CrAg test or culture for *Cryptococcus* at the time of study enrollment, when the patient was attending for the result of their screening CrAg test. The full details of the study methods, participants and relationship between titres, subclinical CM and mortality have been published elsewhere [11].

For this sub-study, we retrieved stored EDTA-plasma samples from 201 study participants and re-tested 194 samples (60 CrAg-positive; 134 CrAg-negative) at an IS0 15189-accredited reference medical laboratory. Seven samples were excluded after they were visually assessed and found not suitable for re-testing due to a high lipid content, insufficient volume, or high viscosity. All additional CrAg testing was performed as per the manufacturers' instructions. Trained laboratory personnel (N.P.B. and I.R.) performing the SQ and CryptoPS assays were blinded to previously recorded LFA qualitative and titre results. All samples were tested once with SQ and CryptoPS assays; repeat testing was later performed if the qualitative result yielded by the semi-quantitative assays was found to be different to those of the LFA. SQ testing was performed by mixing 40 µl of plasma and 1 drop of diluent in a test tube, inserting a test strip into the mixture and reading the results at 10 minutes. For SQ-positive specimens, the SQ interpretation card was used as an aid to scoring from 1 to 5 based on the presence or absence of T1 and T2 lines. For the CryptoPS assay, 20 µl of plasma and 3 drops of diluent were added to the sample well of the cassette and results

were read after 10 minutes. The presence of a T1 line was interpreted as a positive result and both T1 and T2 lines recorded as a strong-positive result. All samples were also tested using a CrAg enzyme immunoassay (EIA) (Immy), for which results were obtained using an absorbance microplate reader at 450nm and 630nm wavelengths, BioTek EL808 (BioTek instruments, Inc., Winooski, VT). Samples with optical density (OD) readings of ≤0.265 were interpreted as negative, and positive if the OD reading was >0.265.

We calculated the sensitivity and specificity (with 95% confidence intervals [CI]) of qualitative results yielded by the SQ and CryptoPS assays compared to those of the LFA. We used the LFA as the primary reference method because this assay has been validated for use in the national CrAg screening programme. We used EIA results to resolve discrepancies. We also calculated diagnostic likelihood odds ratios. For LFA-positive samples, we compared SQ scores and CryptoPS results to titres. For the sub-set of patients with CSF CrAg results, a receiver operating characteristic (ROC) curve was used to determine a cut-off SQ score for detecting subclinical CM. A chi-square test was used to assess the association between CryptoPS results and subclinical CM. We used a Cox proportional hazards model to determine the association between plasma SQ scores and mortality. A high SQ score was defined as 3 or 4 and a low score as 1 or 2. We plotted Kaplan-Meier survival curves over 6 months of follow-up for the cohort by SQ score category.

Ethics approval was obtained for the cohort study from the University of the Witwatersrand and the London School of Hygiene and Tropical Medicine. Additional ethics approval was granted by Cape Peninsula University of Technology to use the archived plasma samples to evaluate the SQ and CryptoPS assays.

3.3 Results

Of the 194 study participants included in this analysis, CrAg LFA results were positive for 60 and negative for 134. Of the 60 with antigenaemia, 41 (68%) had a titre of ≤160 and 19 (32%) a titre >160.

The sensitivity and specificity of qualitative results from the SQ assay was 98.3% (59/60 95% CI 91.1- 100) and 100% (134/134 95% CI 95.97-100) compared to the LFA (Table 1). A single LFA-positive/SQ-negative sample had a CrAg titre of 20 and was also EIA-negative. Of 41 participants with a CrAg titre of ≤160, 40 had SQ scores of 1, 2 or 3 and 1 had a negative SQ result. Of the 19

with titres of >160, 18 had SQ scores of ≥3 and 1 had an SQ score of 2 (Figure 1). Of 60 participants with antigenaemia, 50 had a lumbar puncture performed (Table 2). Of 10 who were diagnosed with subclinical CM, 5 had titres ranging from 1280 to 163840 and an SQ score of 4. The other 5 had titres that ranged from 80 to 327680 with an SQ score of 3. Of the 40 without subclinical CM, 22 (55%) had an SQ score of <3 and 18 (45%) had a score of ≥3. The area under the ROC curve for the association between SQ score and subclinical CM was 0.85 (95% CI, 0.75-0.95) (Figure 2). The optimal cut-off SQ score for predicting subclinical CM was ≥4 with 93% specificity and 50% sensitivity (Table 3 and Figure 2). Patients with a plasma SQ score of ≥3 had a hazard ratio for death within 6 months of 2.20 (95%CI, 0.79-6.34; p=0.13) compared to those with a low score, after adjusting for CD4 count (Figure 3 and 4).

