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An evaluation of the analytical performance of the Dynamiker ® Fungus (1-3)- ß-D- Glucan assay in comparison to the Cape Cod Fungitell ® 1,3 beta-D glucan assay for the diagnosis of invasive fungal infections

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AN EVALUATION OF THE ANALYTICAL PERFORMANCE OF THE DYNAMIKER® FUNGUS (1-3)-*B*-D-GLUCAN ASSAY IN COMPARISON TO THE CAPE COD FUNGITELL® 1,3 BETA-D-GLUCAN ASSAY FOR THE DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS.

Dr Shalini Pillay

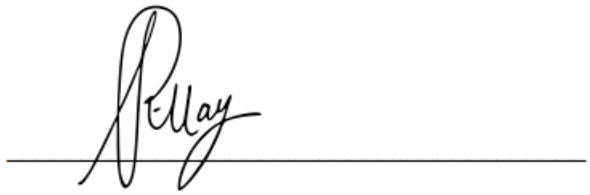
Student number 1238856

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Medicine in Clinical Pathology

Johannesburg, April 2021

Declaration

I Shalini Pillay declare that this Research Report is my own, unaided work. It is being submitted for the Degree of Master in Medicine in Clinical Pathology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

23 day of April 2021 in Johannesburg

Dedicated to my parents,

Kriba and Lilly

Presentations arising from research project

The content of this research project has not been presented at a recognised congress.

Publications arising from research project

The content of this research project has not been published in an accredited journal at present. The author and supervisors wish to submit this work for publication in the future.

Abstract

Background:

Left untreated invasive fungal infections (IFI) carry a high mortality rate. The broad fungal biomarker 1,3 beta-D-glucan (BDG) has become increasingly recognized as an adjunct diagnostic test, with the capability of producing results earlier than gold standard culture and is useful in directing clinicians to appropriate initiation of therapy. The Associates of Cape Cod Fungitell® is a widely used BDG assay however it serves predominantly larger laboratories. Alternative options should be sought to implement at lower throughput laboratories to avoid a delay in diagnosis.

Objectives:

We aimed to evaluate the performance of Dynamiker® Fungus assay in comparison to Fungitell® and to assess its precision in our setting.

Methods:

In this prospective laboratory-based study 100 patient serum samples were analysed on both Fungitell® and Dynamiker®. Quantitative and qualitative results were compared to Fungitell®. Additionally, both assays were assessed against gold standard culture and/ or *Pneumocystis jirovecii* PCR (PJP PCR) (60/100). The Dynamiker® precision was determined over 5 days, using the manufacturer's standard material.

Results:

The observed agreement was 74% (80% with a cut-off of 96 pg/ml), with a moderate categorical agreement (Kappa= 0.582, $P < 0.05$). Sensitivity and specificity were 63.64% and 92.86% respectively when compared to Fungitell®, and 75% and 69% for fungal culture respectively. Dynamiker® had excellent repeatability and reproducibility with no significant difference to the manufacturer's means.

Conclusion:

Although Dynamiker® was found to be less sensitive than Fungitell®, it is precise with moderate overall categorical agreement. Further clinical studies are warranted.

Acknowledgements

I would like to acknowledge the Clinical Microbiology and Infectious Disease (CMID) technologists (Thebe Sephapo and Simphiwe Mbatha) for their support and participation in the study. In addition, I would like to thank Dr. Vindana Chibabhai for her patience and supervision during the write up.

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Nomenclature

IFI	Invasive fungal infections
BDG	1,3 beta-D-glucan
Fungitell	The Associates of Cape Cod Fungitell® 1,3 beta-D-glucan assay
Dynamiker	Dynamiker® Fungus assay
PCR	Polymerase chain reaction
PJP PCR	*Pneumocystis jirovecii* polymerase chain reaction
CMID	Clinical Microbiology and Infectious Disease
HIV	Human Immunodeficiency Virus
TB	Tuberculosis
TAT	Turn-around-time
POCT	Point of care test
EORTC/ MSG	European organization for research and treatment of cancer/ invasive fungal infections cooperative group and the national institute of allergy and infectious disease mycoses study group
FDA	Food and Drug Administration
CFDA	Chinese Food and Drug Administration
OD	Optical density
pg/ml	Picograms per millilitre
NHLS	National Health Laboratory Service
IBM SPSS	International Business Machines Corporation Statistical Package for the Social Sciences
CV	Coefficient of variation
SD	Standard deviation
CI	Confidence interval
*S. pneumonia*e	*Streptococcus pneumoniae*
ICU	Intensive care unit
ul	Microlitre

CHAPTER 1

1. Introduction:

The incidence of invasive fungal infections (IFI) has increased worldwide[1–3]. The true burden of IFI in South Africa is likely underestimated due to lack of access of relevant diagnostic tests to clinicians based in peripheral hospitals[4]. IFI in South Africa are largely due to the relationship with HIV co-infection, tuberculosis (TB) and immune suppression secondary to malnutrition as result of poverty[5]. Invasive fungal infections (IFI) secondary to *Candida* species, *Pneumocystis jirovecii* (causing *Pneumocystis jirovecii* pneumonia or PJP), *Cryptococcus* species and opportunistic mould infection including *Aspergillus* species are among the commonest causative organisms in South Africa[5]. Risk factors include long-term steroid use, immunosuppressive drugs or radiotherapy, haematopoietic stem cell transplant, solid organ transplant, diabetes mellitus and HIV[1,5,6].

Currently, the diagnosis of IFI remains challenging. The clinical features and radiological signs may be non-specific or absent depending on the stage of disease, the immune response mounted by the patient and site of infection. In addition, the gold standard, culture-based diagnostics, are associated with a long turn-around-time (TAT) and fungal cultures may only be positive in ~50% of cases of candidiasis and aspergillosis[1,7]. Although histological diagnosis by means of invasive procedures has improved sensitivity, it is often technically difficult and thus only performed by trained individuals[2,8] .

If left untreated, IFI may be associated with an increase in mortality. Delays in reaching a diagnosis has been shown to impact the time to appropriate initiation of therapy ultimately leading to an increase in mortality rates[1,2,9]. Clinicians may therefore have a lower threshold to institute or de-escalate empiric antifungal therapy in high risk patients whilst awaiting fungal culture results, contributing towards the global increase seen in antifungal resistance[2,9], collateral damage[9], increase in hospital care costs[9] and possible delay in establishing the true diagnosis.

