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Comparison of sequencing the D2 region of the large subunit ribosomal RNA gene (MicroSEQ®) versus the internal transcribed spacer (ITS) regions using two public databases for identification of common and uncommon clinically relevant fungal species

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ABSTRACT

Context: Fungal infections cause considerable morbidity and mortality in immunocompromised patients. Rapid and accurate identification of fungi is essential to guide accurately targeted antifungal therapy. With the advent of molecular methods, clinical laboratories can use new technologies to supplement traditional phenotypic identification of fungi.

Objective: The aims of the study were to evaluate the sole commercially available MicroSEQ® D2 LSU rDNA Fungal Identification Kit compared to the in-house developed internal transcribed spacer (ITS) regions assay in identifying moulds, using two well-known online public databases to analyze sequenced data.

Design: 85 common and uncommon clinically relevant fungi isolated from clinical specimens were sequenced for the D2 region of the large subunit (LSU) of ribosomal RNA (rRNA) gene with the MicroSEQ® Kit and the ITS regions with the in house developed assay. The generated sequenced data were analyzed with the online GenBank and MycoBank public databases.

Results: The D2 region of the LSU rRNA gene identified 89.4% or 92.9% of the 85 isolates to the genus level and the full ITS region (f-ITS) 96.5% or 100%, using GenBank or MycoBank, respectively, when compared to the consensus ID. When comparing species-level designations to the consensus ID, D2 region of the LSU rRNA gene aligned with 44.7% (38/85) or 52.9% (45/85) of these isolates in GenBank or MycoBank, respectively. By comparison, f-ITS possessed greater specificity, followed by ITS1, then ITS2 regions using GenBank or MycoBank. Using GenBank or MycoBank, D2 region of the LSU rRNA gene outperformed phenotypic based ID at the genus level. Comparing rates of ID between D2 region of the LSU rRNA gene and the ITS regions in GenBank or MycoBank at the species level against the consensus ID, f-ITS and ITS2 exceeded performance of the D2 region of the LSU rRNA gene, but ITS1 had similar performance to the D2 region of the LSU rRNA gene using MycoBank.

Conclusion: Our results indicated that the MicroSEQ® D2 LSU rDNA Fungal Identification Kit was equivalent to the in-house developed ITS regions assay to identify fungi at the genus level. The MycoBank database gave a better curated database and thus allowed a better genus and species identification for both D2 region of the LSU rRNA gene and ITS regions.

1. Introduction

Invasive fungal infections are associated with high morbidity and mortality, and accurate identification of the organism is needed to guide judicious antifungal therapy. Identification of fungi remains a major challenge and with roughly 560 species of currently known clinically relevant fungi causing morbidity and mortality in the world, and novel species continually being discovered (Caston-Osorio et al., 2008; Cleveland et al., 2015; de Hoog et al., 2015; Taylor et al., 2001), we must keep expanding the techniques used in the Clinical Diagnostic Laboratories to identify fungi. Commonly encountered fungi such as *Aspergillus* sp. and *Candida* sp. can be identified, usually to the species level, with phenotypic methods. However, novel or rarely encountered fungi are challenging to identify and a genus level identification can

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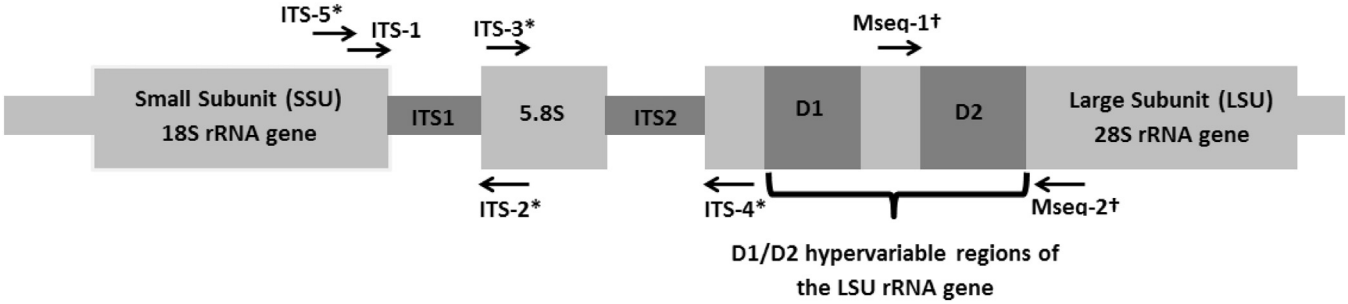

Fig. 1. Diagram of the ribosomal gene complex (not drawn to scale). The organization of this complex includes a sequence coding for the 18S rRNA gene, an internal transcribed region 1 (ITS1), the 5.8S rRNA gene coding region, another ITS region called ITS2 and the sequence coding for the 28S rRNA gene in moulds (25/26S rRNA in yeasts). Arrows indicate approximate positions of primers used to amplify regions of interest.
*The sequence of each primer is given in Table 2.
†The sequences of the primer pair (Mseq-1 and Mseq-2) used for the amplification of the D2 region of the large subunit of the ribosomal RNA gene were not provided by the manufacturer of the MicroSEQ® Fungal Identification Kit.

even be difficult to determine. In moving forward with guiding antifungal treatments, fast, consistent, and accurate identification is imperative, as many of these novel/rare fungal pathogens will carry their own antifungal resistance mechanisms.

