Validation of a Pan-fungal polymerase chain reaction (PCR) assay for the detection and identification of medically important fungi isolated in a diagnostic laboratory.

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Part A: Declaration

DECLARATION

I, **Sindile Venessa Ntuli**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.This work has not been reported or published prior to registration for the above mentioned degree

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Part B: Abstract

The laboratory diagnosis of fungal infection is complicated, based on the microscopic detection, culture isolation, and detection of serological response. In recent years, there has been an increase in the utilization of molecular diagnostic techniques for the detection and accurate identification of fungal pathogens. This was a laboratory accuracy study evaluating the performance of a selected pan-fungal PCR using 70 previously identified reference fungal isolates.

The DNA yield and purity of three different DNA extraction methods was assessed, using 6 representative fungal isolates. The ZR Fungal/Bacterial DNA MicroPrep™produced a median concentration of 17.28 ng/µl,which was significantly higher (p value = 0.0079) than the MagNA pure LC DNA Isolation Kit III and QIAamp DNA Mini Kit, which produced median yields of 11.08ng/µl and 3.54 ng/µl, respectively.

The selected pan-fungal PCR was optimized PCR and successfully performed on 62 of the 70 reference isolates. A selection of 56 amplicons were submitted for DNA sequence determination. Of all the sequences queried on the NCBI and Ribosomal Development Project (RDP) databases, 95/111(86%) were concordant with the results obtained from the reference laboratory.

Study findings have shown that the selected pan-fungal PCR, coupled with DNA sequence analysis is an excellent diagnostic tool for the identification of medically relevant fungi. This assay, in combination with conventional culture, is useful for the rapid and accurate identification of fungal isolates. Future work will involve evaluating the utility of this assay for the detection and identification of medically relevant fungi in deep tissue biopsies.

Part C: Protocol

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Introduction

The detection and identification of deep tissue fungal infections in a diagnostic laboratory relies conventionally on histology and/or culture and microscopy techniques, which are subjective and laborious diagnostic tools requiring an experienced laboratory technician [1, 2]. Laboratory confirmation of deep fungal infections is important, as the clinical features may mimic those of other common opportunistic infections, resulting in delayed diagnosis and inappropriate treatment, sometimes with serious fatal consequences. The diagnosis of invasive fungal infections is complex and is based on careful analysis of patient risk factors, clinical findings – including CT imaging, histological examination of clinical samples, and microbiological findings. Classical detection of fungal infection relies on histological examination and/or microbial culture, with identification being based on phenotypic characteristics [3]. However, histology has limited specificity, while fungal culture is slow with limited sensitivity and specificity, and phenotypic identification can be subjective, requiring experience [4].

More recently, molecular methods such as PCR and DNA sequence analysis have been used to improve the detection and identification of fungal infections. These techniques can identify the presence of non-viable organisms which have failed to grow in culture, may or may not have been present on direct microscopy, or may be fastidious, requiring complex culture techniques not available in a routine diagnostic laboratory[2, 5, 6]. PCR-based methods for fungal identification can be species-specific, genus-specific, or broad range. To-date, several molecular formats exist for the detection and identification of fungi. the internal transcriber spacer regions (ITS)PCR, coupled with DNA sequence analysis, provides superior identification of fungal isolates, compared to culture and microscopy, with up to 100% correct identification being documented for a panel of well characterized isolates, of different species, obtained from a national reference laboratory [2].

The ‘pan-fungal’ PCR is a commonly used sequence based fungal detection and identification assay. Universal fungal primers, representing conserved DNA regions, have been designed to target the 18S rDNA subunit, 5.8S rDNA subunit, or 28S rDNA region (Figure1). These primers

allow for the amplification of 18S, 5.8S, and 28S rDNA genes, as well as ITS1 and ITS2 regions[1-3, 5, 7, 8].

Several studies have evaluated the utility of a pan-fungal assay for the detection and identification of fungi in clinical specimens. The pan-fungal PCR assay has been shown to perform well on blood, cerebrospinal fluid, bronco-alveolar lavage, tissue biopsies and formalin fixed paraffin embedded tissue (FFPE) [1, 9-11]. A prospective multi-centre comparison of pan-fungal PCR, microscopy and culture demonstrated high concordance between PCR and conventional methods [11].There are few studies which have evaluated the assay on fresh tissue biopsy [7, 8].
Molecular assays are helpful in rapidly diagnosingsuspected invasive fungal infections when microscopy and culture are negative. This is performed in conjunction with other investigations. This will allow for rapid identification, and guided therapy.

Validation protocol

To provide rapid detection and identification of human fungal infections, we proposed to introduce a pan-fungal PCR, coupled with DNA sequence analysis, for the identification of fungal isolates in a routine diagnostic laboratory.

Aim

The study aimed to evaluate the performance of a selected pan-fungal PCR, coupled with DNA sequence analysis, targeting the ITS1 – 2regionof fungi, for the rapid detection and identification of medically relevant fungi, isolated in a routine diagnostic laboratory.

Objectives

1. To evaluate manual kit-based, and automated methods for fungal DNA extraction, from cultured reference isolates.
2. To assess the performance of a selected pan-fungal PCR assay, using DNA extracted from cultured reference isolates.
3. To identify a suitable DNA sequence reference database, for confirming fungal identities.

Methodology

Description of the method

Stored fungal isolates were used for this validation. Of 81 stored fungal isolates, 28 were isolated from clinical specimens and 53 were quality control isolates obtained from a South African reference laboratory (NICD) (Appendix Table 1).

Preparation of isolates

Fungal isolates were routinely sub-cultured onto brain heart infusion (BHI) agar (incubated at 35°C) or Sabouraud dextrose (SD) agar with or without amikacin (incubated at 25°C and 35°C)[12]. Plates were incubated for up to 4 weeks. All fungal growth were examined with lactophenol cotton blue and Gram stain microscopy for purity, using the diagnostic laboratory standard protocols[12, 13].

DNA extraction

DNA with high structural integrity and purity is required for many molecular tests, particularly for PCR, to ensure reliable and reproducible results. DNA of inferior quality or purity, or DNA of unknown concentrations can produce inaccurate results. To ensure the best quality DNA was obtained for use with this assay, three DNA extraction kits were evaluated for DNA yield (concentration), DNA purity (260:280 ratio), and final structural integrity (gel electrophoresis).Six fungal isolates were selected for DNA extraction evaluation namely: *Candida albicans, Sporothrix schenckii, Emmonsia* spp. (yeast and mould phases)*, Trichophyton rubrum* and *Aspergillus fumigatus.*

Kits compared:

- **QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany)**

A silica-membrane based manual nucleic acid extraction kit which was found to be suitable for use with different specimen types, including tissues. The extraction procedure was carried out using spin columns in a standard micro-centrifuge or with a vacuum manifold.

- **ZR Fungal/Bacterial DNA MicroPrep™(Zymo Research Corp,United States)**

A DNA extraction method using fast-spin column technology was ideal for the isolation of DNA from tough-to-lyse fungi. A simple, rapid method using bead beating lysis.

- **MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche)**

The MagNA pure LC DNA isolation kit III was an automated DNA extraction procedure. The kit was used with the MagNA pure LC instrument (Roche) to extract high quality fungal and bacterial DNA from several clinical specimens, including cultured isolates and tissue biopsies. The isolation procedure is based on magnetic-bead technology. To increase the lysis efficiency for fungi, lysis buffer and lysozyme are added, and the sample incubated overnight at room temperature.

Pan-fungal PCR evaluation

The pan-fungal PCR was based on PCR amplification and DNA sequencing of the highly variable ITS1 – 2 region, found within the fungal ribosomal RNA (rDNA) gene cassette. This region comprises the 18S, 5.8S and 28Sribosomal gene sub-units, as well as the internal transcriber spacers, ITS 1 and ITS2 (Figure 1)[14].This was one of the most widely used PCR assays for fungal identification [1-3, 5, 7, 8]. Primers targeting the entire ITS1 – 2 regions will be tested: Universal forward (**ITS1**) 5'-TCC GTA GGT GAA CCT GCG G-3' and Universal reverse (**ITS4**) 5'-TCC TCC GCT TAT TGA TAT GC-3'[14].

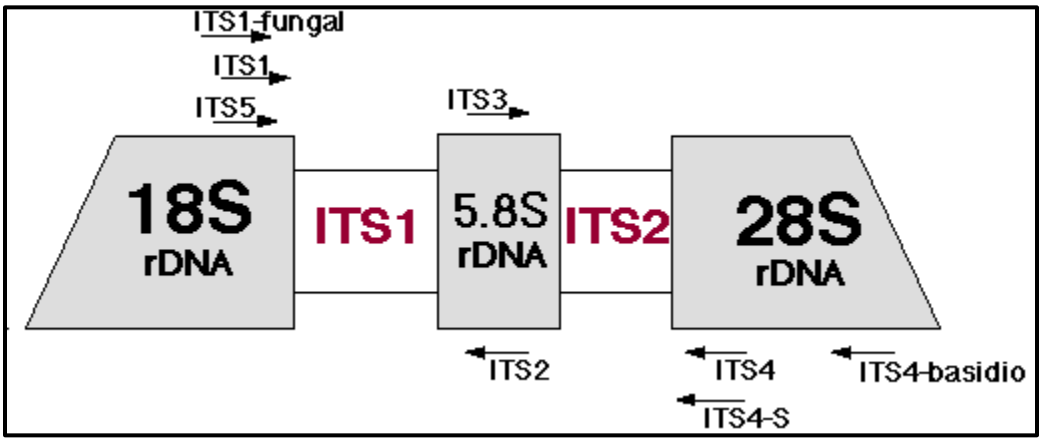

Figure1. A schematic representation of the internal transcriber spacer regions (ITS), the fungal DNA region targeted for pan-fungal PCR and DNA sequencing for identification [15].

Primer analysis

To ensure that all requirements of primer design have been fulfilled, primer specificity was checked using bioinformatics tools such as primer-BLAST at NCBI [16].

PCR reagents

Conventional multiplex-PCR was carried out using a 25µlreaction volume, with Go*Taq* Flexi DNA Polymerase (M829) (Anatech). This PCR-kit allows for $MgCl_2$ concentration optimization, and includes a green PCR buffer which allows for direct PCR to gel loading.Genomic DNA from *C. albicans* ATCC90028was used as a positive control. A no-template control was run through extraction, amplification, and sequencing to detect possible contamination.

PCR cycling

PCR thermocycling conditions included an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 seconds, primer annealing at 53°C for 30 seconds, and strand extension at 72°C for 90 seconds, with a subsequent final elongation at 72°C for 10 min. The expected amplicon size was approximately600 base pairs(bp) [10].

PCR analysis

The PCR reaction mix (10µl) was loaded on a 2.0 % w/v agarose gel, stained with ethidium bromide, and run in 1 X Tris Acetic Acid-EDTA buffer, at 70 – 120 V. The gel was visualized using a UV transilluminator (Gel Doc, BioRad).

PCR proficiency

<u>Limit of detection (LOD)</u>

To determine the analytical limit of detection of the assay, DNA extracted from a cloned plasmid harbouring the *C. albicans* ATCC90028 ITS1 – 2 PCR amplicon, was serially diluted and run using the optimized PCR conditions. The lowest concentration of DNA for which a reliable PCR product and DNA sequence could be obtained was considered the lower limit of detection. The limit of detection was determined at a95%confidence interval.

Precision

Repeatability and reproducibility was determined from the limit of detection experiments.

Accuracy

Identification results obtained from the pan-fungal PCR assay were compared to those obtained from the reference laboratory, for the same isolates.

DNA sequencing data analysis

PCR positive samples were submitted to an external sequencing facility for purification and DNA sequence determination. Bidirectional sequencing was performed on selectedPCR amplicons and the individual raw data files inspected visually for integrity before querying on a suitable reference database.

Selection of reference database

Several ITS sequence reference databases are available in the public domain. DNA sequence similarities were assessed for homology in two reference databases at the following locations: http://www/ncbi.nlm.nih.gov/BLAST/, and http://rdp.cme.msu.edu/. Sequence similarities higher than 99% over a range of at least 90% of the ITS1 – 2 region were considered reliable and reported [7]. The NCBI database is used by the reference laboratory.