The landscape of the diagnostic microbiology laboratory is ever evolving, and non-culture-based diagnostic tests have grown in favour by offering an alternative less invasive sampling method with favourable diagnostic accuracy and rapid TAT. They have been shown to improve patient management and antimicrobial stewardship[3,10]. Fungal diagnostics have moved towards this advancement, with the emergence of molecular tests and biomarkers such as the 1,3 beta-D-glucan (BDG). In a local South African study assessing the sensitivity of BDG for candidaemia, the investigators also established the tests earlier time to positivity (averaging 2.5 days) when compared to culture-based diagnostic tests[11], elucidating one of the test's major advantages in promoting antifungal stewardship in the critical care setting[10].

BDG test is a broad fungal biomarker used as an adjunct in the diagnostic workup of IFI, and has been included in the European organization for research and treatment of cancer/ invasive fungal infections cooperative group and the national institute of allergy and infectious disease mycoses study group (EORTC/ MSG) revised definitions for probable IFI.[12]

The fungal cell wall is comprised of polysaccharides, of which glucan is the most abundant. The principle of the BDG test exploits the coagulation cascade of the North American horse-shoe crab amoebocyte. Glucan present in the test sample initiates the clotting pathway by activating coagulation factors. The rate of production of the chromogen released downstream is directly proportionate to concentration of BDG in the specimen [8]. Beta-D glucan levels may be elevated in invasive candida infection (with highest sensitivities for *C. albicans* and lowest for *C. parapsilosis*)[13], aspergillosis, *Pneumocystis jirovecii* (PJP), but due to the lack of BDG in the cell walls of some fungi, is not diagnostically useful in Zygomycetes, Blastomyces dermatitidis and cryptococcosis[8]. Caveats of BDG include false positive results due to antibiotics (beta-lactam/ beta-lactamase inhibitor combinations such as piperacillin-tazobactam and amoxicillin-clavulanate), bacteraemia caused by *S. pneumoniae*, BDG containing surgical gauze, blood tubes, cellulose-containing haemodialysis membranes and blood products such as immunoglobulin and plasma[8]; and false negatives may occur secondary to interferences (haemolysis, icterus or lipaemia)[14], low fungal load in the case of

early infection and concurrent use of antifungal therapy[14], and candidaemia secondary to *C. parapsilosis*[13].

The first BDG glucan assay to be Food and Drug Administration (FDA) approved was the Fungitell® 1,3 beta-D glucan assay (Associates of Cape Cod, Massachusetts, USA) (Fungitell), which has the capability to run 96 well plates in a single run (21 patient samples).

One alternative BDG, the Dynamiker® Fungus assay (Dynamiker Biotechnology, Tianjin, China) (Dynamiker) is Chinese Food and Drug Administration (CFDA) approved, and works on a similar test principle as Fungitell, with the additional advantage of a positive control with each batch, the ability to run smaller sample numbers on 12 x 8 well breakable plates[15] and improved cost per test.

In a local study at a tertiary public-sector laboratory, BDG testing had increased 6-fold from 2009 to 2017[16]. Increasing the availability of the test at peripheral sites may be valuable for patient care as well as reduce the need for transport of specimens to referral laboratories, the backlog of testing and batching of samples, which ultimately compromises TAT. The introduction of the single patient BDG test Fungitell STAT™ (Associates of Cape Cod)[17], has been a reminder of the continued vested interest in seeking alternative diagnostic assays which can improve TAT (Fungitell STAT™ TAT of 70 minutes).

There have been two previous studies comparing the performance of Dynamiker to Fungitell. In a clinical and method comparison study, White *et al* found that Dynamiker correlated well with Fungitell with an overall sensitivity and specificity of 81.4% and 78.1% respectively for cases of proven and probable invasive fungal disease[18]. In a second laboratory-based study conducted in China, 72 clinical serum samples were evaluated on both Dynamiker and Fungitell assessing the former to have a sensitivity of 82.9% and specificity of 94.6%[19].

To our knowledge, there had been no published data from Southern Africa evaluating the Dynamiker assay. In view of this we aimed to evaluate the analytical performance of the Dynamiker in comparison to the Fungitell assay in our laboratory setting as well as assessing the diagnostic accuracy by comparing both assays to blood culture, fungal culture and *Pneumocystis jirovecii* PCR (PJP PCR) as crude surrogate clinical performance estimates.

CHAPTER 2

2. Materials and methods:

2.1 Study design:

This was a prospective laboratory-based study conducted over five days (from 28 May to 01 June 2018) at the National Health Laboratory Service (NHLS) Microbiology Laboratory based at Charlotte Maxeke Johannesburg Academic Hospital, a 1088-bed tertiary level institute in Gauteng province. The Fungitell was validated for use upon introduction in 2009. Majority of the BDG specimens seen are referred from within the province as well from centres across the country. A study conducted at the same centre reported majority of samples referred from paediatric and adult medical and high-care wards[11].

Training involved only 2 technologists (who were previously deemed competent on the Fungitell bench), to reduce disruption to routine workflow within the laboratory, and was based on the standard operating procedure provided by Dynamiker Biotechnology. The training encompassed preparing of reagents, controls and clinical samples for test runs. The user was deemed competent to participate in the study once quality control had passed on the test run and was required to rotate on the bench on alternative days. Patient specimens and quality control materials were then prepared according to the respective manufacturers instructions [15,20].

Dynamiker shared the same principle as Fungitell as a kinetic colorimetric assay, therefore, integrated with the existing platform (no new instrumentation was required) apart from having to install reader software. The patient sample volume differed slightly. Fungitell required 5ul of patient serum, whereas Dynamiker required 20ul. Both methods run a five-point standard curve as well as patient samples in duplicate, therefore a 96-well plate may allow maximum 41 patients (routine average of 21 patients per batch). The time taken from reagent preparation, specimen incubation (40 minutes), plate reading up until result reporting was approximately 80 minutes for both methods depending on the user. Reagent and standard material could be reconstituted and stored at -20ºC for up to 30 days according to the insert package.

The run was accepted if the standard curve was linear, the negative control was less than the lower standard optical density (OD) value, the R^2 value close to 1 (> 0.98), and the positive control was within acceptable range (batch specific)[15,20]. Qualitative results were interpreted based on manufacturer cut-off values (Table 1).