In contrast, the CryptoPS assay had 90% sensitivity (54/60; 95%CI, 79.49-96.24) and 94.8% specificity (127/134; 95%CI, 88.66-97.41) compared to LFA for qualitative results (Table 1). Thirteen samples had discordant LFA and CryptoPS qualitative results. Of the 6 CryptoPS-negative/LFA-positive samples, 3 had titres of 10, 1 had a titre of 5 and 2 had titres of <5. The EIA confirmed 5 of these samples as CrAg positive. The sixth sample was EIA-negative and had a titre of <5. Of the 7 CryptoPS-positive/LFA-negative samples, all sample dilutions were LFA-negative and the EIA was negative. Of 41 participants with a positive LFA result and a titre of ≤160, 6 had negative CryptoPS results, 29 had positive results and 6 had strong positive results. Of the 19 with a positive LFA result and a titre of >160, 15 (78%) had strong positive CryptoPS results; the other 4 had positive CryptoPS results (Table 4). Nine of ten patients with subclinical CM had a strong-positive CryptoPS result versus 10/40 without subclinical CM (p<0.001). Compared to a lumbar puncture diagnosis of subclinical CM, the sensitivity and specificity of a strong-positive CryptoPS result was 90% (9/10; 95% CI, 55.5-99.7) and 75% (30/40; 95%CI, 58.8-87.3) respectively.

3.4 Discussion

In this re-evaluation of stored plasma samples from a prospective cohort study, the SQ assay was simple and rapid to perform with identical testing steps to a single-strip qualitative LFA and an equivalent accuracy for CrAg detection in stored plasma. Using a cut-off score of ≥3, the SQ assay detected all cases of subclinical CM but had very low specificity. A cut-off score of ≥4 reduced the sensitivity of this assay substantially but was much more specific for CM. Although the

CryptoPS assay did not perform well as a qualitative assay compared to the LFA, a strong-positive result was a sensitive screening test for subclinical CM; this was also not specific.

As previously reported, the SQ assay accurately detected CrAg compared to the LFA [12-13]. In our study, only one low-titre sample had a negative SQ result. In contrast, the CryptoPS assay incorrectly classified a higher proportion (5/33; 15%) of low-titre samples as negative and also yielded 7 false-positive results. All 7 participants with false-positive CryptoPS results did not receive antifungal treatment and none progressed to CM during 6 months of follow-up. In an evaluation of the same assay in Botswana, a lower sensitivity (61%) but higher specificity (97%) was reported. In this study, 29 patients with false-positive CryptoPS results were followed up for 3 months and none developed CM [14].

Since a blood CrAg titre cut-off of >160 had previously been identified as a threshold for subclinical CM [3,11], we evaluated the accuracy of the two semi-quantitative assays in categorising plasma samples with a titre ≤160 and >160. All samples but one with an SQ score of <3 had a titre of <160. All samples with a score of >3 had a titre of >160. However, a score of 3 did not clearly distinguish between these two titre categories. Although a strong-positive CryptoPS result identified 76% of samples with a CrAg titre of >160, 18% of samples with a titre of ≤160 also had strong-positive results. In their evaluation, Tenforde et al also reported a single LFA-negative plasma sample with a strong-positive CryptoPS result.[15]

We went on to investigate the relationship between plasma semi-quantitative assay results and subclinical CM. Semi-quantitative CrAg assays performed on blood are not a substitute for lumbar puncture which is universally recommended for all patients with a new diagnosis of antigenaemia. However, such assays could be used to refine the pre-test probability of subclinical meningitis, especially at the primary healthcare level where patients need to be referred to hospital for a lumbar puncture or in settings where a high proportion of patients decline lumbar puncture. A score of ≥4 was highly specific for concurrent CM, though half of the cases in our study were missed at this threshold. A lower threshold (score ≥3) included all cases of CM but also included 18/40 (45%) of cases without subclinical CM. Based on a sample of 189 screened patients, approximately one third of who had lumbar punctures, the aforementioned Botswana study reported a strong association between plasma SQ score and central nervous system involvement at baseline (a