Result interpretation

Negative results

Negative results may be due to:

- Failed DNA extractions
- Failure to amplify target DNA due to
    - Inhibition due to the presence of inhibiting molecules
    - Low DNA concentrations (below LOD)
- DNA which does not contain the appropriate binding sites (novel species)
- Failure to produce a reliable DNA sequence (technical error)
- Mixed samples containing more than one organism
- Failure to find a significant homology match in the reference database (novel species)

Positive results

- Fungal DNA present in the sample which produces a reliable and interpretable DNA sequence (true positive)
- Cross- contamination or cross-reactivity (false positive)

Data management

Data was collected using a standardized data entry sheet. The data was checked and verified before being exported to STATA 11 for statistical analysis.DNA concentration means and medians of the three kits were calculated. Comparison analysis of the three DNA extraction methods was performed using STATA 11.Normality was assessed with a histogram and the Shapiro-Wilk test. For comparison analysis of the three kits, non-parametric Ranksum and Wilcoxon signed-rank tests were used.

Limitations

The limitations of this study included:

- Possible contamination of samples during sampling, handling or processing. This may lead to detection of environmental and/or non-pathogenic fungi, making it may be difficult to determine the clinical significance of these results.
- The identification of fungi is limited by the sequences available in the current reference database, which may not be representative of local and emerging pathogens.

Envisaged outputs/outcomes

The results of the study were disseminated through presentation at academic meetings. Planning a submission for publication in appropriate peer reviewed journals.

Impact

We have postulated that direct pan-fungal PCR will be a reliable, rapid and sensitive method for diagnosis and identification of fungi involved in deep fungal infections and that it could be applied

in a diagnostic laboratory setting. This method has the potential to significantly improve laboratory diagnosis of fungal infections, and reduce result turn-around-time.

Ethics

The study was approved by University of Cape Town (UCT) Human research ethics committee (HREC REF: 758/2013).

Approximate budget

Item	Cost per specimen	Number of specimens	Total cost
Consumables	R30.00	200	**R6000**
DNA extraction	R30.00	200	**R6000**
Primers	R125.00	2	**R250**
Sequencing	R200.0	70	**R14000**
TOTAL per specimen:	R285.00	70	**19950**
15% for repeat testing and additional specimens			**R977**
Stationary and printing			**R200**
TOTAL cost:			**R47377**

<u>Consumables include:</u>

- Sterile specimen containers
- Saline for specimen transport
- Sterile gloves
- PCR tubes
- Reagents
- *Taq* DNA polymerase
- Double distilled water
- PCR filter tips: 10ul, 50ul, 200ul
- Expender tubes

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Part D: Acknowledgement

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Introduction

Laboratory detection and identification of fungal infections relies traditionally on histology and/or culture and microscopy techniques [1, 2]. Histology has limited specificity, while fungal culture is slow with limited sensitivity and specificity [3, 4]. Laboratory confirmation of deep fungal infections is important, as the clinical features may mimic those of other common opportunistic infections, resulting in delayed diagnosis and inappropriate treatment, sometimes with serious, even fatal consequences.

Molecular methods, such as PCR and DNA sequence analysis, have been used to improve the detection and identification of fungal infections [8, 17, 18]. These molecular techniques have been found to be more sensitive, able to identify the presence of non-viable organisms that may have failed to grow in culture [2, 5, 6, 10]. Molecular diagnostics have enhanced our understanding of invasive fungal infections.

Fungal pathogens and invasive fungal infections (IFI)

Fungi are aerobic eukaryotes which can be sessile in the environment, widely distributed in soil, plant debris and organic material, or part of the normal flora of humans and animals[19]. There are more than 100 000 species of fungi have been described in literature, of which only 500 are known to cause disease in humans[20].

Human fungal infections are mostly considered to be opportunistic in nature and are a recognized cause of morbidity and mortality in immunocompromised patients such as transplant recipients, patients with haematological malignancies, bone marrow suppression, diabetes, invasive procedures or surgery, HIV or other immunodeficiency disorders[21-24]. Increased use of corticosteroids, chemotherapy and broad-spectrum antibiotics has substantially widened the range of fungal species pathogenic to humans [20, 21]. There is also an increased awareness of fungi as significant pathogens [5, 25].

Fungi causing disease in humans can be divided into yeast, moulds and dimorphic fungi. Fungal infections can present as superficial, sub-cutaneous and deep, or invasive. The following fungi have been implicated as a cause of invasive fungal infection (IFI): Yeasts (*Candida* spp., *Cryptococcus* spp.), moulds (*Aspergillus* spp*., Fusarium* spp*., Scedosporiumprolificans (Lomentospora prolificans), Mucor*, *Rhizopus)* and dimorphic fungi (*Histoplasma capsulatum, coccidioidesimmitis, Blastomycesdermatitidis, Paracoccidioides*spp*., Sporothrix spp. and Penicillium marneffii (Talaromyces marneffii*). Rarely isolated yeasts such as *Saccharomyces* spp*., Trichosporon*spp*., Malassezia*spp*., Geotrichumcandidum, Rhodotorula*spp. *and Picchia*spp. can also cause IFIs[22, 26].A study reviewing IFI in organ transplant recipients found that invasive candida infections were the most common fungal infection followed by invasive aspergillosis and cryptococcosis; less common fungi included other dermatiatious moulds and Mucorales[27].Because of the devastating consequences of fungal infections a proposal has been made to include infections by certain fungal species in the list of "Neglected tropical diseases". These infections include cryptococcal meningitis, Pneumocystis pneumonia, histoplasmosis, sporotrichosis and blastomycosis[25].

Invasive fungal infections in sub-Saharan Africa

There has been an increase in the number of opportunistic fungal infections due to the HIV pandemic in sub-Saharan Africa. In South Africa, *C. neoformans* is the most commonly detected fungal pathogen causing meningitis among HIV infected patients[28, 29]. Cryptococcal meningitis is an AIDS-defining illness associated with high mortality[30]. The development of the lateral flow assay, to detect the cryptococcal antigen in cerebrospinal fluid and blood, has improved the laboratory diagnosis of cryptococcal meningitis. This test has also demonstrated good results when used as a screening test to detect asymptomatic disease[30].

Certain endemic fungal pathogens are a major cause of morbidity in patients with AIDS. *Histoplasma capsulatum* is a dimorphic fungus found in the environment. Infection with this fungal pathogen has been linked with soil excavation, exposure to caves and bat excreta[25]. In sub-Saharan Africa, there are two different varieties of *Histoplasma capsulatum*: var. *capsulatum*

and var. *duboisii*. Most of the infections described in Africa are caused by *H. capsulatum var. duboisii*[25, 31].Infection with *H. capsulatum* develops after inhalation of the microconidia or hyphal elements which then convert into yeasts in the lungs. Clinical manifestations range from asymptomatic infection to progressive disseminated histoplasmosis[31].

New and emerging fungal pathogens causing invasive disease in HIV-infected patients in sub-Saharan Africa have been described as well [31, 32].Anovel *Emmonsia* spp. that was thought to be closely related to *E. pasteuriana* was described in 2013. The fungus was recognized as a pathogen in 13 HIV-infected persons with disseminated infection in South Africa[32].The cases reported were seen between July 2008 and July 2011.This was thought to be an underestimation of the true prevalence, since *Emmonsia* sp. is thought to be more widespread and difficult to detect and identify.Recently, molecular sequencing has confirmed that the*Emmonsia*-like fungi which cause disseminated disease in AIDS patients,belongs to a genus to Emergomyces and further phylogenetic analyses have motivated the name change to *Emergomyces africanus*[33].

Fungal pathogens developing resistance to available antifungal agents are a devastating threat in hospitalised patients. *Candida auris* is an emerging fungus describes as resistant to the three majorantifungal drug classes[34]. Recent reports have indicated that this fungus can persist in the hospital environment causing large scale outbreaks in hospitals [35]. Of major concern is that this fungus was, for a long period, misidentified by most automated biochemical identification methods because its biochemical profile has not been known or included in most identification platforms[34].Accurate identification was achieved through the use molecular methods such as PCR and sequence analysis of the internal transcribed spacer region (ITS) [34, 35].The increase in the numbers of patient who are susceptible to invasive fungal infections has highlighted that more efforts should be made in improving the laboratory diagnosis of fungal infection, to limit spread, and assist with correct treatment options for better clinical outcomes.

Diagnosis of Invasive fungal infection

The diagnosis of fungal infections, as with all infections, is based upon a combination of clinical diagnostic features and laboratory investigations. Some infectionsproduce suggestive typical lesions, which aid in the diagnosis[19].

Most clinicians utilizes the term invasive fungal infection (IFI) when describing severe, systemic infection with yeasts or mould, or the presence of fungal elements either in deep tissues of biopsy or needle aspirates that is confirmed on culture isolation and histological findings[22].The currently accepted definition of IFI has been developed by members of the European Organization for Research in the Treatment of Cancer–Invasive Fungal Infection Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group. The definition of IFI's takes into consideration the host factors, clinical features and culture confirmation[26].

IFI are defined aseither proven, probable, or possible. IFI is considered proven in a host with an illness consistent with an endemic mycosis, along with one of the following: isolation in culture from a specimen obtained from the affected site or from blood, histopathologic or direct microscopic finding of typical features of known fungi[26]. A 'proven' case of IFI should have positive fungal culture or histological finding of fungal elements. Probable and possible IFIs are further defined on the basis of host factors, clinical features of fungal infection, and mycological evidence from culture and microscopic analysis and indirect tests, such as a positive antigen test result from urine, blood, or CSF [26].

Laboratory diagnosis of invasive fungal infection

Laboratory methods for the diagnosis of fungal infections remain based on three broad approaches[19]: (i) microscopic detection of the etiologic agent in clinical material or histopathology in which hyphae and yeast cells are observed accompanied by evidence of associated tissue damage; (ii) isolation of fungi from a normally sterile site, blood culture and deep tissue biopsy along with clinical and radiological findings in keeping with an infectious

disease process [22, 23], (iii) detection of either a serologic response or some marker of due to the presence of a fungal pathogen, such as a fungal cell constituent or metabolic product[19].

Microscopic detection of Fungi

Rapid diagnosis of fungal infection can be made by the direct microscopic detection of fungal elements in clinical specimen. Microscopic examination of skin scrapings or deep tissue material can reveal fungal organisms rapidly. This method is very helpful to guide treatment decisions, to determine whether an organism recovered later in culture is a contaminant or a pathogen, since fungal cultures are prone to contamination during specimen processing. Some fungal pathogen may fail to growth[19].

Histopathologic inspection of tissue sections is a reliable method of establishing the diagnosis of subcutaneous and systemic fungal infections[19]. However, it requires a skilled laboratory technician to recognize the distinct appearance in tissue, such as presence of yeast or hyphae, size and features of hyphae and observation of tissue invasion. Detection of pathogens depends on the specimen size, site and processing technique. Several stains are available to visualize fungal elements in tissue, including hematoxylin and eosin, methenamine-silver, periodic acid-Schiff (PAS) and calcofluor white stain.

Direct microscopic detection in clinical material can detect fungal pathogens rapidly and assist to guide treatment, and confirmatory test decisions[19]. However, it has low sensitivity and culture confirmation should be performed on clinical materials [19].

Fungal culture

Fungal isolation using cultureis a gold standard to established the diagnosis of fungal infection[19, 36]. Most pathogenic, mycelialfungi are slow growing and the development of the structures used in fungal identification can be poor[19]. For these reasons, most laboratories use several standard and differential culture media and incubation conditions for the recovery of fungal agents[19, 37, 38], for example, for routine fungal isolation, Sabouraud's dextrose agar and potato dextrose agar are used, whereas dimorphic fungi such as *H.capsulatum* in the yeast phase will require the use

of media such as brain heart infusion agar[38]. A variety of chromogenic agars are available commercially for the detection and preliminary identification of *Candida* spp.[19, 37].

The best growth temperature for most pathogenic fungi is around 30°C. For patients with superficial infection culture plates should be incubated at 25–30°C, because dermatophytes grow best at that temperatures[19, 36, 38].For subcutaneous or deep sites infections, culture plates should be incubated at two temperatures, 25–30°C and 35°C. Several important pathogens, including *H. capsulatum, B. dermatitidis*, *S. schenckii* and *Emmonsia spp.* (*Emergomyces africanus)* are dimorphic with the mycelial phase growing best at 25–30°C on Sabouraud's dextrose agar and the yeast phase growing well on enriched media such as brain heart infusion agar incubated at 35 °C[19, 36, 38].