Table 1. Cut-off levels as determined by the manufacturers in pg/ml.

	Fungitell	**Dynamiker**
Negative	< 60	< 70
Positive	>= 80	>= 96
Indeterminate	60 - 79	70 - 95

2.2 Diagnostic accuracy:

To evaluate the diagnostic performance of the Dynamiker assay, we analysed 105 consecutive patient serum specimens. The samples were run in parallel on both assays (initially on the Fungitell assay and thereafter on the Dynamiker assay) within two hours of each other, so as to avoid interferences introduced through freeze-thaw cycles of samples in storage. The specimens were capped and stored on the bench in between runs. Each clinical sample was run in duplicate on both assays, with only the means and categorical classification used in the comparison analysis. Categorically discordant samples (between the duplicate results on Fungitell) or those with CV of >20% were excluded (n = 5) from the

analysis, as they required retesting according to the local laboratory protocol. As a result, only 100 samples were reported for the purpose of this evaluation (Figure 1).

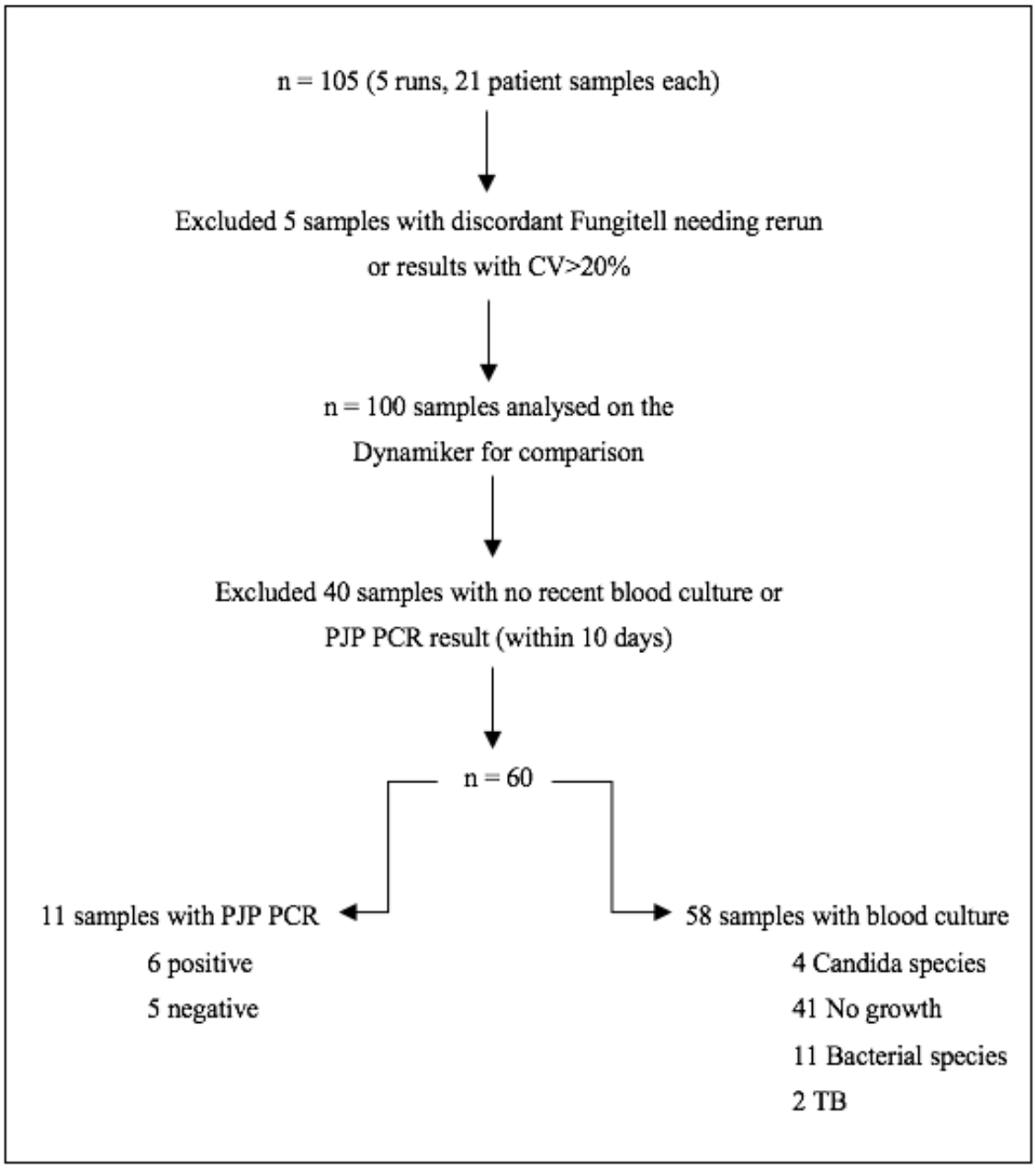


Figure 1. Study design.

To determine the diagnostic accuracy, we compared both the quantitative results as well as the categorical correlation between the two assays. We also compared each assay with blood

culture, fungal culture (blood) or qualitative PJP PCR (specimen type information unknown) results for each of the patients, if these had been submitted at the time of or within 10 days of sample collection for BDG analysis. The samples were identified with a laboratory accession number and additional microbiological data linked to the medical record number of that sample was accessed via. the laboratory information system (TrakCare® Intersystems). Dynamiker results were not reported to the clinician, even in the instance of discordant results with Fungitell, as the assay had not completed verification.

2.3 Precision experiment:

To assess the reliability of Dynamiker in our local setting, standard material provided by the manufacturer was used to establish a five-point calibration curve, inclusive of medical decision points. The experiment was run over five days. Repeatability and reproducibility were reported for each of the standards over each day, and across different days and separate users respectively. Linearity and correlation were reported.

2.4 User-friendliness:

This was determined crudely based on user experience communicated verbally with the investigator and through a written survey.

3. Statistical analysis:

The data was captured in Microsoft Excel for Mac version 16.25 (2019) and exported into IBM SPSS statistics version 24.0 (2019) for analysis.