composite endpoint of microbiologically-confirmed CM and/or clinical signs of meningitis).[12] Individuals with SQ scores of 4 were classified as being at highest risk of central nervous system involvement and mortality, with a recommendation for inpatient management. In our study, a strong-positive CryptoPS result picked up 9 of 10 cases of subclinical CM but also 10/40 (25%) of those without CM. The lack of a clearer association between the semi-quantitative CrAg results and subclinical CM in our study might be explained by a smaller sample size since we restricted our analysis to patients who had a lumbar puncture. We found that CrAg-positive patients with an SQ score of ≥3 had a more than two-fold increase in mortality though the 95% CI was wide and spanned 1. In contrast to Jarvis et al, we did not find that the hazards of death increased with each step-wise increase in SQ score. Again, this may be related to a smaller sample size.

Compared to testing serial sample dilutions to obtain LFA titres, the SQ assay was a much less laborious method of obtaining a semi-quantitative CrAg result using a single test strip. SQ testing was easy to perform but reading the SQ test strip was more complex and the result interpretation card was needed to obtain an SQ score. For instance, for a CrAg-negative result, only a control line is positive with the LFA, whereas with the SQ assay, both the control and the T2 lines are positive. Moreover, grading of scores 1, 2 and 3 is determined by comparing the intensity of the T1 and T2 bands. Through an inter-laboratory comparison, we have identified reading and interpretation errors for the LFA in the national CrAg screening programme and expect that the complexity of reading the SQ test strips would increase the proportion of erroneous readings. Using automated readers to read the SQ test strips could be considered to prevent such errors. Automated readers may also offer an advantage of interfacing CrAg results to the laboratory information system, and therefore reduce transcription errors. In contrast, the CryptoPS cassette design provided a simple method of semi-quantitative CrAg testing without using tubes. Results were available within 10 minutes and interpretation was very simple.

The strengths of this study included a prospective cohort design and enrolment of consecutive eligible CrAg-positive individuals. Patients with asymptomatic antigenaemia were enrolled after excluding those with clear symptoms and signs of CM and a relatively large proportion of patients (25%) had a baseline lumbar puncture. The main limitation was a relatively small sample size. In addition, we tested stored rather than fresh plasma. To determine if freezing and thawing of plasma had an effect on SQ scores, fresh plasma samples that were tested using the SQ assay were stored

in a -70ºC freezer for >6 months. The samples were re-tested after 6 months and fresh and frozen-thawed plasma SQ results were compared. There was excellent result concordance between fresh plasma SQ scores and frozen-thawed SQ scores (unpublished data, NICD).

3.5 Conclusions

Although not a substitute for lumbar puncture, semi-quantitative assays offered a simple rapid means of estimating plasma CrAg concentrations. However, the associations between the semi-quantitative assay results and concurrent subclinical CM and mortality were not clear in our study. These assays should not be implemented in routine screening programmes until more prospective data have accumulated from diagnostic intervention studies.

3.6 Conflicts of interest: Nil

3.7 Author contributions:

- Conceptualisation: R.W., N.P.G., N.P.B.
- Laboratory testing: N.P.B., I.R.
- Student supervision: N.P.G., Y.P.
- Data analysis and interpretation: N.P.B., R.W., N.P.G.
- Drafting and revising manuscript: N.P.B., N.P.G.
- Manuscript review: All authors

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3.11 Tables and figures

Table 1: Sensitivity and specificity of the SQ and CryptoPS assays for detection of CrAg in frozen-thawed plasma from HIV-seropositive patients with a CD4 count of <100 cells/µL compared to a reference lateral flow assay

Statistic	**SQ assay**		**CryptoPS assay**	
		95% CI		95% CI
Sensitivity (%)	**98.33**	91.06- 99.99	**90**	79.49-96.24
Specificity (%)	**100**	97.28-100	**94.1**	89.5-97.9
Positive likelihood ratio	**>100**		**15.19**	7.71-29.90
Negative likelihood ratio	**0.02**	0.00-0.12	**0.11**	0.05-0.23

Note: Cryptococcal antigen (CrAg) enzyme immune-assay (EIA) results for the 194 participants were positive for 58 and negative for 136. Compared to the EIA, the sensitivity and specificity of the semi-quantitative (SQ) qualitative results were 100% (58/58) and 99% (135/136) whilst Crypto PS had 91.4 % (53/58) sensitivity and 93.4% (127/136) specificity.