Cultures are routinely incubated for several weeks to maximize the recovery of slow-growing fungi[36]. A minimum incubation period of 5 – 7 days been recommended for yeasts, and 4 – 6 weeks for moulds [38]. Prolonged incubation period of 6–12 weeks is recommended when a dimorphic fungus is suspected to be causing disseminated infection [36]. Culture plates should be read at least twice per week from the second week. Prolonged incubation with repeated inspection increases the laboratory workload and technologist time costs. Culture isolation is followed by laboratory identification by means of colony characteristic and microscopic morphology, biochemical tests and more recently molecular diagnostic technique.

Fungal Identification

Yeasts are routinely identified using colony morphology and biochemical characteristics [36, 37, 39].Other morphologic characteristics includes presence of a capsule around the cells, the production of hyphae or pseudohyphae, and the production of chlamydospores. Modern biochemical tests consist of assimilation and fermentation of certain carbohydrates, and assimilation of nitrate and urea [37]. Commercially available yeast identification systems, such as the Vitek II Yeast Biochemical Card (bioMérieux Vitek, Taipei, Taiwan) and API 20C (bioMérieux), are useful and convenient. However, there is a delay in the identification [37].Another limitation

of the biochemical identification system for yeast is that the biochemical profile database is not comprehensive, and unusual or rare yeast speciescould bemissed or misidentified[19, 36, 38].

Traditional identification methods for moulds are based on a combination of macroscopic and microscopic morphology. Macroscopic morphology, such as colonial form, surface colour and pigmentation are often helpful along with lactophenol cotton blue smear staining[19].The smear of the mould is viewed under a microscope, often providing sufficient information on the form and arrangement, of the presence of segmented or unsegmented hyphae, conidia, and other structures they have produced aiding in the identification of the fungus. Lactophenol cotton blue smear staining relies on visualising spore-bearing structures, therefore reliant on the organism's ability to sporulate, for identification[19].

In most cases, the traditional phenotypic identification methods for mycelial fungi are usefulin identifying a fungal genus and/or species. The main limitation that it relies upon growthon solid media and the microscopic detection of reproductive structures (conidia, conidiophores, ascospores, sporangiospores and sporangia) produced during incubation on appropriate culture media, using specific culture conditions [40]. Phenotypic based methods can fail to identify a pathogen in the following circumstances: isolates displaying atypical morphology, isolates which fails to sporulate, the need for prolonged incubation or incubation on specialized media not available to the laboratory[40].The limited performance of phenotypic based methods as a diagnostic tool has been improved by adopting genotypic or molecular approaches.

Molecular diagnosis of fungal infection

There has been an increase in the use of molecular diagnostic techniques for the rapid detection and accurate identification of microbial pathogens in clinical microbiology [1, 2, 7, 8, 10]. Molecular methods designed to detect fungal DNA are an excellent diagnostic tool, since they have been shown to be more sensitive at detecting a pathogen when performed directly on clinical specimens, andaids in the identification of cultured isolates [6, 8].Molecular methods available for fungal detection and identification can be sequence based, 'other', or non-sequence based.

Numerous commercial kits and researcher designed PCR assays are available for DNA extraction, detection and identification of fungi, but these may not be globally accessible, and limit standardisation.

Non-DNA sequence based molecular methods

Non-sequencing methods are used to detect and identify a DNA sequence of interest without sequence analysis. It includes DNA probe hybridization, conventional and real-time PCR.

Genprobe A.ccuprobe test

DNA probe hybridization methods have been used to detect some fungi. This is a longstanding molecular method, which utilizes short species-specific DNA oligonucleotide fragments, labelled with a chemical tag. After restriction enzyme digestion of the purified DNA, the DNA is hybridized onto a fixed to a membrane with the labelled probe. Specific binding of a probe to its target is visualized as colour development at the position(s) of probe binding. The test has been used for the identification of *H. capsulatum, Coccidioides* species, and *B. dermatitidis*[41].

- Conventional PCR

Many laboratories use "in house "conventional, targeted PCR methods which generate specific DNA products which are visualized on an agarose gel or after capillary electrophoresis[42]. These PCR-based methods can be species-specific, genus specific or broad range. Fungal identification is based on the presence of the PCR product fragment, the size of the fragment, or the specific fragment size. The sensitivity of the PCR is partly related to the ability to visualize the fragment(s) and the specificity depends on the distinctiveness of the fragment size for a given species[42].

- Real-time PCR

Real-time PCR is a technique which monitors the amplification of a targeted DNA sequence, using fluorescently labelled probes which bind or are cleaved during each PCR cycle. Real-

time PCR is rapid, reducing turnaround times, displaying high sensitivity and specificity[43]. Melt curve analysis, or a multiplexed reaction using different fluorophores for each probe, can be used to differentiate multiple amplification products in a single reaction[36].

Other methods:

1. PCR-restriction fragment length polymorphism(RFLP)

In PCR-RFLP, the first-round amplicon is digested with restriction enzyme(s) and species identification can be made using the specific restriction digest fragment pattern. This method has been used to identify various species of *Penicillium*, *Aspergillus*, and *Cladosporium*[44]. This method is considered cumbersome and not cost-effective to implement in a diagnostic laboratory.

2. PCR-enzyme immunoassay

PCR enzyme immunoassay (EIA) produces a first-round amplicon which is hybridized to several short probes that are specific for the species of interest. The probes have been pre-labelled with fluorescent molecules so that the specific PCR product and probe complex can be visualized.

3. Microarray-based methods

The DNA microarray makes use of a glass slide, nylon membrane, or microchip with numerous unlabelled probes for detection and identification. This is an ideal method for high throughput identification of multiple pathogens. The method makes use of universal primers targeting a conserved region of the 18S and 28S genes (ITS1 – 2). This technology is based on the hybridisation of fluorescent labelled target to the oligonucleotide probes. It has been used to identify *Candida* and*Aspergillus* species, and is able to detect and accurately identify a pathogen within 4 hours after DNA extraction[37].

DNA sequence-based molecular methods

Recent developments in technology and the accessibility of whole genome sequences for many fungi has enabled the use of DNA sequence-based methods in research and clinical diagnostic laboratories[5, 18, 37, 40].The accuracy of sequence-based methods for the identification of medically significant fungi is influence by the choice of target region to be amplified, the ease of interpretation of the results and availability of a reliable and comprehensive sequence database for comparison[40].

The term “pan-fungal PCR” is used to describe a broad-range PCR targeting a conserved region among most fungi which allows for detection of most genera of fungal pathogens. The pan-fungal PCR is coupled with DNA sequence analysis for genus and/or species level identification. The PCR target is a multicopy, ribosomal DNA (rDNA) gene or operon, which contains a highly-conserved region comprised of the18S, 5.8S, and28S rDNA subunits, separated by one or more variable domains of internal transcribed spacer (ITS) 1 and 2 regions (Figure 1). These primers allow for the amplification of 18S, 5.8S, 28S rDNA genes, as well as ITS1 and ITS2 regions[18, 37]. The internal transcribed spacer (ITS) 1 and ITS2 regions have been shown to contain sufficient sequence variability to provide differentiation at the species level[8, 17, 42, 45]. This region, located between the small and large ribosomal subunits, and the nucleotide D1/D2 region is part of the large 28S ribosomal subunit used mostly for species identification for Mucorales [46]. Ribosomal genes exist in 50–500 copies per cell and are detected with better sensitivity than a single copy gene using PCR [5].

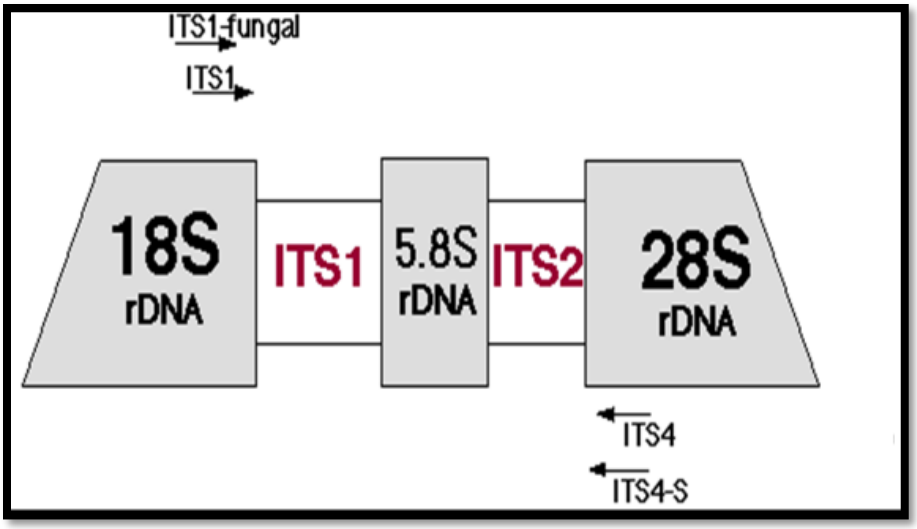

Figure1. A schematic representation of the fungal ribosomal genes, showing the internal transcriber spacer regions (ITS) 1 and 2. ITS1 represents the primer binding site for the selected forward primer, and ITS4 the target for the selected reverse primer[18].

Several studies have evaluated the utility of a pan-fungal assay for the detection and identification of fungi in clinical specimens. The pan-fungal PCR assay has been shown to perform well on blood, cerebrospinal fluid, bronco-alveolar lavage, tissue biopsies and formalin fixed paraffin embedded tissue (FFPE) [1, 9-11]. A prospective multi-centre comparison of pan-fungal PCR, microscopy and culture has demonstrated a high concordance between PCR and conventional methods [11]. There are a few studies which have evaluated the assay on fresh tissue biopsy [7, 8]. In one such study the assay was found to be highly sensitive and useful for the detection and identification of fungi from both fresh biopsy (>97% sensitivity) and formalin fixed paraffin embedded tissue (68% sensitivity) when compared to culture-confirmed invasive fungal infection [8]. A prospective study evaluating the diagnostic performance of pan-fungal PCR for the detection of fungal pathogens on microscopy-negative samples showed that the assay was 57% sensitive with a specificity of 97% and a positive and negative predictive value of 80% and 91%, respectively[4].

The assay will be helpful in diagnosing fungal infections when microscopy and cultures are negative. Pan-fungal PCR, coupled with DNA sequence analysis, provides superior identification

of fungal isolates, with up to 100% correct identification being documented for a panel of well characterized isolates, of different species, obtained from a national reference laboratory [2, 18].PCR is suitable for fast and accurate identification of cultures of morphologically similar species of yeasts and filamentous fungi[8, 17, 42, 45]. The greatest advantage of PCR based methods over culture is that it is rapid, enables early initiation of empirical antifungal therapy and detection of non-viable organisms. The biggest limitation of this method in low resource setting is cost.

Fungal DNA sequence database

For sequence-based identification methods for fungi the crucial step to accurate identification is comparison of the DNA sequence of the unknown isolate with DNA sequences in a database. The basic local alignment search tool (BLAST) database is routinely used to identify regions of similarity between two sequences. BLAST searches yield a pair-wise alignment of the queried sequences matched with sequences in the database and represented as ranked scores. The BLAST database is an easily accessible, GenBank sequence database, maintained by the US National Library of Medicine[40, 47]. Users can submit DNA sequences or search for sequences at no cost. The main advantage is that it comprises a repository of sequences from various sources around the world. The limitation of the database is that manual curation of the records is not performed on all sequences uploaded to the database. One study reported that 20% of the entries for fungi identifications were incorrect[40, 47].The finding showed that the family and genus names were incorrect. During sequence query, the BLAST algorithm returns several high-level matches based on sequence alignment, which appear to refer to multiple different species, and may lead to confusion. Species identification of the unknown isolate can be accepted onlyif there is a high sequence similarity (99–100%) to one or more reliable sequences in the database.

The Ribosomal Database Project (RDP) fungal sequence database is another widely used database which has been updated to enable phylogenetically mapping of fungal pathogens, it contains references sequences for medically important and environment fungi. The database provides alignment of fungal 28SrRNA gene sequences, ITS1 and 2, and a fungal training set for

the RDP classifier search[48].The RDP database has been manually curated making it more accurate than the NCBI database, for medically important and environment fungi. The search result identification is reported according to the taxonomic classification, making results interpretation better than the NCBI database. The output does not include scoring data.