The diagnostic comparison samples were not preselected, therefore the values obtained were random and non-parametric in distribution. We therefore described the median, interquartile ranges and Wilcoxon rank sum test for quantitative data (where a *Z* score close to 0 indicates no difference in data being compared), and the percentage agreement and Cohen's kappa for categorical data. We assessed 3 variables (negative, positive and indeterminate) and 2

variables (i.e., by adopting a higher cut-off viz. 80 pg/mL and 96 pg/mL on the Fungitell and Dynamiker respectively, the indeterminate results were interpreted as negative).

Sensitivity and specificity and the 95% confidence intervals (95% CI) were determined using 2-by-2 tables. The diagnostic accuracy of the Dynamiker was assessed in comparison to Fungitell and both assays were evaluated against blood culture and PJP PCR. For the precision study we reported the standard deviation (SD), CV and student paired *t*-test. We accepted a CV of <20% as reliable and a *P* value of <= 0.05 was considered significant.

For the purpose of the comparison study all mean values were converted to whole numbers and values outside the reliable linear range (according to the manufacturers recommendation) were converted to upper/ lower most reliable value.

4. Ethics statement:

As this was primarily a laboratory-based study, no patient clinical details were obtained. The laboratory information system was utilised to extract blood culture, fungal culture or PJP PCR results for each of the patients, if these had been submitted. Permission to conduct the study was granted by the University of Witwatersrand Human Research Ethics Committee (Clearance Certificate M171025).

CHAPTER 3

5. Results:

5.1 Diagnostic accuracy:

Descriptive data:

The quantitative results descriptive statistics are summarised in Table 2 including the median and interquartile ranges for each category (positive, negative and indeterminate).

Majority of samples fell within the negative category (n = 48 on Fungitell, n = 61 on Dynamiker), with the indeterminate category being the least well represented (n < 10 for both Fungitell and Dynamiker). Wilcoxon rank sum test showed no significant difference when comparing each category ($P > 0.05$) with Z scores < 0 (Table 3).

Table 2. Quantitative descriptive statistics for each category on Fungitell and Dynamiker.

Categories	Fungitell negative	Fungitell positive	Fungitell indeterminate	Dynamiker negative	Dynamiker positive	Dynamiker indeterminate
Total	48	44	8	61	32	7
Median	35	380	69	32	378	70
IQR	18,48-42,43	147,83-524,00	63,55-74,73	17,30-47,00	180,25-629,00	58,80-79,30

Table 3. Wilcoxon rank sum test results comparing Dynamiker and Fungitell quantitative results for the 3 categories.

	***Z* score**	***P* value**
Negative	- 0.636	0.525
Positive	- 0.860	0.390
Indeterminate	- 0.338	0.735

Quantitative agreement:

The relationship between the quantitative results is represented graphically in scattergram (Figure 2). Data is concentrated towards the negative values in a non-linear distribution. There were scanty discordant results between the two assays, mirroring qualitative agreement (described below).

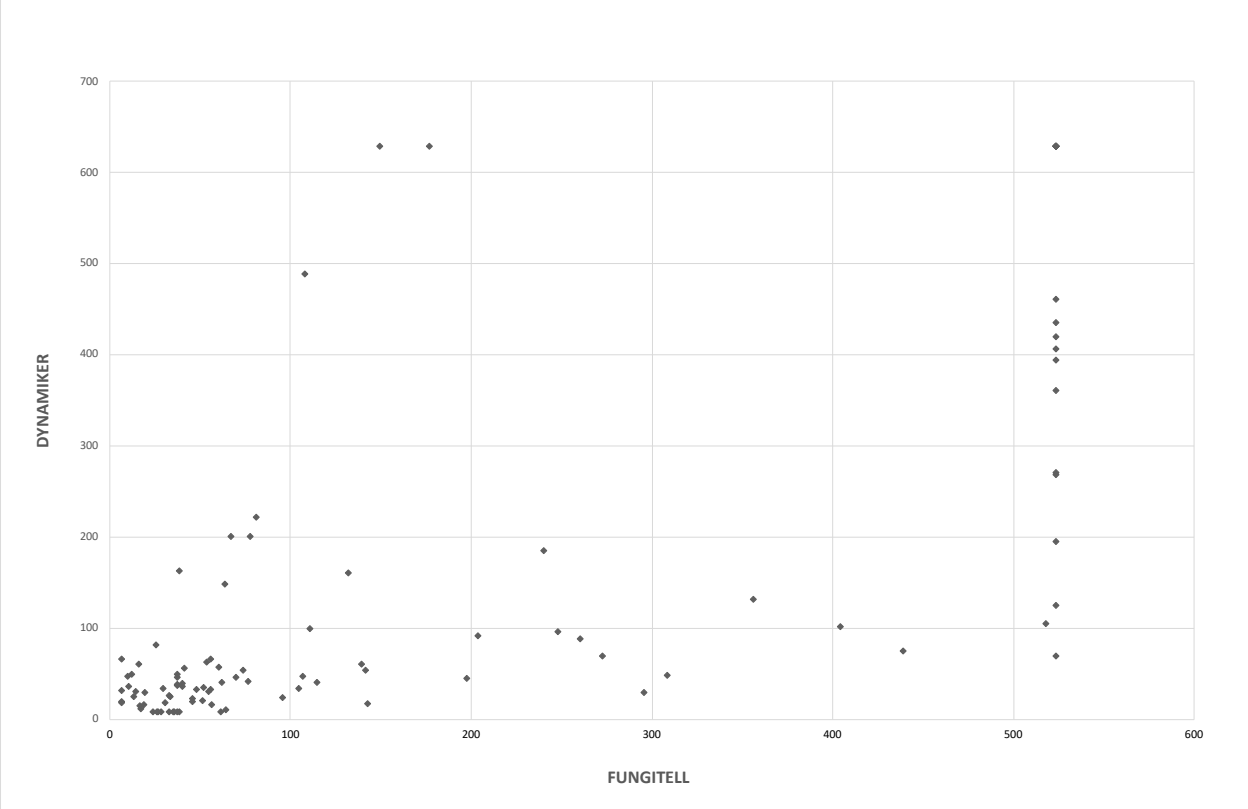


Figure 2. Scatterplot of quantitative values (pg/ml).