Table 2: Association between SQ assay scores, titres and subclinical cryptococcal meningitis (CM) for 50 patients with antigenaemia and lumbar puncture results

SQ assay score	Samples n=50	CrAg LFA titre ≤160 (n=33)	CrAg LFA titre >160 (n=17)	Subclinical CM (n=10)	No subclinical CM (n=40)
Negative	0	0	0	0	0
1	14 (28 %)	14 (42%)	0	0	14 (35%)
2	8 (16%)	7 (21%)	1 (6%)	0	8 (20%)
3	20 (40%)	12 (37%)	8 (47%)	5 (50%)	15 (37%)
4	8 (16%)	0	8 (47%)	5 (50%)	3 (8%)

Table 3: Sensitivity and specificity of SQ assay score cut-offs from ≥1 to ≥4 for detecting subclinical cryptococcal meningitis among asymptomatic CrAg-positive patients with lumbar puncture results (n=50)

Cut point	**Sensitivity %**	**Specificity**	**Positive Likelihood Ratio**	**Negative Likelihood Ratio**
≥1	100	0	1.0000	
≥2	100	35	1.5385	0.0000
≥3	100	55	2.2222	0.0000
≥4	50	92.5	6.6667	0.5405
5	0	100		1.0000

Receiver Operating Characteristic area = 0.85 (95% CI 0.75-0.95)

Table 4: Association between CryptoPS, lateral flow assay titres and subclinical CM for 50 patients with antigenaemia and lumbar puncture results.

CryptoPS assay results	**Samples (n=50)**	**CrAg LFA titre ≤160 (n=33)**	**CrAg LFA titre >160 (n=17)**	**Subclinical CM (n=10)**	**No subclinical CM (n=40)**
Negative	5 (10%)	5 (15%)	0	0	5 (12%)
Positive	26 (52%)	22 (67%)	4 (24%)	1 (10%)	25 (63%)
Strong positive	19 (38%)	6 (18%)	13 (76%)	9 (90%)	10 (25%)

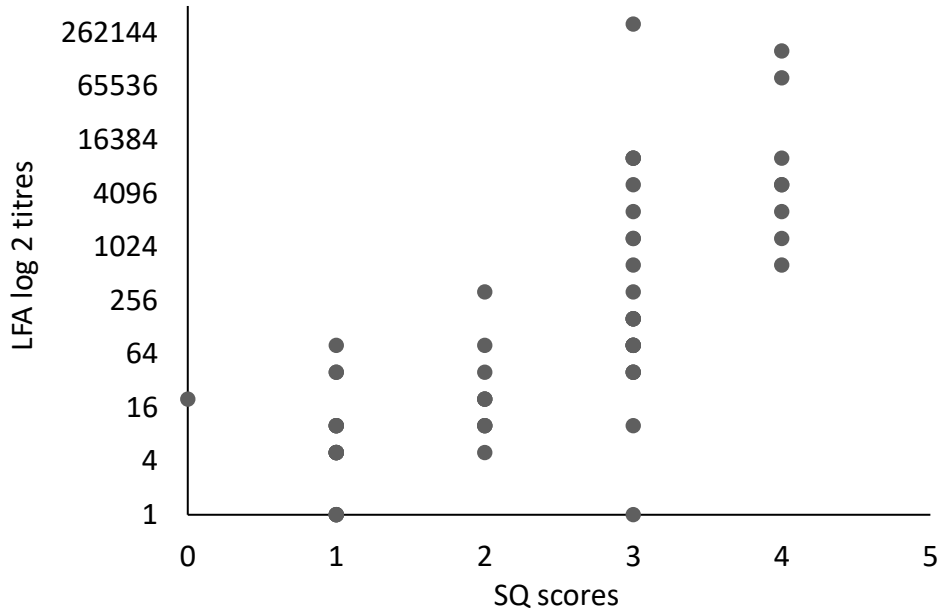

Figure 1: SQ assay scores compared to lateral flow assay (LFA) log2 titres from HIV-seropositive patients with a CD4 count of <100 cells/µl