Commercially available databases such as the MicroSeq D2 LSU fungal sequencing kit also provides curated sequences to query for organism identification. The system provides reagents for sequencing the D2 region. It has been evaluated and found to contain a comprehensive list of fungal sequences[47].

DNA extraction in fungi

The fungal cell is encased within a rigid cell wall, moistly composed of chitin and glucan[19]. Lysis of this thick cell wall is a very important step in releasing the cell DNA.

High quality, intact pure DNA is required for molecular tests, particularly for PCR to ensure reliable and reproducible results. Poor quality DNA or DNA of unknown concentrations can lead to problems during analysis leading to inaccurate results. DNA extraction on tissue sample is more challenging than on fungal isolates because of the need to remove possible PCR inhibitors. A study evaluating DNA extraction yield of six commercial kits found a huge difference in the yield and no single extraction method was optimal for all organisms [19]. The limitation of this study was that only *Candida albicans* and *Aspergillus fumigatus* were assessed, and the kits used are not available in South Africa.

Conclusions

The diagnosis of invasive fungal infections is complex and is based on careful analysis of clinical findings and laboratory investigations, including histological examination of clinical specimens, microbiological detection and identification techniques. Microbiology techniques include traditional and molecular methods.

The need for rapid and reliable methods for the diagnosis of fungal infections has resulted in an increase in the use of molecular diagnostic methods. It is of great significance that such methods be validated and subjected to suitable quality assurance, to ensure accuracy. Sequence-base molecular techniques require a reliable and accurate database for identification. The existing databases, both public and commercial should be improved by adding validated sequences of clinically

relevant fungal pathogens and closely related genera. The queried sequence results should be reported according to the taxonomic classification for the ease of interpretation.

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Part H: Journal manuscript

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The evaluation of a Pan-fungal PCR assay for the detection and identification of medically important fungi isolated in a diagnostic laboratory

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Abstract

The laboratory diagnosis of fungal infection is complicated, based on the microscopic detection, culture isolation and detection of serological response. In recent years, there has been an increase in the utilization of molecular diagnostic techniques for the detection and accurate identification of fungal pathogens. This was a laboratory based study, evaluating the performance of a selected pan-fungal PCR, using 70previously identified fungal isolates, with 6 representative isolates selected for the evaluation of three DNA extraction methods.

The DNA yield and purity of the three DNA extraction methods was assessed. The ZR Fungal/Bacterial DNA MicroPrep™ had a median concentration of 17.28 ng/µl with the MagNA pure LC DNA Isolation Kit III and QIAamp DNA Mini Kit producing median yields of 11.08ng/µl and 3.54 ng/µl, respectively. The ZR Fungal/Bacterial median concentrations were significantly higher than the median yield from the two other kits (p value 0.0079).

The optimized PCR was successfully performed on 62 of the 70 reference isolates. A selection of 56 amplicons were sent for DNA sequence determination. Of all the sequences queried on the NCBI BLAST and Ribosomal Development Project (RDP) databases, 95/111(86%) were concordant with the results obtained from the reference laboratory. Study findings have shown that the selected pan-fungal PCR coupled with DNA sequence analysis is an excellent diagnostic tool for the rapid identification of medically relevant fungal isolates. This assay has the potential to be applied to the detection and identification of medically relevant fungi present in deep tissue biopsies.

INTRODUCTION

The clinical diagnosis of invasive fungal infections is complex, requiring careful analysis of patient risk factors, clinical findings and laboratory investigations. Laboratory confirmation of deep fungal infections is important, as the clinical features may imitate those of other common opportunistic infections, resulting in delayed diagnosis and inappropriate treatment, with poor clinical outcome.

Traditional laboratory methods for the diagnosis of fungal infections are based on three broad approaches: (i) microscopic detection of the pathogen in clinical material or histopathology in which hyphae and yeast cells are observed, accompanied by evidence of associated tissue invasion or damage; (ii) isolation of fungi from a normally sterile body site, blood culture and deep tissue biopsy along with clinical and radiological abnormalities consistent with an infectious disease process [1,2];(iii) detection of serologic response to the pathogen or some biomarker, such as the cell constituent.

Direct microscopic detection of fungal elements in clinical material such as skin scrapings or deep tissue biopsy can reveal fungal pathogens promptly and guide on treatment initiation decisions. However, direct microscopy has low sensitivity and subsequent culture confirmation should be performed [3]. Culture of fungal pathogens from clinical samples is the gold standard for the diagnosis of fungal infections. The main advantage of culture is that it may yield a pathogen for further investigation, and susceptibility testing can be performed for certain isolates. However, fungal culture isolation is an imperfect gold standard since a negative culture does not rule out the presence of a fungal infection and most dimorphic fungi and some mycelial fungi are slow growing. In addition, fungal identification maybe poorly performed; it is relatively subjective requiring considerable training and experience[3,4].Phenotypic methods for identifying mycelial fungi often fail to identify the causal organism because of atypical features, failure to sporulate, the need for prolonged incubation period or requirements for specialized media which may not be available in the routine laboratory[5].The limitations of phenotypic based methods as a diagnostic tool has led to the adoption of supplementary genotypic or molecular approaches.

There has been an increase in the use of molecular diagnostic techniques for the rapid detection and accurate identification of fungal pathogens in clinical microbiology, unfortunately most of these molecular methods have not yet been fully implemented as routine diagnostic tests [6-10].Most molecular approaches for the detection and identification of fungal pathogens utilise the polymerase chain reaction (PCR). PCR assays are available in different variations: a simple conventional PCR assay generates a DNA amplicon which can then be visualized on an agarose gel, while real-time PCR simultaneously amplifies and indicates amplification using fluorescence. PCR assays can be designed with primers targeting a single genus or species of fungi, or can be broad range, using primers targeting a conserved region among most fungi.

The term "pan-fungal PCR" is used to describe a broad-range PCR targeting a conserved region among most fungi which allows for detection of most genera of fungal pathogens. The pan-fungal PCR is coupled with DNA sequence analysis for genus and/or species level identification. The PCR target is a multicopy, ribosomal DNA (rDNA) gene or operon, which contains a highly-conserved region comprised of the18S, 5.8S, and28S rDNA subunits, separated by one or more variable domains of internal transcribed spacer (ITS) 1 and 2 regions (Figure 1). The ITS regions display species-specific differences in sequence composition [5-7, 11-13].

Several studies have evaluated the utility of a pan-fungal PCR assay for the detection and identification of fungi in clinical specimens. The pan-fungal PCR assay has been shown to perform well on blood, cerebrospinal fluid, bronco-alveolar lavage, tissue biopsies and formalin fixed paraffin embedded tissue (FFPE) [6,14]. A prospective multi-centre comparison of pan-fungal PCR, microscopy and culture demonstrated a high concordance between PCR and conventional methods [16]. There are a few studies which have evaluated the assay on fresh tissue biopsy [10,17]. In one such study the assay was found to be highly sensitive and useful for the detection and identification of fungi from both fresh biopsy (>97% sensitivity) and formalin fixed paraffin embedded tissues (68% sensitivity), when compared to culture-confirmed invasive fungal infections[17].Numerous commercial kits and researcher designed PCR assays are available for

DNA extraction, detection and identification of fungi, but these may not be globally accessible, and limit standardisation

High quality, purified DNA is required for molecular testing, particularly for PCR, to ensure reliable and reproducible results. Inferior quality DNA or DNA with very high or low concentrations can lead to a failed molecular test, due to inhibition and failure to bind to the target. Fungal DNA extraction is complex, due to the hardiness of the fungal cell wall, mostly composed of chitin and glucan[3]. Several chemical and mechanical lysis methods of DNA extraction have been used to extract fungal DNA; these include cell wall lysis with detergents and proteases, manual crushing of cells, or the use of a sonicator.Most of these methods have been found to be cumbersome and not practical for use in a routine diagnostic laboratory. There are very few studies in available literature which have evaluated methods of extracting fungal DNA[19,].DNA extraction on clinical specimens is more complex because of the presence of PCR inhibitors which may affect PCR performance which is not a problem encountered with cultured isolates.

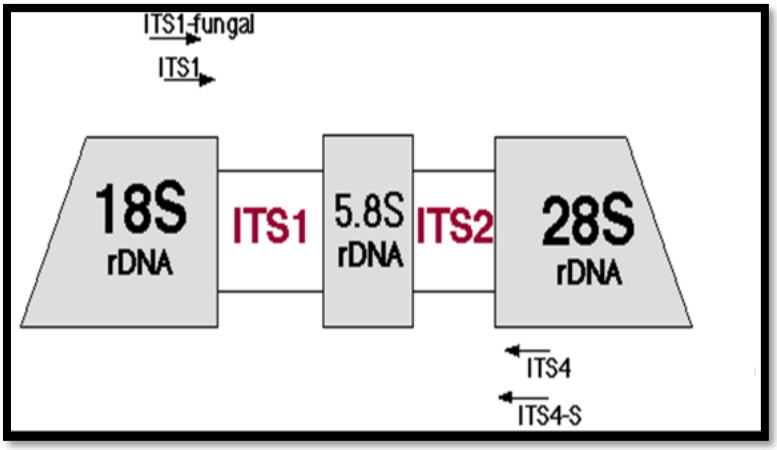

Figure1. A schematic representation of the fungal ribosomal operon, showing the ribosomal genes and internal transcriber spacer regions (ITS) 1 and 2[21]. ITS1 indicates the primer binding site for the selected forward primer, and ITS4 the site for the selected reverse primer.

This study aimed to evaluate the performance of a selected pan-fungal PCR, targeting the ITS1 – 2 region of fungi, coupled with DNA sequence analysis for the detection and identification of

medically relevant fungi, in a routine diagnostic laboratory. The study included the following objectives: identification of a suitable fungal DNA extraction method for cultured isolates, performance of the selected pan-fungal PCR on DNA extracted from a collection of cultured isolates, and validation of a suitable DNA sequence reference database for confirming fungal isolate identification.This study is based on cultured isolates and that clinical specimen validation will addressed in future work.

MATERIALS AND METHODS

This was a laboratory diagnostic accuracy study evaluating the performance of a selected pan-fungal PCR using a collection of previously identified fungal isolates. The study was conducted at Groote Scbuur hospital microbiology laboratory where only phenotypic identification methods were available. Yeasts were routinely identified using colony morphology and biochemical characteristics with a turnaround time estimated to be 3- 4 days , dimorphic and mycelia mould identification were based on a combination of macroscopic and microscopic morphologysuch as colonial form, surface colour and pigmentation are often helpful along with lactophenol cotton blue smear staining which took 14-21day to have a final identification for some fastidious mould it would take up 6 weeks for the final identification

The study was performed by the Masters in medicine microbiology student and supervised by a senior medical scientistand a pathologist in the division.A total of 81 stored reference isolates were used, of which 28 were isolated from clinical specimens, and 53 were external quality assurance program isolates. Identification of all isolates had previously been determined at a South African reference laboratory. Reference strains were identified using both phenotypic and molecular testing methods.

A similar PCR is available at the mycology reference unit but has a lengthy turnaround time for results.

A selection of 6 representative isolates were used to evaluate DNA extraction methods. Funding was provided by the Division of Medical Microbiology at the University of Cape Town, and the

National Health Laboratory Service Research Trust. The study was approved by University of Cape Town (UCT) Human research ethics committee (HREC REF: 758/2013).

Preparation of isolates

Specimen manipulations and DNA extractions were performed in a biosafety class II laminar flow cabinet (Laboratory and air purification system Co.). Fungal isolates were routinely sub-cultured onto Sabouraud dextrose (SD) agar (Oxoid) (incubated at 25°C and 35°C) or brain heart infusion (BHI) agar (incubated at 35°C), for 4 weeks (8 weeks for dimorphic fungi). To ensure purity, all fungi cultured were inspected using lactophenol cotton blue and Gram stain microscopy, according to the diagnostic laboratory protocols [22,23].