Qualitative agreement:

Categorical correlation using the manufacturer recommended cut-off for the 3 categories (negative, positive and indeterminate) was 74%, showing improvement to 80% when comparing 2 categories viz., positive and negative (indeterminates were considered as negative). Cohen's kappa assessing categorical agreement demonstrated a moderate agreement (K= 0.582, P value < 0.001).

Sensitivity and specificity:

Based on the 2-by-2 (Table 4), the sensitivity and specificity of Dynamiker compared to Fungitell qualitative results (positive and negative categories) were 63.64% (95% CI: 63.18 - 64.09) and 92.86% (95% CI: 92.64 – 93.07) respectively.

Table 4. 2-by-2 table Dynamiker categorical results compared to Fungitell (n = 100)

		Fungitell	
		Positive	Negative
Dynamiker	Positive	28	4
	Negative	16	52

Comparison of Fungitell and Dynamiker to blood culture and PJP PCR:

60/100 samples had additional paired samples or recent results for blood culture/ fungal culture (n = 58) and/ or PJP PCR (n = 11) and were evaluated further. Results of the assessment can be seen in Table 5*a* and 5*b*. Of the 58 samples with culture, 54 were negative for fungi, of which 41 had no growth of an organism (Fungitell 15 positives, 26 negatives; Dynamiker 11 positives, 30 negatives), 11 had growth of bacterial species (Fungitell 7 positives, 4 negatives; Dynamiker 6 positives, 5 negatives) and 2 samples were positive for *Mycobacterium tuberculosis* (Fungitell and Dynamiker were negative on both occasions) (see Figure 1). When compared to blood cultures positive for fungal growth the sensitivity and specificity for Fungitell were 100% and 59.26% (95% CI: 58.84 – 59.68) respectively, and 75% (95% CI: 73.64 – 76.36) and 68.52% (95% CI: 68.12 – 68.92) for Dynamiker respectively. The 4 samples positive for fungi included 1 *Candida famata* and 3 *Candida albicans*. Of those 4 samples, Dynamiker was associated with 1 indeterminate result which had been interpreted as negative (for *Candida albicans*) and Fungitell was positive for all 4 cases.

Of the 11/100 samples with additional qualitative PJP PCR, 6 were positive and 5 were negative (Figure 1). The Dynamiker was associated with 2 indeterminate results which was interpreted as negative (wherein the Fungitell read positive) and 1 negative result (wherein the Fungitell was in agreement and also read negative). The sensitivity and specificity for PJP PCR was 83.33% (95% CI: 82.38 – 84.29) and 80% (95% CI: 78.88 – 81.12) for Fungitell respectively, and 50% (95% CI: 48.72 – 51.28) and 60% (95% CI: 58.63 – 61.37) for Dynamiker respectively.

Table 5. 2-by-2 table Fungitell and Dynamiker compared to (5*a*) blood culture positive for fungal growth and (5*b*) PJP PCR.

Table 5*a*

		Fungitell		Dynamiker	
		Positive	Negative	Positive	Negative
Blood culture	Positive	4	22	3	17
(n = 58)	Negative	0	32	1	37

Table 5*b*

		Fungitell		Dynamiker	
		Positive	Negative	Positive	Negative
PJP PCR	Positive	5	1	3	2
(n = 11)	Negative	1	4	3	3

5.2 Precision experiment:

The overall mean, standard deviation and CVs are summarised in Table 6 for the Dynamiker precision study. The CVs were acceptable for all 4 levels, across the different days and operators. The lowest standard material having a poor overall CV of >20%. When comparing the overall means with the manufacturers expected values, student paired *t*-test shows no significant difference (*P* values > 0.05). The standard curves were found to be linear with R^2 close to 1.

Table 6. Dynamiker precision study. Overall mean, SD and CV over 5 days.

	Manufacturer's mean	Mean (*P* value)	SD	CV %
Standard A	600	597,99 (0,65)	20,46	3,42
Standard B	300	300,55 (0,86)	13,38	4,45
Standard C	150	154,87 (0,19)	11,42	7,37
Standard D	75	74,68 (0,75)	4,48	5,99
Standard E	38	34,90 (0,20)	8,75	25,06

5.3 User-friendliness:

Users reported no overall difference in experience between Fungitell and Dynamiker.

6. Discussion:

The Dynamiker BDG assay performed diagnostically well in comparison to Fungitell, with an observed agreement of 74% comparable to that described in previous evaluations (75.5% reported by White *et al* using a higher cut-off and 89% by Wang *et al* using a lower cut-off)[18,19]. We noted an improvement in observed agreement to 80% when optimising the cut-off to 96 pg/ml (i.e., indeterminate results re-classified as negative). In our study we chose a higher cut-off because of the doubtful significance of an indeterminate result in clinical practice, prompting further investigation and serial monitoring of patient's BDG levels. The previous studies do not report on the agreement using alternative cut-off levels, however, White *et al* had described an improvement in sensitivity at a lower cut-off of 70 pg/ml at the expense of specificity[18].

When compared to Fungitell, the Dynamiker showed a moderate categorical agreement with a Kappa of 0.582, less than that reported by Wang *et* al ($K = 0.77$)[19]. White *et al* had noted a similar finding (moderate agreement $K = 0.5$)[18]. The authors attributed this to possible sampling handling and storage between the sequential runs on Fungitell and Dynamiker, and this shortcoming may have contributed to the discrepancies seen in our study as well.

Wilcoxon rank sum test results show that quantitatively the 2 assays are comparable within each category with P values > 0.05. The Z scores were < 0 suggesting the median values on Fungitell were numerically smaller than the Dynamiker, however their linear range is known to be smaller, therefore a direct quantitative comparison between the two assays is of questionable significance.

The sensitivity and specificity of the Dynamiker were 63.64% and 92.86% respectively when evaluated against the Fungitell. The specificity was higher than that described by White *et al*

(78.1%)[18], and similar to the Wang study group (94.6%)[19], but sensitivity was noted to be lower than both studies (81.4% and 82.9% reported by White and Wang respectively)[18,19].