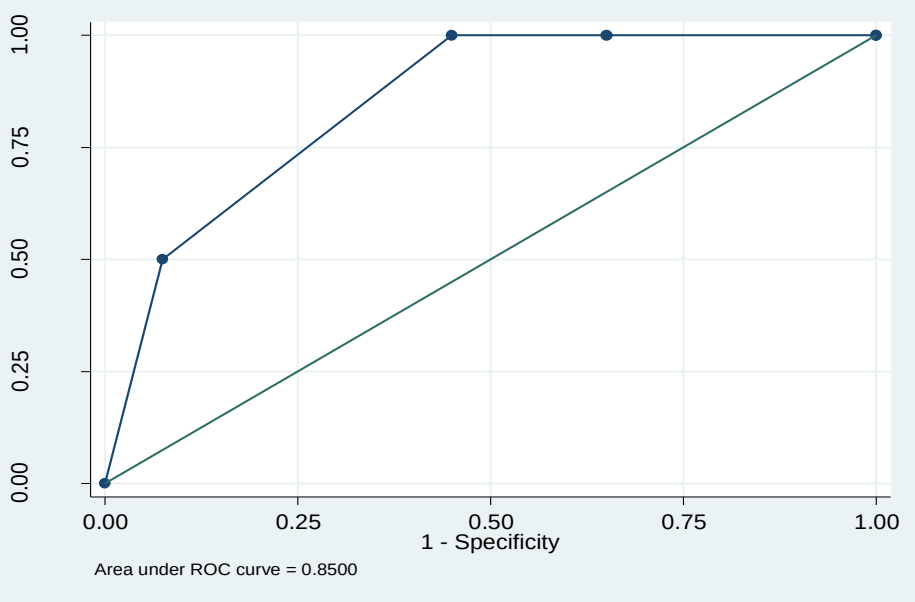

Figure 2: A receiver operative characteristic curve for plasma semi-quantitative (SQ) scores and subclinical cryptococcal meningitis among 50 asymptomatic cryptococcal-antigen positive patients.

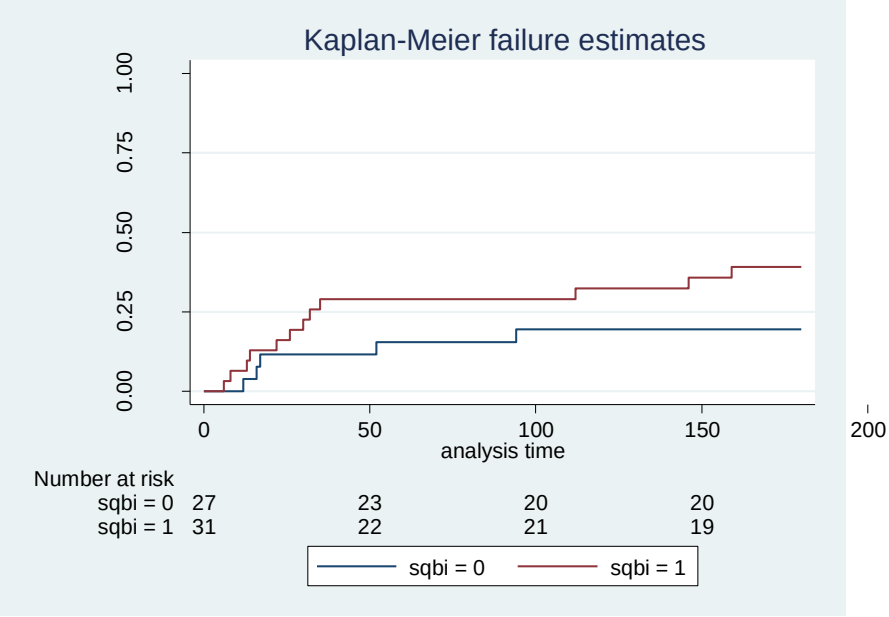

Figure 3: Kaplan-Meier survival estimates in 58 CrAg-positive patients by plasma SQ assay score category (0: score of ≥3 and 1: score of <3)