DNA extraction method validation

Three yeast and three mould species, representative of the 4 medically important phyla of fungi, were selected to evaluate the DNA extraction methods. These included *Candida albicans, Sporothrix schenckii, Emmonsia* spp. (yeast and mycelial form)*, Trichophyton rubrum* and *Aspergillus fumigatus*. Both *Sporothrix schenckii and Emmonsia* spp*.* are thermally dimorphic allowing for growth in the yeast phase on BHI agar, incubated at 35°C for 2 weeks. Three DNA extraction kits, namely the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), the ZR Fungal/Bacterial DNA MicroPrep™ (Zymo Research Corp, United States) [19], and the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche) were evaluated for the yield, purity and DNA integrity of the extracted DNA. DNA was extracted over three days, in triplicate, by the same individual.

To determine the extraction efficiency of each kit, the starting inoculum was standardized for each organism. For the yeast cultures, a loopful of fresh culture was suspended DNAse free water, the cells manually counted using a hemocytometer, and standardised to 42000 cells/100µl[19]. For the mycelial fungi, 2cm^2 of mycelial lawn was cut from the agar plate, suspended in 1ml of nuclease free water and then vortexed thoroughly[19]. A 100µl aliquot of the standardised suspensions were extracted using the different kits. All experiments were conducted in triplicate,

over three days. The subsequent DNA concentrations and purity (260:280 ratio) were measured spectrophotometrically (Biodrop). Each sample was measured in triplicate and the data averaged. DNA was loaded on a 2.0 % agarose gel stained with ethidium bromide and electrophoresed in 1 X TAE buffer, at 100 V for 60 minutes.

Validation of selected pan-fungal PCR.

The selected pan-fungal PCR was based on conventional PCR amplification and sequencing of the highly variable ITS1 – 2 region in the fungal ribosomal RNA (rDNA) gene cassette (Figure 1). Primers targeting the entire ITS1 – 2 region, Universal forward (ITS1) 5'-TCC GTA GGT GAA CCT GCG G-3' and Universal reverse (ITS4) 5'-TCC TCC GCT TAT TGA TAT GC-3',selected from published literature were used [21,].Primer specificity was confirmed using primer-BLAST at NCBI [17].

A similar PCR is available at the Mycology reference laboratory unit, at the South African NICD, but has a lengthy turn- around-time for results.

PCR reaction optimization.

Conventional PCR was carried out in 25 µl reaction volumes, using Go*Taq* Flexi DNA Polymerase (Anatech) with green buffer for direct PCR to gel loading.PCR optimization was performed using the checker board system with 25ng of *C. albicans* ATCC 90028 DNA template added to a 25 µl reaction containing a range of primer concentrations (0.125 – 1.5 µM), $MgCl_2$ concentrations (1.5 mM, 2 mM) and annealing temperatures of53°C or 55°C. PCR thermocycling conditions included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C or 55°C for 60 seconds, and extension at 72°C for 60 secs, with a subsequent final elongation at 72°C for 6 min. The expected amplicon size was approximately 600 base pairs (bp)[24].

Analytical PCR validation.

The ITS1 – 2 region of *C. albicans*ATCC 90028 was cloned onto a T-tailed plasmid vector (pTZ57R/T) (Thermo Scientific), and the resulting plasmid DNA transformed into competent*E.coli*JM109cells (Inqaba). Extracted plasmid DNA was confirmed with DNA sequence

analysis, before serially diluting ten-fold from 10^8–10^1 target copies/reaction. The PCR was performed using the optimized PCR conditions and the lowest concentration of DNA which produced a reliable PCR product and which could be sequenced was considered the analytic limit of detection. Precision, repeatability and reproducibility were determined from the limit of detection results.

DNA sequencing data analysis.

Amplicons were submitted to an external sequencing company for purification and Sanger sequence determination (Inqaba Biotech). Bidirectional sequencing was performed and the individual raw data files were visually inspected for integrity before querying on a suitable reference database.The final amplicons were randomly selected to include representatives for all the major phyla.

Selection of reference database.

Several ITS sequence reference databases are available in the public domain. DNA sequence similarities were assessed with a search for homology in two reference databases at the following sites: http://www/ncbi.nlm.nih.gov/BLAST/last accessed on the 18th of April 2017 and http://rdp.cme.msu.edu/[25-26] .Sequence similarities ≥98% over a range of ≥90% of the ITS1 – 2 region was considered reliable and were reported [10]. The two databases were selected because they are more easily accessible and a there is robust information

Data analysis.

DNA concentration means and medians of the three kits were calculated. Comparison analysis of the three DNA extraction methods was performed using STATA 11.Normality was assessed with a histogram and the Shapiro-Wilk test. For comparison analysis of the three kits, non-parametric Ranksum and Wilcoxon signed-rank tests were used.

PCR accuracy

The 81 stored fungal isolates were subcultured as described and DNA extraction performed using the optimized conditions. For 25 isolates a heat inactivation step at 56°C for 2hours [51]was

introduced prior to extraction to kill the fungal cells to reduce the risk of exposure to infectious particles. All heat inactivated isolates were subsequently subcultured to assess viability. PCR was performed using optimized conditions; accuracy was determined by comparing identification results obtained from the pan-fungal PCR assay to those obtained from the External Quality Assurance report, final laboratory report, and Mycology reference laboratory results.A similar PCR is available at the Mycology reference laboratory unit, at the South African NICD, but has a lengthy turn- around-time for results

RESULTS AND DISCUSSION

DNA extraction evaluation

Six fungal isolates were used for the DNA extraction evaluation, including 2 yeast and 2 mould isolates, and both the yeast and mould phase of *Emmonsia* spp*.* Selection of the three DNA extraction kits for testing was based on feasibility of use in a diagnostic laboratory, local availability, and reported evidence from published literature. Initial experiments yielded low DNA yields with the MagNA pure and QIAamp kits, likely due to poor lysis, since both kits lack a mechanical lysis step. The bead beating step from the ZR fungal/bacterial kit was added to the MagNA pure and QIAamp methods before proceeding according to the manufacturer's instructions (data not shown).

For the kit evaluation, a total of 9 extractions were performed per kit. Overall, the median DNA concentrations for the 3 yeast and 3 mycelial fungi, for all three kits combined were9.33ng/µl for yeast and 11.86 ng/µl for mycelial fungi. These results indicated that the yield of the three kits was comparable for yeast and fungi overall. The yields were low on average, but this was likely due to a low starting inoculum and not reflective of the kit extraction efficiency, since much higher concentrations were obtained using higher starting inocula (data not shown).The overall DNA purity produced a median absorbance ratio at 260 and 280 nm ($A_{260/280}$) for yeast at 1.69 and 1.71 for mould. A 260:280 ratio of 1.8 – 2.0 is considered very pure, but values that are between 1.6 and 1.8 are acceptable for conventional PCR. Based on these results the overall 260:280 ratio was considered adequate for use.

The DNA yield and purity of the three DNA extraction methods were also assessed individually. The ZR fungal/bacterial kit had a median concentration of 17.28 ng/µl with the MagNA pure and QIAamp producing median yields of 11.08ng/µl and 3.54 ng/µl, respectively. The Kruskal-Wallis equality-of-populations rank test indicated a statistically significant difference between median DNA concentrations of the three kits (p value< 0.001).These differences are not evident in the overall comparison. When evaluating the DNA purity using the median$A_{260/280}$ratio, the ZR fungal/bacterial kit yielded a value of 1.66, with MagNA pure and QIAamp producing median ratios of 1.72 and 1.74 respectively.

The overall performance of the different kits in extracting yeast and mould DNA indicated that the ZRFungal/Bacterial DNA kit produced the highest median DNA concentration of 16.19ng/µl and 17.26 ng/µl, for mould and yeast respectively, when compared to the other 2 kits. The MagNA pure LC DNA isolation kit produced a median DNA concentration of 13.47 ng/µl for mould and 9.51 ng/µl for yeast. The QIAamp had the lowest yield, with median concentrations of 4.62 ng/µl for mould and 2.25 ng/µl for yeast (Figure 2).The ZR Fungal/Bacterial median concentrations for both mould and yeast were statistically significantly higher than the median yields obtained from the other 2 kits(p value 0.0079 for mould and < 0.0001 for yeast). The MagNA pure had median concentrations within acceptable limits for PCR. The QIAamp had the lowest median concentrations which would be inadequate for a conventional PCR assay.

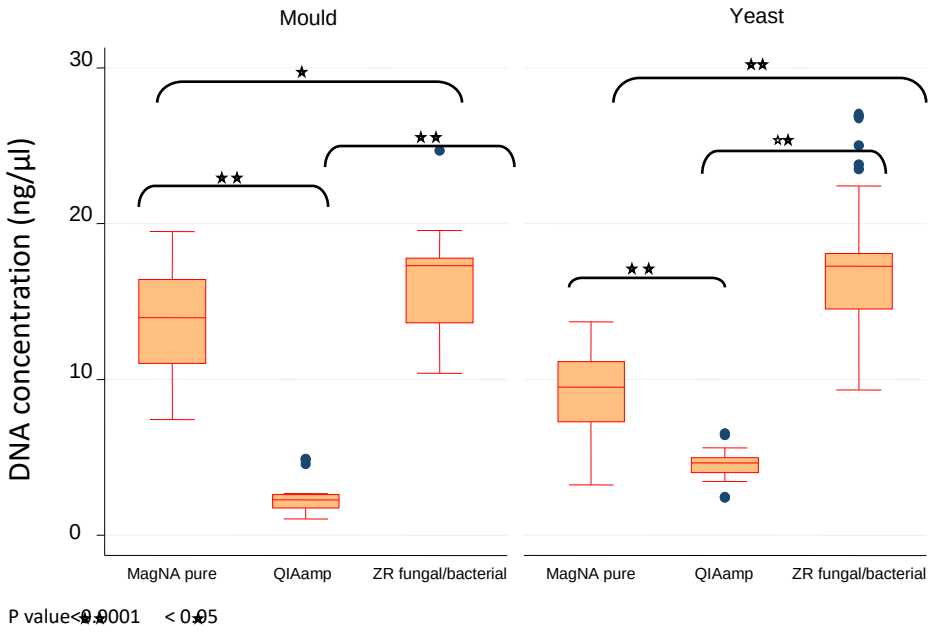

Figure 2. DNA concentrations of the three extraction methods for mould and yeast.

When comparing the purity (260:280 ratios) of the three methods for yeast and mould extractions (Figure 3), the ZR Fungal/Bacterial DNA MicroPrep™ kit produced DNA elutes with a median ratio of 1.63 and 1.66 for yeast and mould respectively, the MagNA pure had a ratio of1.83 for yeast and 1.72 for mould and QIAamp shows median ratios of 1.68 and 2.17 for yeast and mould. All the kits produced DNA with 260:280 ratios that were acceptable for conventional PCR.

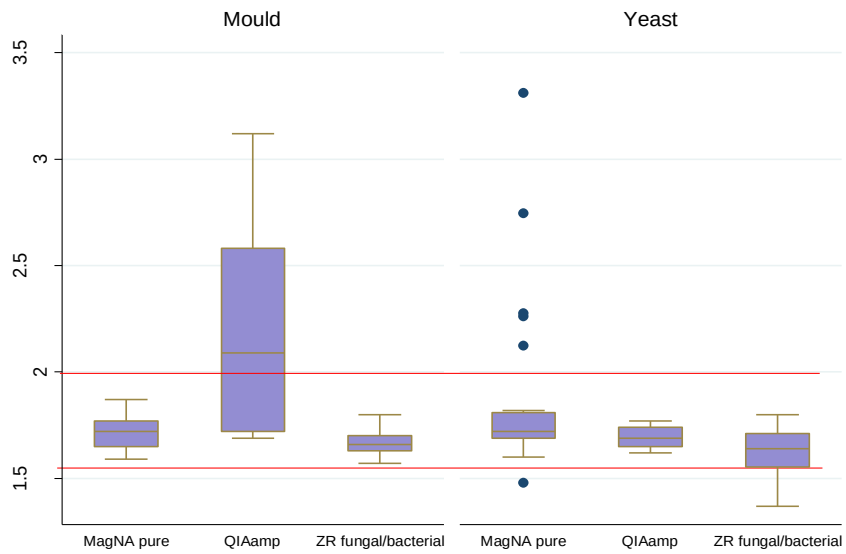

Figure 3. 260:280 ratios of the three extraction methods for yeast and mould. Red horizontal lines indicate the 1.6 – 2.0 zone , considered sufficient for conventional PCR.