There are numerous factors which may have contributed to the discrepancy (lower sensitivity and higher specificity) reported in our study. Firstly, White *et al* study cohort included intensive care units (ICU), post-abdominal surgery and haemoncology patients, all of which are associated with a higher false positive rate therefore lowering BDG specificity[18]. Secondly, the sensitivity of the test has been found to be better in high risk patients[1,6–8]. We, however, did not restrict sampling to critical care wards, which may have resulted in the lower sensitivity we report in our analysis. Lastly, and most importantly, there is no single conclusive cause for the differences seen in diagnostic accuracy observed among many previous study groups analysing different BDG assays. In a recent Cochrane review assessing the diagnostic accuracy of BDG assays, the authors report large differences among statistical results likely due to differences in study design, study populations chosen, sample handling, possible interfering factors, fungal species included and differences in optimal cut-off levels[21].

A large metanalysis assessing different BDG platforms had reported a pooled sensitivity of 76.8% and specificity of 85.3% for IFI [14]. We report the sensitivity and specificity for Dynamiker for candidaemia as 75% and 68.52% respectively. The sensitivity and specificity for Fungitell were 100% and 59.26% respectively as assessed in this study but was found to be 79% and 71-100% respectively in a study conducted at the same institute in 2019[11].

The Dynamiker was found to have a better specificity for candidaemia compared to specificity for PJP (60%). This phenomenon was also reported in the White *et al* paper with a sensitivity of 93.3% for candidaemia vs 50% for PJP, although the PJP cohort was admittedly small[18]. The sensitivity and specificity did improve to 87% and 70% in a follow-up study by the same author[22] but not sufficiently to reliably exclude Pneumocystis pneumonia.

Dynamiker did have a slightly higher number of false negative results than Fungitell, however the number of cases with fungal growth on culture (4 candidaemia) and PJP PCR (11) samples were too small to draw any reliable conclusions. We did not have clinical information to exclude concurrent use of antifungal therapy which may have contributed towards false negative BDG[2,14]. Additional confounding factors to consider would be the low yield of fungal culture for candida[7] and the lack of a gold standard microscopy, quantitative PJP PCR results (cycle threshold) and clinical or radiological information to assess if the patients with positive PCR did have disease viz., Pneumocystis pneumonia. The latter may explain why both Fungitell and Dynamiker were negative for 1/6 sample in which the PCR was found to be positive. We also did not have access to patient HIV results, which is important to factor in, as the sensitivity of the test for PJP is lowered in HIV-negative populations[22].

We report the Dynamiker to have a higher specificity with lower sensitivity for candidaemia (based on fungal culture) when compared with the Fungitell. Our study also demonstrated poor diagnostic accuracy for Pneumocystis pneumonia (using surrogate marker PJP PCR), which concurs with both studies conducted by White *et al*[18,22]. Although the findings of this report suggests that Dynamiker is a good rule-in test, especially for candidaemia, our sample numbers were small, and further clinical studies including long-term studies assessing serial BDG tests are warranted.

In the context of South Africa's high incidence of tuberculosis, it is worthwhile to note that neither assays were associated with false positive BDG results for patients with *Mycobacterium tuberculosis* infection. To our knowledge this is the first study to comment on this finding. However, our case numbers were small (2) and further prospective studies would be of value in the South African context.

We proved the Dynamiker assay to be precise over the 5-day period, observing excellent repeatability (no significant difference between our average daily means and the manufacturers means (student paired *t*-test *P* values > 0.05)) and reproducibility (CV over the study period reported as <20% for the higher values). The variability was >20% for Standard E, due to one outlier, however higher CVs can be expected at the lower levels.

The Dynamiker kit allows technical flexibility of running smaller batches of tests on the breakable plate. The reagent and standard material are stable at 2-8ºC for 12 months and can be reconstituted and stored at -20ºC for up to 30 days. These features may be advantageous to laboratories in reducing medical waste and financial pressure to perform large test batches, thus improving TAT. Although the manufacturer has claimed to be less expensive than other assays, we did not find a great difference between the 2 assays cost per test. However, the study was conducted over 5 days, and the overall expense to the laboratory may only be appreciated if followed up long-term. The inclusion of positive control material with every new batch added further quality assurance that each run would be precise. In addition, we found Dynamiker to be user-friendly with sufficient reagent material in each test kit to run over the course of the experiment, and ease of transition between both assays.

Strengths:

Our study was performed prospectively, thus eliminating interferences introduced during the freeze-thaw cycles of stored serum samples. We did not preselect patient populations and we correlated with blood culture and PJP PCR results to assess diagnostic accuracy. Our BDG laboratory is partitioned away from routine benches and airflow, and the temperature was regulated over the study period.

Study limitations:

1. We do acknowledge that in the absence of relevant clinical and radiological information, we could not classify our samples into proven, probable or suspected IFI based on the EORTC/ MSG definitions[12] in which case the Dynamiker was compared to an imperfect gold standard (culture) and the current in-use assay (Fungitell).

2. Not every patient had additional specimens submitted for culture or PJP PCR.
3. Serum samples were not paired, and patients were not followed up longitudinally, therefore discrepant categorical results (between the 2 assays) were not assessed for trends in the BDG level. A clinical study would be required to assess if kinetics of BDG would change with time, and correlate with culture results.
4. Values outside of the reliable measurable range (according to manufacturers' specifications) were not diluted. These results were converted to the highest/ lowest reliable value, thus introducing potential bias.
5. Other causes for potential bias- we did not deduplicate samples, and discordant Fungitell samples that were subjected to reruns were not included in the study.
6. Quantitative PJP PCR (cycle threshold) data unavailable, and specimen type, would have been useful in assessing whether patients had true disease rather than false positives due to commensal fungi.
7. Prevalence studies were not conducted.
8. Small samples volumes with only 2 cases of TB and 4 fungi. Further studies would be recommended to assess clinical diagnostic accuracy in the South African setting.

CHAPTER 4

7. Conclusion:

The BDG is a rapid non-culture based broad fungal marker that has established a role as an adjunctive test in the management of critically ill patients evidenced by the increasing test volumes seen in the last decade at our tertiary centre[16]. There is sufficient evidence of the positive impact it has on patient care, reducing overall cost and improving anti-fungal stewardship[10].

We found the Dynamiker to be a reasonable alternative to the more commonly used Fungitell, with the potential of being implemented in laboratories with lower sample throughput. This would enable near point of care testing and offer clinicians a guide to the

early initiation of appropriate therapy. Our study serves as a baseline evaluation for other laboratory-based studies, however the poorer sensitivity for candidaemia compared to Fungitell and diagnostic accuracy for Pneumocystis pneumonia requires further clinical investigation.