DNA sequence-based identification, when used in conjunction with phenotypic methods, often provides greater resolution of an unknown fungus's identity (Hinrikson et al., 2005; Kurtzman and Robnett, 1998; Petti, 2007). Two sequences of significance being investigated are the D1-D2 hypervariable region and the Internal Transcribed Spacers (ITS) regions (Fig. 1). The D1-D2 hypervariable region encodes for a ribosomal RNA expansion segment of the large-subunit (28S for moulds and 25/26S for yeasts). The ITS regions lie between the small subunit (SSU) ribosomal DNA and the large subunit (LSU) ribosomal DNA coding regions. It is divided into ITS1 and ITS2, which are separated by the gene encoding for 5.8S ribosomal DNA. Both, the D1-D2 region and the ITS regions contain mosaics of conservative and variable sequence regions (CLSI, 2008) that permit the utilization of universal primers that produce sequences with sufficient variability to define a given fungal species. Investigation into the use of only segments of these sequences, such as the D2 from the D1/D2 complex and ITS1 or ITS2 from the full ITS (f-ITS) region (ITS1 + 5.8S + ITS2), has been shown to provide adequate identification of many fungi (Balajee et al., 2009; Hall et al., 2003; Leaw et al., 2006; Rakeman et al., 2005).

Implementation of fungal identification by DNA sequencing can be challenging for smaller laboratories with limited molecular expertise, leaving them with only one option, and that is to purchase a kit with prepackaged reagents and a prewritten assay protocol that eliminates the need for assay development. To our knowledge, in the United States, no commercial kits are available for the full sequence of the D1-D2 hypervariable region or the internal transcribed spacer regions. One commercial product from Applied Biosystems (Foster City, CA), is available for sequencing of the D2 region of the D1/D2 complex and research has gone into determining its effectiveness as a taxonomic identifier, but has primarily focused on a few commonly encountered fungal genera (Fell et al., 2000; Hinrikson et al., 2005; Kurtzman and Robnett, 1998; Leaw et al., 2006). This has left further investigation of the MicroSEQ® D2 LSU rDNA Fungal Identification Kit's effectiveness in identifying many clinically encountered fungi, and its comparison to sequencing of ITS regions, largely unexplored.

Another challenge is the choice of the databases used to analyze the generated sequencing data. Multiples private fungal sequence databases like SmartGene Fungi (Lausanne, Switzerland), and MicroSEQ® ID Fungal Gene Library (Applied Biosystems, Foster City, CA) offer better curated databases compared to online public databases like GenBank and MycoBank, but they are relatively expensive to acquire and to justify in laboratories that process only a few specimens a year (lowest price of $11,700 for MicroSEQ® ID Fungal Gene Library as of 3/2017). In the past decade, reliable online public databases dedicated to fungal identification have been developed that can be used by laboratories for identification of fungi. National Center for Biotechnology Information (NCBI) GenBank is the oldest and the most commonly used online public database but is not curated. MycoBank is a well-known online fungal public database, substantially used by the mycological community, remotely curated, and offers the most comprehensive search options on molecular data alone (Prakash et al., 2017).

Thus, we focused on two highly accessible databases that continually improve with community support, and help introduce newcomers to sequence-based means of identification.

The objective of this study was to evaluate the performance of the MicroSEQ® D2 LSU rDNA Fungal Identification Kit (Applied Biosystems, Foster City, CA) at identifying fungi compared to the laboratory developed ITS regions sequencing assay, using two public online databases GenBank and MycoBank to analyze generated sequences of common and uncommon clinically relevant fungi.

2. Materials and methods

2.1. Clinical isolates

A total of 78 moulds and 8 yeasts, previously identified in the University of Minnesota Medical Center's Infectious Diseases Diagnostic Laboratory (IDDL), were analyzed in this study. The IDDL used current techniques that rely on morphological (macroscopic and microscopic features) and physiological (growth temperature and various media) characteristics to assign a genus and species to fungal isolates (Ashbee, 2015). Once identified, the isolates were sub-cultured on BBL™ Sabouraud Dextrose Agar (Emmons) (Becton Dickinson and Company, Franklin Lakes, New Jersey) and incubated at 30 °C for up to one week before DNA extraction and sequencing. Seventy-seven moulds were investigated (Table 1). One mould could not be identified by any approach and thus was excluded from the analysis.

2.2. Nucleic acid extraction

Extraction of the fungal DNA was performed using the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA). A small amount (7–20 mg) of a fungal colony was suspended into a 2.0-ml microcentifuge tube containing 200 µl of PrepMan® Ultra Sample Preparation reagent. The tube was vortexed for 10 to 30 s and incubated for 10 min at 100 °C in a heating block. The tube containing the lysates was then centrifuged at 14,000 RPM for 3 min to settle the cell debris and 100 µl of the supernatant was transferred to a new microcentrifuge tube and stored at 4 °C. The fungal DNA concentration was determined by using the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific™, Waltham, MA).

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