To evaluate the repeatability and reproducibility of the three DNA extraction methods, the extractions were repeated in triplicate, over three days. The daily median DNA concentrations of the three kits were similar over the 3 days (Figure 4). These results indicated very low levels (statistically insignificant) of daily variation of the median DNA yields for each kit, with good repeatability and reproducibility, and low levels of technical error.

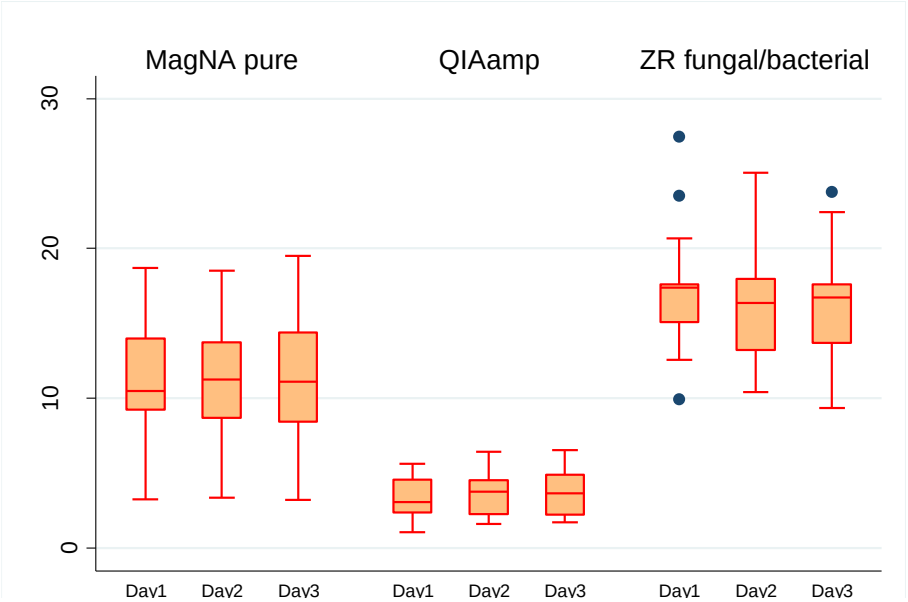

Figure 4. DNA concentrations of the three kits performed over three days.

The findings from these experiments indicated that the ZR Fungal/Bacterial DNA MicroPrep™ kit performed the best in our study. This method uses fast-spin column and with bead beating for cell lysis which was found to be effective in lysing the fungal cell wall. This DNA extraction method was found to be simple and rapid to perform in a diagnostic laboratory. Modification of the MagNA Pure LC and QIAamp DNA Mini kits to incorporate a bead bashing step improved the overall DNA yields of the other kits (data not shown). Even though the DNA yield of the modified method for the MagNA pure was low, it was also within acceptable limits for conventional PCR, and could be used as an alternative extraction method for this assay.

PCR OPTIMIZATION

To determine the optimal reaction conditions for the assay,PCR optimization was performed using the checker board system. A starting amount of 25 ng of *C. albicans* ATCC 90028 DNA template was added to a 25 µl reaction volume containing a range of primer and $MgCl_2$ concentrations, and tested using 2 annealing temperatures (53 and 55 °C) (Figure 5).The optimal PCR conditions were selected to yield a reliably visible PCR amplicon, without compromising on the specificity of reaction. The optimal amplification conditions chosen were a primer concentration of 0.25 µM, $MgCl_2$ concentration of 1.5 mM, and an annealing temperature of 53°C.

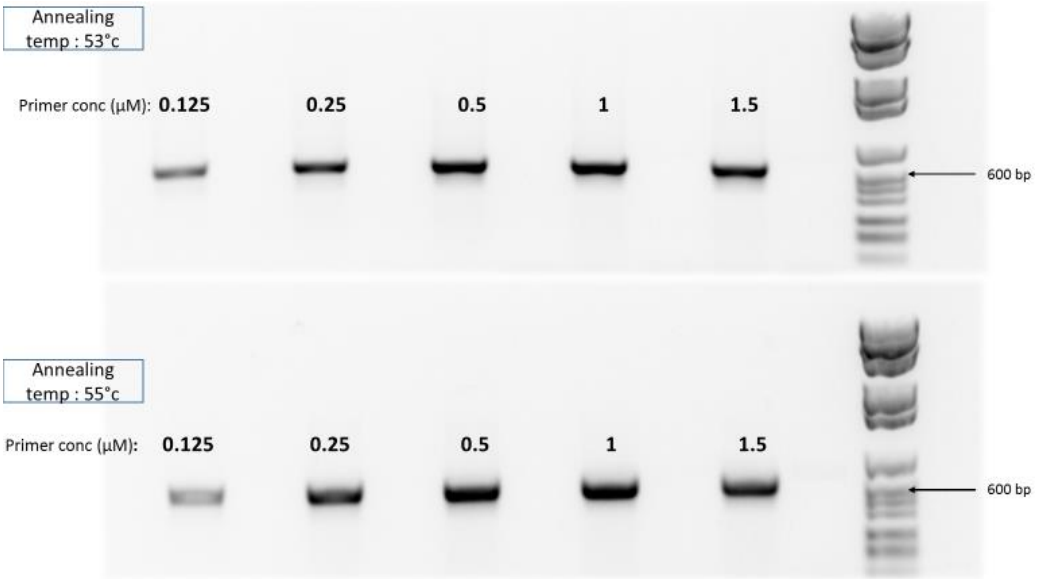

Figure 5. Agarose gel electrophoresis following PCR optimization experiments. Numbers above the lanes indicate primer concentrations (μM), $MgCl_2$ concentration at 1.5 mM for both gels. Empty adjacent lanes indicate corresponding no template controls.

Since the cellular ITS copy number differs for each species and each cell, a cloned single copy target is needed to determine the analytical limit of detection (LOD).The analytical LOD was determined by performing PCR reactions, with the optimised conditions, using a control plasmid containing the ITS1 – 2 PCR amplicon from *C. albicans*, serially diluted ten-fold from 10^8 –10^1 target copies/reaction. The results indicated the analytical LOD to be approximately10^4or10 000 target copies per reaction (Figure 6). Using the estimated LOD, the approximate mass of fungal chromosomal DNA needed for each reaction was determined by dividing the mass of the average *C. albicans* genome by the estimated LOD, which was equivalent to 7,4594× 10^{-16} ng of fungal genomic DNA per reaction. Since the analytical LOD and corresponding mass of DNA needed were well below the amount of DNA to be used in the optimised PCR, and considering that this is a multicopy target, the assay would be sufficiently sensitive to detect even low positive samples.

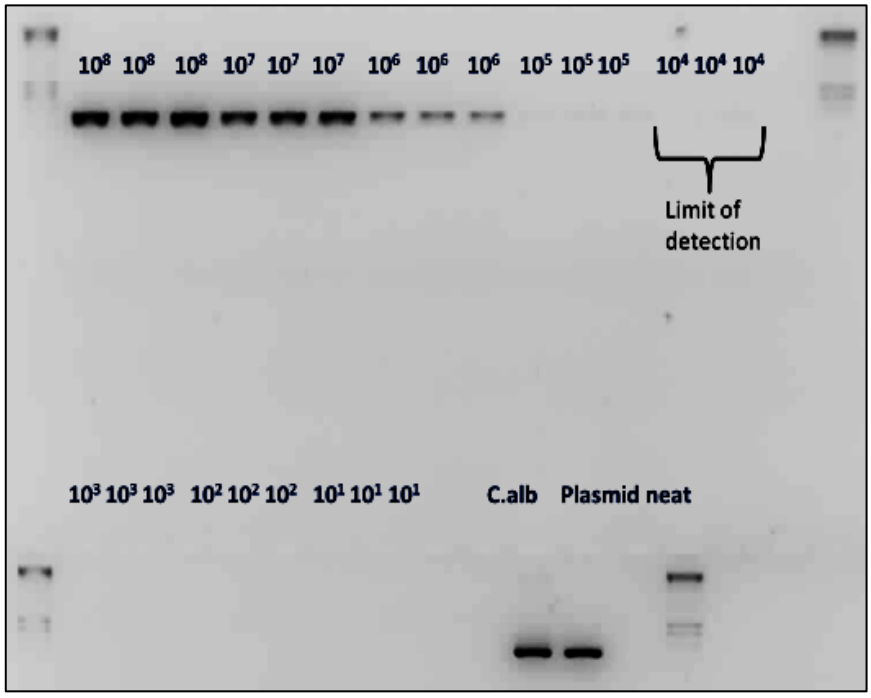

Figure 6. Agarose gel electrophoresis of the limit of detection experiments using a control plasmid containing the ITS1 – 2 PCR amplicon from *C. albicans*, serially diluted ten-fold from 10^8–10^1target

copies/reaction, negative control, positive controls: *C. albicans* DNA, and undiluted plasmid extract.

Accuracy

Of the 81 stored fungal isolates, 70 were successfully subcultured. For the DNA extraction from yeast cells, a 10 µl loopful of colonies from a fresh culture, and a 4 cm^2cutting of mycelial growth were used for the extractions. The heat inactivation step performed on 25 selected isolates was unsuccessful, as all the isolates were viable upon subculture, and further investigation is required on methods to inactivate infectious fungal cells and spores to make it safe for DNA extraction to be performed on an open bench. The heat inactivation method did not affect the quality of the extracted DNA, since PCR performed using this DNA produced reliable results, and DNA concentrations and purity was within range for PCR. Increasing the starting inoculum for these extractions resulted in an increase in the DNA yield (Appendix Table 1).

PCR was performed using the optimized conditions on DNA extracted from the 70 isolates. The target region was successfully amplified in 62/70 (89%) of the isolates, and the amplicons visualised using agarose gel electrophoresis. The remaining 8 isolates which were not amplified, failed to grow on repeat culture, and were excluded from further analysis. A selection 56 of 62 successful amplicons were submitted for DNA sequence determination.

The PCR protocol was easy to implement in the diagnostic laboratory, with the protocol easy to follow. The PCR results of the amplification were available within 24 hours after DNA extraction. The current method for yeast identification does not cover all medically important yeast and it has turnaround time 3-4 day . The PCR will enable identification of yeasts that is not included in the biochemical platform. DNA extraction can be performed after a day of incubation. For mould there are no biochemical identification platform The PCR produced reliable results when performed with adequate DNA template concentrations. All successful PCR amplicons were subsequently found to be adequate for sequence analysis.

Sequencing analysis

A selection of 56 of the 62 successful amplicons were submitted for bidirectional sequencing, producing 112 sequence results (56 forward and 56 reverse sequences). The subsequent raw data files were visually inspected for integrity using Codoncode aligner software (version 1.6.1), before querying on suitable reference databases. Data on the total length, trimmed length, and quality data was collected to determine if there were differences between the forward and reverse sequences in terms of length, quality, and performance when queried on available databases. For the *Emmonsia* spp. isolate 2, the forward sequence was too short (45 bases) for comparison and it was excluded from further analysis.

The mean sequence length of the forward sequence was 695bp with a 95% confident interval (CI) of 637bp – 752bp, and the reverse was 661bp (624bp – 698bp), while the trimmed sequence means were 511bp (478bp – 544bp) for the forward, and 579bp (519bp – 583bp) for the reverse sequences. The trimmed reverse sequences produced a marginally longer sequence length than the forward. These differences in length did not influence the identification results obtained.

A local alignment for sequence similarities, using the NCBI database was performed on the 111 trimmed sequences. The following search parameters were used: MEGABLAST, search on all microorganisms' databases, and excluding model and uncultured organisms. Data on identification percentage, coverage percent of the query length that is included in the aligned segments and expect value (E-value) were collected. Sequence similarities ≥ 98 %, over a range of ≥ 90 % of the ITS1 – 2 region was considered reliable and reported [8,10].

Of all the sequences queried on the NCBI BLAST database, 95/111(86%) were concordant with the results obtained from the reference laboratory.

Sequence similarity searches were performed on the Ribosomal Database Project (RDP) fungal sequence database using the "new fungal ITS training sets" to classify fungal ITS sequences. The database was last updated on the 30th of September 2016. The RDP classifier database requires sequence of at least 50 bases in length. The database only reports results that achieved 100% identification probability. Results of the RDP search database produced the same results as those

obtained using the NCBI database, with the same 95/111 (86%) results concordant to the reference laboratory results (Appendix Table2).