Future unanswered questions:

There are insufficient case-controlled clinical studies evaluating the performance of the Dynamiker in specific patient populations with specific pathologies (candidaemia, Pneumocystis pneumonia, and in cases of TB), or to establish population-specific cut-off levels and the effect on batched samples subject to non-optimal environmental conditions and storage. BDG cut-off levels should be optimized for the specific population in which is intended for use, which may improve test sensitivity.

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9. Appendices:

A

MMed Research Protocol:

An evaluation of the analytical performance of the Dynamiker® Fungus (1-3)-β-D-Glucan assay in comparison to the Cape Cod Fungitell ®1,3 beta-D glucan assay for the diagnosis of invasive fungal infections.

Investigator: Dr Shalini Pillay
Student No.: 1238856
Clinical Pathology Registrar

Supervisor: Dr Vindana Chibabhai
Consultant Pathologist at the department of Clinical Microbiology and Infectious Diseases



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1. TITLE:

An evaluation of the analytical performance of the Dynamiker® Fungus (1-3)-*β*-D-Glucan assay in comparison to the Cape Cod Fungitell® 1,3 beta-D glucan assay for the diagnosis of invasive fungal infections.

2. INVESTIGATORS:

The principle investigator is Dr Shalini Pillay, a fourth year registrar in clinical pathology currently rotating in microbiology at the National Health Laboratory Service (NHLS). The supervisor is Dr Vindana Chibabhai, a consultant clinical microbiologist at the department of clinical microbiology and infectious diseases.

3. BACKGROUND LITERATURE REVIEW AND CRITIQUE:

Invasive fungal infections contribute significantly to the disease burden in southern Africa, largely due to the relationship with HIV infection and immune suppression. Invasive fungal infection (IFD) secondary to *Candida* species, *Pneumocystis jirovecii* (causing *Pneumocystis jirovecii* pneumonia or PJP) and opportunistic mould infection including *Aspergillus* species are among the commonest causative organisms in South Africa. Risk factors include long-term steroid use, immunosuppressive drugs or radiotherapy, haematopoietic stem cell transplant recipients, solid organ transplant recipients, diabetes mellitus and HIV (1). Currently, the diagnosis of IFD remains challenging. The clinical features and radiological signs may be non-specific or absent depending on the stage of disease, the immune response mounted by the patient and site of infection. In addition, culture based diagnosis can be delayed and fungal blood cultures may only be positive in ~50% of cases of candidiasis and aspergillosis (2). Although histological diagnosis by means of invasive procedures has improved sensitivity, it is often technically difficult and thus only performed by trained individuals (2).

Non-culture based diagnostics have grown in favour by offering an alternative less invasive sampling method with favourable sensitivity, such as the 1,3 beta-D glucan (BDG) assay.

This test is used as an adjunct in the diagnostic workup of IFD. The fungal cell wall is comprised of polysaccharides, of which glucan is the most abundant. The principle of the BDG test exploits the coagulation cascade of the North American horse-shoe crab. Glucan present in the test sample initiates the clotting pathway by activating coagulation factors, the end result of which is the detection of chromogenic substance by spectrophotometry at a specific wavelength (3). This method has the added advantage of compatibility with numerous sample types for analysis (including serum, pleural fluid and bronchoalveolar lavage) (4), and is sensitive in most patients including solid organ transplant recipients and neonates (5, 6). Beta-D glucan levels may be elevated in invasive candida infection (with highest sensitivities for *C. albicans* and lowest for *C. parapsilosis*), aspergillosis, PJP, but due to the lack of BDG in the cells walls of some fungi, is not diagnostically useful in Zygomycetes, *Blastomyces dermatitidis* and cryptococcosis. Caveats of this test method include false positive results due to antibiotics (beta-lactam/ beta-lactamase inhibitor combinations such as piperacillin-tazobactam and amoxicillin-clavulanate), bacteraemia due to *S. pneumoniae*, BDG containing surgical gauze, blood tubes, cellulose-containing haemodialysis membranes and blood products such as immunoglobulin and plasma; and false negatives may occur secondary to interference (haemolysis, icterus or lipaemia) (7).

The NHLS Microbiology Laboratory based at the Charlotte Maxeke Johannesburg Academic hospital is currently the only laboratory serving the public sector that runs the BDG assay and patient samples are transported from across the country for analysis. The test is performed on the Food and Drug Administration (FDA) approved Cape Cod Fungitell ® 1,3 beta-D glucan assay (F-BDG), which has the capability to run 96 well plates in a single run (21 patient samples, 5 standards and negative controls run in duplicate) and costs R700 per test. Other manufacturers of this assay include Endosafe-PTS (Charles River Laboratories), Fungitec-G (Seikagaku Biobusiness), beta-Glucan Test (Waco Pure Chemical Industries), Dynamiker ® Fungus (Biotechnology) and BGSTAR β-Glucan Test (Maruha).

The F-BDG is the only available platform in southern Africa, and although this in itself is an advantage in that results are comparable across medical laboratories, newer automated testing platforms exist that may offer increased output capacity and reduced cost. The Dynamiker ® Fungus is one such instrument which has the advantage of running smaller volumes of samples (12 x 8 well breakable plates) at R331.00 per test. This may be appealing especially

to smaller laboratories which would no longer have to batch samples and specimen transport and logistics would be obviated.

To date, there are two studies comparing the Dynamiker ® Fungus and Cape Cod Fungitell ® 1,3 beta-D glucan assays. In a clinical and method comparison study, White et al found that Dynamiker ® Fungus (1-3)-β-D-Glucan assay (D-BDG) correlated with the F-BDG, with an overall sensitivity and specificity of 81.4% and 78.1% respectively when assessed on clinical samples (8). In a second study conducted in China, when 72 clinical serum samples were compared on both instruments, results showed the Dynamiker ® Fungus to have a sensitivity of 82.9%, specificity of 94.6%, a positive predictive value of 93.5% and negative predictive value of 94.6% (9).

In light of this we hypothesize that the Dynamiker ® Fungus (1-3)-β-D-Glucan assay will be comparable to the Cape Cod Fungitell ® 1,3 beta-D glucan assay when evaluated in the African setting.