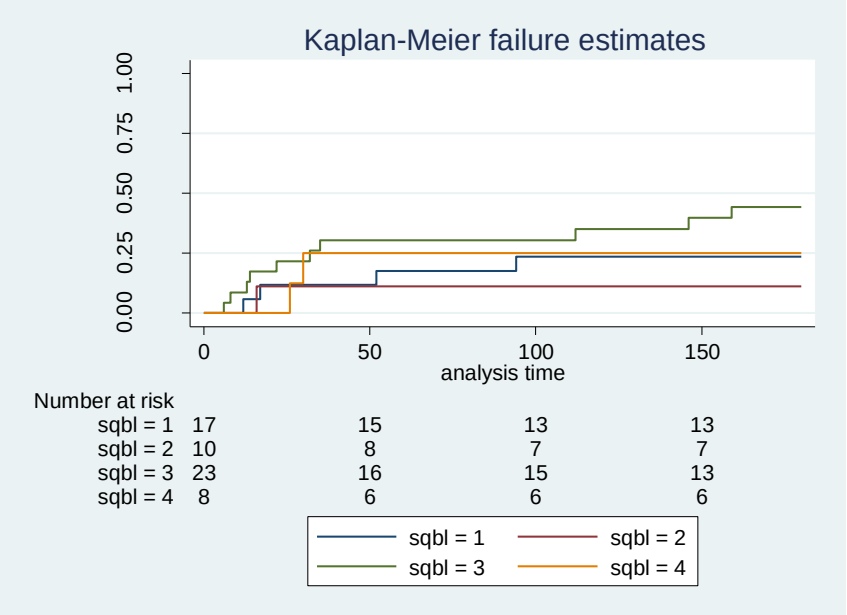

Figure 4: Kaplan-Meier survival curve in 58 CrAg-positive patients by plasma SQ score category, adjusted for CD4 count (hazards ratio of mortality for SQ score of 2 versus 1: 0.45 (95%CI 0.05-4.07); SQ score of 3 versus 1: 2.04 (95%CI 0.63-6.61); SQ score of 4 versus 1: 1.00 (95%CI 0.28-5.85))

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

CM remains one of the leading causes of HIV-associated deaths in sub-Saharan Africa (Park et al., 2009, Rajasingham et al., 2017). Detecting cryptococcal antigenaemia followed by treatment with antifungals reduces progression to CM and mortality (Govender et al., 2019). Commercially available CrAg screening assays can be used to detect the presence of CrAg in blood, but require serial dilutions to quantify CrAg levels. Quantification of blood CrAg levels can help to predict cryptococcal disease status (CM vs no CM), and future progression of disease and future risk of dying. As a result, research is being conducted across the continent to evaluate single-step semi-quantitative assays against the standard lateral flow assay (LFA) (Jarvis et al., 2020, Skipper et al., 2020, Tenforde et al., 2020).

Blood samples that were tested in this research were collected in a cohort study conducted from 2015 to 2017 in Johannesburg, South Africa (Wake et al., 2018). Study participants were CrAg-screened individuals with a CD4 count <100 cells/µl, and were identified from 2 hospitals and assessed for symptoms of CM. Cerebrospinal fluid (CSF) from those with antigenaemia and a headache were tested for the presence of *Cryptococcus* spp.. A relationship between antigenaemia and CM was investigated through determining CrAg lateral flow (LFA) serial dilution titres. A titre >160 had 88.2% sensitivity and 82.1% specificity of predicting CM.

In this sub-study, we evaluated accuracy for testing CrAg in plasma using single-step semi-quantitative CrAg assays, Immy semi-quantitative (SQ) (Immy, Norman, OK, USA) and CryptoPS (Biosynex, Strasbourg, France) assays. The SQ and CryptoPS assays can detect CrAg in plasma, serum, CSF and whole blood; however, we focused on evaluating accuracy of these assays in plasma, a sample type that is tested in the South African CrAg screening programme. Remnant plasma samples from the cohort study were re-tested using the SQ and CryptoPS assays and results were compared to the cohort standard LFA results. The turnaround time for testing and results interpretation for both the SQ and CryptoPS was determined. Since a titre of > 160 predicted subclinical CM and mortality in the cohort, we also tried to establish a similar threshold within the semi-quantitative CrAg assays. For the SQ assay, semi-quantitative results are numerical values of scores from 1 to 5; therefore, a receiver operating characteristic (ROC) curve was used to establish a cut-off score for predicting subclinical CM. Using a ROC curve was not necessary for

the CryptoPS since it has a pre-established cut-off and detects CrAg titres > 160 as Strong positive. Accuracy of Strong positive results was investigated by comparing sensitivity and specificity against titres ≤ 160 and titres >160.

To determine the turnaround time, time taken to detect a CrAg result in plasma using the SQ and CryptoPS assays was compared to time taken to obtain an LFA result and a serial dilution titre (20 minutes). The SQ and CryptoPS assays provided results more rapidly than the LFA titre, testing each sample took approximately 1 minute, and a result was ready in 10 minutes. Initially, result interpretation for the SQ assay was difficult without using the result interpretation card, but became easier with more practise as testing continued. CrAg result interpretation was straightforward with the CryptoPS assay.