The remaining 16/111 (14 %) sequences with discordant identification results (corresponding to 8 isolates) were inspected and summarized in the Appendix Table3. Both databases gave the same incorrect identification for all 16 discordant samples.

The DNA yield and purity of the 8 isolates(Table 1) indicated that 5 isolates had DNA concentrations<5 ng/µl, thus poor quality or low yield DNA was considered to be the most likely reason for incorrect or failed identification. Contamination during DNA extraction or elute mix-up during PCR run was considered a likely reason for the remaining discordant results.Due to budgetary and time constraints further work to investigate discordant results, such as whole genome sequencing, was not possible.

An internal control will be included for clinical sample validation of the PCR

Table1. The DNA yield and purity of fungal isolates with discordant sequence results.

Fungal isolate	**DNA concentration (ng/µl)**	**260:280 ratio**
Aspergillus fumigatus	12.99	1.69
Scedosporium species	11.89	1.99
Rhizopus species	3.49	1.48
Rhizopus species	4.99	1.61
Candida albicans	18.77	1.72
Rhodotorula species	1.211	1.672
Rhodotorula species	1.902	1.543
Scedosporiumprolificans	5.98	1.65

The two sequence databases were found to be simple to use and produced reproducible and reliable results for isolates with sufficient quality DNA. The basic local alignment tool at NCBI was found to be slightly better since it is a more comprehensive database. It contains a wide variety of mycology sequences, including those from non-medical taxa. The NCBI BLAST output includes information on the highest alignment score between the query sequence (Max score) and sum of the alignment scores of all the segments from the same database that match the queried

sequence. The score values are used to assess the relevance of the finding and the overall quality of an alignment. The RDP classifier quickly searches and accurately assigns into taxa with a bootstrap value and does not require full alignment[48].The RDP database has been manually curated to improve its accuracy. Based on these results we recommend that either database be used when querying an unknown fungal sequence, and sequences with unusual or unexpected results confirmed on both databases. If metrics are required for identification, the BLAST algorithm would be a better option than the RDP classifier.

Conclusions

This study has demonstrated that a selected pan-fungal PCR, coupled with DNA sequence analysis was an excellent diagnostic tool for the detection and identification of medically important fungal isolates. The DNA extraction method validation findings indicated that a manual lysis step is required to produce adequate DNA concentrations from cultured isolates. It was shown that implementing the optimized PCR in a routine diagnostic laboratory was feasible and could be performed in combination with conventional culture tests for the rapid and accurate identification of fungal isolates. The current method for yeast identification did not cover all medically important yeast and it had turnaround time 3-4 days. When performed on yeast isolates the panfungal PCR enabled accurate identification of yeasts that are not included in the biochemical platform. For mould there are no biochemical identification platforms the panfungal PCR was able to accurately identify all the mould isolates. In the diagnostic algorithm of the lab the PCR will be performed on yeast isolates failed to be identified by biochemical methods and all mould isolates since there are no biochemical platforms.

PCR is estimated to cost R700 per sample, which is more than the cost of the phenotypic methods. However, it ensures accurate identification of fungal pathogens.

The results need to be correlated with phenotypic appearance, as well as histology/radiology, and clinical data, as this will impact on patient care. Future work will involve evaluating this assay

for use on deep tissue biopsies. The method will even be more rapid when validated for use directly on clinical specimens.

Author contributions

- Conceived and designed: Sindile Ntuli, Clinton Moodley and Colleen Bamford
- Experimental work:Sindile Ntuli and Clinton Moodley
- Contributed reagent/material/analysis tools: Sindile Ntuli and Clinton Moodley
- Data analysis:Sindile Ntuli, Clinton Moodley
- Manuscript preparation: Sindile Ntuli
- Reviewed by: Clinton Moodley and Colleen Bamford

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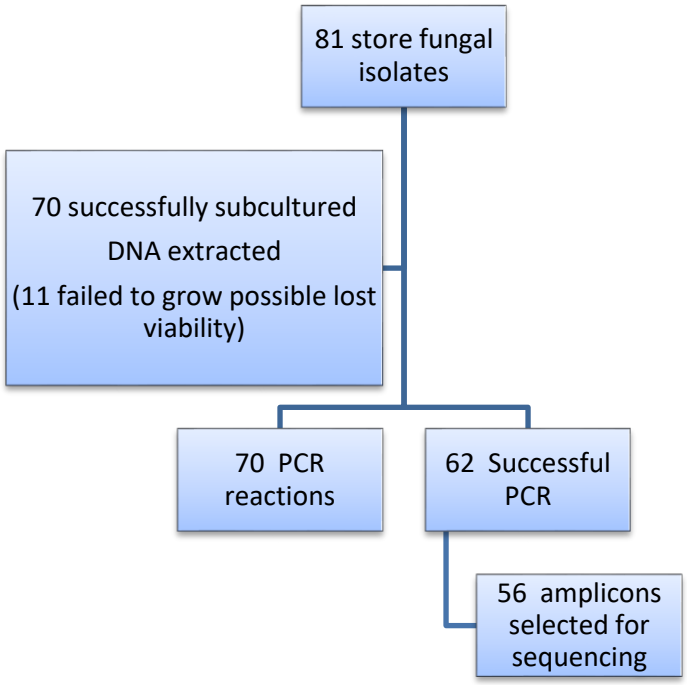
Appendix



Flow diagram1. PCR validation fungal isolates

Table 1. PCR validation fungal isolates

Fungal isolates	DNA concentration (ng/µl)	260:280	Successful PCR	Sequenced
Dimorphic fungi				
Sporothrix schenckii	10.03	1.66	Yes	Yes
Sporothrix schenckii	11.25	1.79	Yes	No
Histoplasma capsulatum	1.47	1.69	No	No
Histoplasma capsulatum	1.25	1.59	No	No
Emmonsia sp.	7.892	1.98	Yes	Yes
Emmonsia sp.1	5.41	1.68	Yes	Yes
Emmonsia sp.2	10.89	1.578	Yes	Yes
Emmonsia sp.3	13.52	1.506	Yes	Yes
Emmonsia sp. 4	9.45	1.45	Yes	Yes
Dermatophytes				
Trichophyton interdigitale	1.47	1.67	Yes	Yes
Trichophyton interdigitale	1.25	1.79	Yes	Yes
Trichophyton interdigitale	14.13	1.63	Yes	Yes
Trichophyton interdigitale	10.33	1.66	Yes	Yes
Trichophyton interdigitale	9.29	1.69	Yes	No
Trichophyton violaceum	8.43	1.59	Yes	Yes
Epidermophytonfloccosum			No	No
Microsporumcanis	9.22	1.66	Yes	Yes
Microsporumcanis	11.41	1.25	Yes	Yes
Microsporumgypseum	4.7	1.635	Yes	Yes
Hyaline hyphomycetes				
Aspergillus fumigatus	12.99	1.69	Yes	Yes
Aspergillus nidulans	11.55	1.65	Yes	Yes
Aspergillus niger	9.22	1.66	Yes	Yes
Aspergillus terreus	7.41	1.75	Yes	No
Aspergillus clavatus	8.7	1.75	Yes	No
Fusarium species	1.404	1.799	Yes	Yes
Fusarium species	1.655	1.745	Yes	Yes
Fusarium delphinoides	12.68	1.782	Yes	Yes
Paecilomyces species	19.33	1.72	Yes	Yes
Acremonium species	37.26	1.62	Yes	
Penicillium species			No	
Penicillium lavendum/Geosmithialavendula	15.88	1.765	Yes	Yes
Pseudolescheria species	9.772	1.79	Yes	Yes
Pseudallescheria boydii	4.44	1.65	Yes	Yes
Scedosporiumprolificans	5.98	1.65	Yes	Yes

Scedosporium species	11.89	1.99	Yes	Yes
Paecilomyces species	6.69	1.72	Yes	Yes
Mucorales				
Actinomucor species	8.663	1.71	Yes	Yes
Mucor species	9.288	1.691	Yes	Yes
Mucor species	7.49	1.74	Yes	Yes
Mucor species	8.288	1.72	Yes	Yes
Mucor species	7.35	1.64	Yes	Yes
Cunninghamella species	28.6	1.536	Yes	Yes
Cunninghamella species	37.26	1.536	Yes	Yes
Rhizopus species	3.49	1.48	Yes	Yes
Rhizopus species	4.99	1.61	Yes	Yes
Yeast				
Candida albicans	18.77	1.72	Yes	Yes
Candida glabrata	4.848	1.64	Yes	Yes
Candida krusei	2.38	1.86	Yes	No
Candida parapsilosis1			No	No
Candida tropicalis	43.96	1.786	Yes	No
Candida utilis ATCC 9950	27.76	1.902	Yes	Yes
Candida parapsilosis2			No	No
Candida lusitanae ATCC 34449	9.615	1.67	Yes	Yes
Candida guillermondi	14.88	1.87	Yes	Yes
Candida auris	19.08	1.719	Yes	Yes
Candida dubliniensis ATCC44508	38.05	1.798	Yes	Yes
Trichosporon sp.	21.08	1.87	Yes	Yes
Cryptococcus neoformans	7.362	1.719	Yes	Yes
Cryptococcus neoformans	3.762	1.719	Yes	Yes
Rhodotorula species	1.211	1.672	Yes	Yes
Rhodotorula species	1.902	1.543	Yes	Yes
Kloeckera Japonica ATCC 58370	12.77	1.76	Yes	Yes
Sporobolomycessalmonicolor MYA-4550	3.198	1.89	Yes	Yes
Trichosporonspp	21.08	1.764	Yes	Yes
Trichosporonucoides ATCC 204094	7.89	1.789	Yes	Yes
Geotrichumspp	15.06	1.71	Yes	Yes
Prototheca ATCC 16529				No
Dematiaceous fungi				
Alternaria species	9.66	1.82	Yes	Yes
Scytalidium species			No	No
Curvularia species	7.892	1.771	Yes	Yes

	70	**62 (successful)**	**56 (sequenced)**

Table2. Concordance results between the two sequence databases and the reference laboratory results.

Sample	*NCBI BLAST Identification*	*RDP Identification*	*Reference laboratory*
1. C4_ITS-1_G09	*Candida guillermondi*	*Candida guillermondi*	*Candida guillermondi*
2. C4_ITS-4_G10	*Candida guillermondi*	*Candida guillermondi*	*Candida guillermondi*
3. 1_ITS-F_B02	*Cryptococcus neoformans*	*Cryptococcus neoformans*	*Cryptococcus neoformans*
4. 1_ITS-R_C05	*Cryptococcus neoformans*	*Cryptococcus neoformans*	*Cryptococcus neoformans*
5. 10_ITS-F_C03	*Paecilomyces sp.*	*Paecilomyces sp.*	*Paecilomyces sp.*
6. 10_ITS-R_D06	*Paecilomyces sp.*	*Paecilomyces sp.*	*Paecilomyces sp.*
7. 11_ITS-F_D03	*Emmonsia sp.*	*Emmonsia sp.*	*Emmonsia sp.*
8. 11_ITS-R_E06	*Emmonsia sp.*	*Emmonsia sp.*	*Emmonsia sp.*
9. 12_ITS-F_E03	*Emmonsia sp*	*Emmonsia sp.*	*Emmonsia sp.*
10. 12_ITS-R_F06	*Emmonsia sp.*	*Emmonsia sp.*	*Emmonsia sp*
11. 13_ITS-F_F03	*Trichosporonsp*	*Trichosporonsp*	*Trichosporonsp*
12. 13_ITS-R_A07	*Trichosporonsp*	*Trichosporonsp*	*Trichosporonsp*
13. 14_ITS-F_G03	*Emmonsia sp.*	*Emmonsia sp.*	*Emmonsia sp.*
14. 14_ITS-R_H06	*Emmonsia sp.*	*Emmonsia sp.*	*Emmonsia sp.*
15. 15_ITS-F_H03	*Trichosporonsp*	*Trichosporonsp*	*Trichosporonsp*
16. 15_ITS-R_G06	*Trichosporonsp*	*Trichosporonsp*	*Trichosporonsp*
17. 16_ITS-F_A04	*Candida auris*	*Candida auris*	*Candida auris*
18. 16_ITS-R_E05	*Candida auris*	*Candida auris*	*Candida auris*
19. 17_ITS-F_B04	*Cunninghamella*	*Cunninghamella*	*Cunninghamella*
20. 17_ITS-R_F05	*Cunninghamella*	*Cunninghamella*	*Cunninghamella*
21. 18_ITS-F_C04	*Geotrichumcandidum*	*Geotrichumcandidum*	*Geotrichumcandidum*
22. 18_ITS-R_G05	*Geotrichumcandidum*	*Geotrichumcandidum*	*Geotrichumcandidum*
23. 19_ITS-F_D04	*Paecilomyces sp.*	*Paecilomycesvariotii*	*Paecilomyces sp.*
24. 19_ITS-R_H05	*Paecilomyces sp.*	*Paecilomycesvariotii*	*Paecilomyces sp.*

25. 2_ITS-F_C02	*Cutaneotrichosporonmucoides*	*Trichosporon sp.*	*Trichosporonmucoides*
26. 2_ITS-R_D05	*Cutaneotrichosporonmucoides*	*Trichosporon sp.*	*Trichosporonmucoides*
27. 20_ITS-F_E04	*Actinomucorelegans*	*Actinomucorelegans*	*Actinomucorelegans*
28. 20_ITS-R_E06	*Actinomucorelegans*	*Actinomucorelegans*	*Actinomucorelegans*
29. 21_ITS-F_F04	*Mucor sp.*	*Mucor sp.*	*Mucor sp.*
30. 21_ITS-R_F06_18	*Mucor sp.*	*Mucor sp.*	*Mucor sp.*
31. 22_ITS-F_G04	*Mucor sp.*	*Mucor sp.*	*Mucor sp.*
32. 22_ITS-R_G06	*Mucor sp.*	*Mucor sp.*	*Mucor sp.*
33. 23_ITS-F_H04	*Mucor sp.*	*Mucor sp.*	*Mucor sp.*
34. 23_ITS-R_H06	*Mucor sp.*	*Mucor sp.*	*Mucor sp.*
35. 24_ITS-F_A05_02	*Mucor sp.*	*Mucor sp.*	*Mucor sp.*
36. 24_ITS-R_A07_01	*Mucor sp.*	*Mucor sp.*	*Mucor sp.*
37. 25_ITS-F_B05_05	*Candida utilis*	*Candida utilis*	*Candida utilis*
38. 25_ITS-R_C08_08	*Candida utilis*	*Candida utilis*	*Candida utilis*
39. 3_ITS-F_D02_11	*Clavisporalusitaniae*	*Clavisporalusitaniae*	*Clavisporalusitaniae(Candida lusitaniae)*
40. 3_ITS-R_E05_14	*Clavisporalusitaniae*	*Clavisporalusitaniae*	*Clavisporalusitaniae(Candida lusitaniae)*
41. 4_ITS-F_E02_14	*Candida dubliniensis*	*Candida dubliniensis*	*Candida dubliniensis*
42. 4_ITS-R_F05_17	*Candida dubliniensis*	*Candida dubliniensis*	*Candida dubliniensis*
43. 5_ITS-F_F02_17	*Microsporumcanis*	*Microsporumcanis*	*Microsporumcanis*
44. 5_ITS-R_G05_20	*Microsporumcanis*	*Microsporumcanis*	*Microsporumcanis*
45. 6_ITS-F_G02_20	*Cunninghamella*	*Cunninghamellaelegans*	*Cunninghamella*
46. 6_ITS-R_H05_23	*Cunninghamella*	*Cunninghamellaelegans*	*Cunninghamella*
47. 7_ITS-F_H02_23	*Acremonium sp.*	*Acremonium alternatum*	*Acremonium sp.*
48. 7_ITS-R_A06_03	*Acremonium sp.*	*Acremonium alternatum*	*Acremonium sp.*
49. 8_ITS-F_A03_03	*Trichophyton interdigitale*	*Trichophyton sp.*	*Trichophyton interdigitale*
50. 8_ITS-R_B06_06	*Trichophyton interdigitale*	*Trichophyton sp.*	*Trichophyton interdigitale*

51. 9_ITS-F_B03_06	*Trichophyton interdigitale*	*Trichophyton sp.*	*Trichophyton interdigitale*
52. 9_ITS-R_C06_09	*Trichophyton interdigitale*	*Trichophyton sp.*	*Trichophyton interdigitale*
53. A2_ITS-1_H05_23	*Aspergillus nidulans*	*Aspergillus nidulans*	*Aspergillusnidulans*
54. A2_ITS-4_H06_24	*Aspergillusnidulans*	*Aspergillus nidulans*	*Aspergillusnidulans*
55. A3_ITS-1_A07_01	*Aspergillus niger*	*Aspergillus sp*	*Aspergillus niger*
56. A3_ITS-4_A08_02	*Aspergillusniger*	*Aspergillus sp*	*Aspergillus niger*
57. ALT1_ITS-1_E11_14	*Alternaria alternata*	*Alternaria sp.*	*Alternaria alternata*
58. ALT1_ITS-4_E12_15	*Alternaria alternata*	*Alternaria*	*Alternaria alternata*
59. C2_ITS-1_E09_15	*Candida glabrata*	*Candida glabrata*	*Candida glabrata*
60. C2_ITS-4_E10_13	*Candida glabrata*	*Candida glabrata*	*Candida glabrata*
61. CH1_ITS-1_F07_16	*Pseudallescheria boydii*	*Pseudallescheria boydii*	*Pseudallescheria boydii*
62. CH1_ITS-4_F08_17	*Pseudallescheria boydii*	*Pseudallescheria boydii*	*Pseudallescheria boydii*
63. CR1_ITS-1_H09_24	*Cryptococcus neoformans*	*Cryptococcus neoformans*	*Cryptococcus neoformans*
64. CR1_ITS-4_H10_22	*Cryptococcus neoformans*	*Cryptococcus neoformans*	*Cryptococcus neoformans*
65. CUR1_ITS-1_G11_20	*Curvularialunata*	*Curvularialunata*	*Curvularialunata*
66. CUR1_ITS-4_G12_21	*Curvularialunata*	*Curvularialunata*	*Curvularialunata*
67. E1_ITS-1_H07_22	*Emmonsia sp*	*Emmonsia sp*	*Emmonsia sp*
68. E1_ITS-4_H08_23	*Emmonsia sp*	*Emmonsia sp*	*Emmonsia sp*
69. E2_ITS-4_A10_01	*Emmonsia sp*	*Emmonsia sp*	*Emmonsia sp*
70. F1_ITS-1_B07_04	*Fusarium sp.*	*Fusarium sp.*	*Fusarium sp.*
71. F1_ITS-4_B08_05	*Fusarium sp.*	*Fusarium sp.*	*Fusarium sp.*
72. F2_ITS-1_C07_07	*Fusarium sp.*	*Fusarium sp.*	*Fusarium sp.*
73. F2_ITS-4_C08_08	*Fusarium sp.*	*Fusarium sp.*	*Fusarium sp.*
74. F3_ITS-1_D07_10	*Fusarium delphinoides*	*Fusarium delphinoides*	*Fusarium delphinoides*
75. F3_ITS-4_D08_11	*Fusarium delphinoides*	*Fusarium delphinoides*	*Fusarium delphinoides*
76. K1_ITS-1_C11_08	*Hanseniasporavalbyensis /Kloeckera sp.*	*Kloeckera sp.*	*Kloeckera sp.*
77. K1_ITS-4_C12_09	*Hanseniasporavalbyensis /Kloeckera sp.*	*Kloeckera sp.*	*Kloeckera sp.*

78. M1_ITS-1_E05_14	*Microsporumcanis*	*Microsporumcanis*	*Microsporum sp.*
79. M1_ITS-4_E06_15	*Microsporumcanis*	*Microsporumcanis*	*Microsporum sp.*
80. M2_ITS-1_F05_17	*Microsporumgypseum*	*Microsporumgypseum*	*Microsporumgypseum*
81. M2_ITS-4_F06_18	*Microsporumgypseum*	*Microsporumgypseum*	*Microsporumgypseum*
82. P1_ITS-1_E07_13	*Geosmithialavendula*	*Geosmithialavendula*	*Geosmithialavendula/ Penicillium lavendum*
83. P1_ITS-4_E08_14	*Geosmithialavendula*	*Geosmithialavendula*	*Geosmithialavendula/ Penicillium lavendum*
84. S1_ITS-1_A05_02	*Sporothrix schenckii*	*Sporothrix schenckii*	*Sporothrix schenckii*
85. S1_ITS-4_A06_03	*Sporothrix schenckii*	*Sporothrix schenckii*	*Sporothrix schenckii*
86. SP1_ITS-1_D11_11	*Sporidiobolussalmonicolor*	*Sporidiobolussalmonicolor*	*Sporidiobolussalmonicolor*
87. SP1_ITS-4_D12_12	*Sporidiobolussalmonicolor*	*Sporidiobolussalmonicolor*	*Sporidiobolussalmonicolor*
88. T1_ITS-1_B05_05	*Trichophyton interdigitale*	*Trichophyton sp.*	*Trichophyton interdigitale*
89. T1_ITS-4_B06_06	*Trichophyton interdigitale*	*Trichophyton sp.*	*Trichophyton interdigitale*
90. T2_ITS-1_C05_08	*Trichophyton erinacei*	*Trichophyton erinacei*	*Trichophyton erinacei*
91. T2_ITS-4_C06_09	*Trichophyton erinacei*	*Trichophyton erinacei*	*Trichophyton erinacei*
92. T3_ITS-1_D05_11	*Trichophyton violaceum*	*Trichophyton violaceum*	*Trichophyton violaceum*
93. T3_ITS-4_D06_12	*Trichophyton violaceum*	*Trichophyton violaceum*	*Trichophyton violaceum*
94. C3_ITS-1_F09_18	*Pichia kudriavzevii/ Candida kruise*	*Pichia sp.*	*Candida krusei*
95. C3_ITS-4_F10_16	*Pichia kudriavzevii/ Candida kruise*	*Pichia sp.*	*Candida kruise*

Table 3.Discordantsequence identification results.

Sample	NCBI BLAST Database 1	Query cover	Identification %	RDP Database 2	Reference lab Identification
1. A1_ITS-1_G05_20	*Trichophyton rubrum*	99	99	*Trichophyton sp*	*Aspergillus fumigatus*
2. A1_ITS-4_G06_21	*Trichophyton rubrum*	98	95	*Trichophyton sp*	*Aspergillus fumigatus*
3. C1_ITS-1_D09_12	*Pichia kudriavzevii*	98	95	*Pichia sp.*	*Candida albicans*
4. C1_ITS-4_D10_10	*Pichia kudriavzevii*	99	93	*Pichia sp.*	*Candida albicans*
5. R1_ITS-1_B09_06	*Meyerozymaguilliermondii*	96	93	*Meyerozyma sp.*	*Rhizopus sp. 1*
6. R1_ITS-4_B10_04	*Meyerozymaguilliermondii*	96	93	*Meyerozyma sp.*	*Rhizopus sp. 1*
7. R2_ITS-1_C09_09	*Meyerozymaguilliermondii*	90	88	*Meyerozyma sp.*	*Rhizopus sp. 2*
8. R2_ITS-4_C10_07	*Meyerozymaguilliermondii*	91	87	*Meyerozyma sp.*	*Rhizopus sp. 2*
9. RH1_ITS-1_A11_02	*Sporobolomyces roseus*	99	97	*Sporobolomycessp.*	*Rhodotorula sp. 1*
10. RH1_ITS-4_A12_03	*Sporobolomyces roseus*	99	99	*Sporobolomyces sp.*	*Rhodotorula sp. 1*
11. RH2_ITS-1_B11_05	*Sporobolomyces roseus*	99	99	*Sporobolomyces sp.*	*Rhodotorula sp. 2*
12. RH2_ITS-4_B12_06	*Sporobolomyces roseus*	98	97	*Sporobolomyces sp.*	*Rhodotorula sp. 2*
13. SC1_ITS-1_G07_19	*Candida parapsilosis*	100	93	*Candida parapsilosis*	*Scedosporium sp.*
14. SC1_ITS-4_G08_20	*Candida parapsilosis*	99	93	*Candida parapsilosis*	*Scedosporium sp.*
15. SC2_ITS-1_F11_17	*Candida parapsilosis*	99	93	*Candida parapsilosis*	*Scedosporium sp.*
16. SC2_ITS-4_F12_18	*Candida parapsilosis*	99	93	*Candida parapsilosis*	*Scedosporium sp.*