4. STUDY OBJECTIVES:

To evaluate the performance of the Dynamiker ® Fungus (1-3)-*β*-D-Glucan assay in comparison to the Cape Cod Fungitell ® 1,3 beta-D glucan assay, the following parameters will be investigated:

a) Accuracy- categorical correlation
b) Precision including reproducibility and repeatability
c) Linearity

5. METHODS:

This laboratory-based study will be run prospectively as an evaluation study as both instruments are IVD (in vitro diagnostics) marked. Procedures will be based on the Clinical and Laboratory Standards Institute (CLSI) EP9 A3E and EP10 A3E guidelines on the evaluation of quantitative measures using patient samples (10, 11) and the manufacturer's instructions (12, 13).

Firstly, standard material provided by the supplier will be run on the Dynamiker ® Fungus to assess analytical competency. Standard material will be serially diluted out five times in order to obtain values at critical cut-offs and will be run twice daily, viz. in the morning and evening. In addition, positive control sample will be run neat and in dilution twice daily. This will be repeated over a period of 5 days (*Table 1*). We will assess precision, including within run repeatability, reproducibility (within day, between day, between user and between batch) and linearity at medical decision limits. The precision study will be performed by two operators, thereby allowing us to assess inter-user reproducibility.

Reproducibility:

- Within day (between morning and afternoon runs on the same day).
- Between day (assess runs comparing each day's analysis over different days).
- Between-user (user to be changed on alternate days)
- Between batch (change in reagent on day 4).

Repeatability: standards will be run in duplicate on day 2 (morning or afternoon) to assess within run repeatability.

Linearity: as the standard material is diluted out (standard 1 to 5), the BDG value should expectedly decrease.

Secondly, in a correlation study, clinical samples will be run in parallel on the Cape Cod Fungitell ®, which is the current method in use, and on the Dynamiker ® Fungus. A sample number of 100 (5 batches) will be run in duplicate on both instruments, within the space of 2 hours. The test will be conducted on consecutive routine samples as to avoid contamination that may occur with the storage and freeze-thaw cycles of samples. We will assess the correlation of the Dynamiker ® Fungus to Cape Cod Fungitell ® by comparing the results of each into clinically relevant categories as per the manufacturer's package insert (*Table 2*).

Table 1. Precision study to be run over 5 days (*n*=50).

Day		Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
1	AM					
	PM					
2	AM					
	PM					
3	AM					
	PM					
4	AM					
	PM					
5	AM					
	PM					

Table 2. Categorical ranges

	Negative	Indeterminate	Positive
Cape Cod Fungitell ®	<60 pg/ml	60-79 pg/ml	≥80 pg/ml
Dynamiker ® Fungus	<70 pg/ml	70-95 pg/ml	≥96 pg/ml

6. DATA ANALYSIS:

Data will be analysed on Stata data analysis and statistical software (version13), where precision variables will be assessed using the student's *t*-test (assess the mean, standard

deviation, coefficient of variation), and accuracy will be determined by calculating Pearson's correlation coefficient.

7. ETHICS:

An application for ethics approval has been submitted to the Wits Human Research Ethics Committee (HREC). Processing of samples will only commence once ethics has been approved.

8. STUDY PERIOD:

TASK	MONTHS (2017 – 2018)									
	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Literature review	■	■								
Preparing protocol	■	■								
Protocol assessment, Ethics approval			■							
Training and R&D			■	■						
Data collection and processing				■	■	■				
Data Analysis							■	■		
Write up & manuscript								■	■	■

9. FUNDING:

All costs including that of consumables (e.g. reagents, QC material, sample trays) will be covered by the supplier, Reliable Diagnostic Supplies. Other items, e.g. stationary will be purchased on the principle investigator's cost centre (NHLS, Haematology department).

10. LIMITATIONS:

a) Small sample size
b) Reproducibility of the comparative instrument may be variable due to fluctuations in the laboratory environment (e.g. temperature)
c) Range of samples may not be representative of all medically important categories (running consecutive samples to avoid freeze-thawing and contamination)
d) Lack of clinical samples to assess precision

11. DEFINITIONS (10, 11):

***Precision*-** closeness of agreement between measured values obtained by repeated measurements.

***Accuracy*-** closeness of a measured value to its true value.

***Linearity*-** ability to obtain measurements within a certain range that are directly proportional to concentrations in the measurand.

***Verification*-** procedure of obtaining evidence to support manufacturer's claim or to specified requirements.

***Reproducibility*-** ability to obtain closeness of repeated measurements despite changes in a set of conditions.

***Repeatability*-** closeness of the agreement of repeated consecutive measurements (of the same measurand) obtained under the same conditions.

***Correlation coefficient*-** measure of the linear relationship between two random variables.

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B

R14/49 Dr Shalini Pillay

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M171025

NAME: (Principal Investigator)	Dr Shalini Pillay
DEPARTMENT:	Clinical Pathology - School of Pathology Charlotte Maxeke Johannesburg Academic Hospital Department of Clinical Microbiology and Infectious Disease
PROJECT TITLE:	An Evaluation of the Analytical Performance of the Dynamiker ® Fungus (1-3)-B-D-Glucan Assay in Comparison to the Cape Cod Fungitell ® 1,3 beta-D Glucan Assay for the Diagnosis of Invasive Fungal Infections
DATE CONSIDERED:	27/10/2017
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Vindana Chibabhai
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	18/12/2017

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 Research Office Secretary on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised 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 to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review October and will therefore be due in the month of October each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

23/04/2021

Dr S Pillay
School of Pathology
Department of Clinical Microbiology and Infectious Diseases
Medical School
University

Sent by e-mail to: shal.pillay@gmail.com

Dear Dr Pillay

Re: **Protocol Ref No:** M171025
Protocol Title: *An evaluation of the analytical performance of the Dynamiker Fungus (1-3)-B-D-Glucan Assay in comparison to the Cape Cod Fungitell 1,3 Beta-D Glucan Assay for the diagnosis of invasive fungal infections*
Principal Investigator: Dr S Pillay

Thank you for your letter of 09/04/2021.

I confirm that we have noted and approve of your proposal to analyse and report on the comparison of the results achieved by the two biomarker assays against those achieved using culture and PCR results.

Thank you for keeping us informed.

Yours Sincerely

J. Burns
.....
Mr I Burns
For the Human Research Ethics Committee (Medical)

CB Penny
.....
Dr CB Penny, Chairperson, Human Research Ethics Committee (Medical)

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