Further investigations of semi-quantitative CrAg assays included determining the stability of semi-quantitative results by testing and comparing fresh plasma and frozen-thawed plasma. The SQ showed excellent result concordance between fresh plasma SQ scores and frozen-thawed plasma SQ scores implying that this assay may not be affected by freezing and thawing of specimens. For the CryptoPS assay, the freezing and thawing effect was not investigated, as it would have been challenging to identify increasing or decreasing Strong positive results.

Even though the main goal of this study was to assess accuracy of semi-quantitative assays in quantifying CrAg in plasma, qualitative accuracy was also very important. A good semi-quantitative CrAg screening test should be reliable in detecting CrAg qualitatively and semi-quantitatively. Moreover, the routinely used standard LFA is very reliable and detects CrAg in plasma qualitatively with high sensitivity and specificity (> 98%) compared to the EIA (Jarvis et al., 2011). Looking at the performance of both the SQ and the CryptoPS, the assay that demonstrated high accuracy close to the LFA was the SQ. The CryptoPS assay detected CrAg far less accurately.

This research provided an opportunity to learn about blood antigen quantification using rapid semi-quantitative assays. A pattern demonstrating a relationship between LFA titres, semi-quantitative results and CM was identified from the results, suggesting that semi-quantitative CrAg assays could be used to stratify the risk of developing CM in patients where lumbar punctures are not possible. However, findings from the study were obtained from a small sample size; therefore,

more studies are recommended to evaluate accuracy of SQ and CryptoPS assays in fresh plasma and other sample types.

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APPENDICES

Appendix A: Ethics certificate, University of Witwatersrand

R14/49 Dr Rae Wake et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M141177

NAME: (Principal Investigator)	Dr Rae Wake et al
DEPARTMENT:	National Institute of Communicable Disease Tambo Memorial Hospital, Helen Joseph Hospital, Free State Primary Care HIV Clinics and Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Optimising the Clinical Impact of Cryptococcal Antigen Screening and Management of Asymptomatic Human Immunodeficiency Virus Infected Individuals
DATE CONSIDERED:	28/11/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Nelesh Governder, Prof Thomas Harrison and Dr Joseph Jarvis
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	22/06/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature

Date 22.6.15

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: Ethics Certificate, Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)

Registration Number NHREC: REC- 230408014

P.O. Box 1906 □ Bellville 7535 South Africa

Symphony Road Bellville 7535

Tel: +27 21 959 6917

Email: simonsy@cput.ac.za

5 December 2019

REC Approval Reference No: CPUT/HW-REC 2019/H28

Dear Ms Nozuko Precious Kinase

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Ms Nozuko Precious Kinase for ethical clearance on 5 December 2019. This approval is for research activities related to student research in the Department of Informatics of this Institution.

Title: **Evaluation of new semi-quantitative cryptococcal antigen Immy (immunochromatographic) SQ (semi-quantitative) and Biosynex tests in plasma for detection of subclinical cryptococcal meningitis in HIV positive patients with CD4 < 100**

Supervisor: Ms Y Prince, Dr S Hector and Dr N Govender

Comment

Approval will not extend beyond 6 December 2020. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Dr. Navindhra Naidoo
Chairperson – Research Ethics Committee

Faculty of Health and Wellness Sciences

Appendix C: Ethics certificate (renewal) Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HWS-REC)

Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa

Symphony Road Bellville 7535

Tel: +27 21 959 6917

Email: simonsy@cput.ac.za

22 April 2021

REC Approval Reference No: CPUT/HW-REC 2019/H28/renewal

Dear Ms Nozuko Precious Kinase

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Ms Nozuko Precious Kinase for ethical clearance on 5 December 2019. This approval is for research activities related to student research in the Department of Informatics of this Institution.

Title: **Evaluation of new semi-quantitative cryptococcal antigen Immy (immunochromatographic) SQ (semi-quantitative) and Biosynex tests in plasma for detection of subclinical cryptococcal meningitis in HIV positive patients with CD4 < 100**

Supervisor: Ms Y Prince, Dr S Hector and Dr N Govender

Comment

Approval will not extend beyond 23 April 2022. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Ms. Carolynn Lackay
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences