Evaluation of the QIAcube® and the MicroSEQ® D2 LSU rDNA system for the identification of fungal isolates from medical products

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ABSTRACT

Fungi have been implicated in multiple infections and outbreaks stemming from the use of drugs and medical devices. FDA routinely tests medical products for the presence of fungi. Any isolates recovered are identified by sequencing the D1-D2 and ITS regions of the ribosomal RNA gene (rDNA) using the method outlined in ORA-LAB.017. This method comprises numerous manual steps and requires significant user input for data analysis.

This study aimed to streamline fungal identification by automating the DNA extraction and data analysis steps. The QIAcube®, a robotics platform, was evaluated for DNA extraction. The MicroSEQ® Fungal Identification Kit, targeting the D2 region of the rDNA gene, was evaluated along with the MicroSEQ® proprietary fungal DNA library for the identification of fungal isolates. Public databases were used when the MicroSEQ® library failed to provide an identification within the established parameters.

A total of 99 mold and yeast isolates were analyzed by ORA-LAB.017 and MicroSEQ® and the results were compared. The QIAcube® yielded DNA comparable to manual extraction and suitable for sequencing. MicroSEQ® and ORA-LAB.017 identified 97/99 isolates as the same genus. Seventy-one isolates (71/99) were identified as the same species. Twenty-seven isolates (27/99) were identified by one or both methods as multiple species of the same genus. One isolate (1/99) was identified as *Sarocladium killiense* by ORA-LAB.017 but as *Nectria mauritiicola*, a closely related species, by MicroSEQ®. Another isolate (1/99) identified by ORA-LAB.017 as *Phialophora americana* could not be identified by MicroSEQ® due to its low percent match. ORA-LAB.017 correctly identified 42/42 culture type strains included in the study. MicroSEQ® properly identified 39/42 isolates. Three isolates (3/42) were identified as other closely related species.

Keywords: fungi, sequencing, MicroSEQ, QIAcube

INTRODUCTION

Fungi are common contaminants of medical products. Between 1998 and 2006 yeast and mold were responsible for 23% of non-sterile and 7% of sterile pharmaceutical product recalls[1]. A more recent analysis conducted over the years 2004-2011showed that yeast and molds were cited in 21% of recalls of non-sterile products[2]. Fungi have been implicated in multiple infections and outbreaks stemming from the use of medical products including oral and injectable drugs, as well as, ophthalmic products[3-5].

In 2012, a fungal meningitis outbreak linked to contaminated steroid injections manufactured at a compounding pharmacy led to more than 750 cases of illness and 60 deaths in 20 states[6]. In response to the outbreak, FDA increased its oversight of compounding pharmacies, including increased inspections and environmental monitoring (EM) sample collections. A typical EM inspection may include 100 or more environmental samples. Because fungi are commonly found in the environment, EM sample collections have greatly increased the number of fungal isolates recovered.

ORA laboratories currently apply a ribosomal DNA (rDNA) sequencing method, ORA-LAB.017, to identify fungi isolates recovered from medical products. The method starts with manual extraction of the DNA, a time consuming and labor-intensive step. This is followed by amplification and sequencing of the D1-D2 and ITS regions of the rDNA gene. The DNA sequences obtained are then trimmed and checked for quality in a process that involves considerable user input. Finally, the trimmed sequences are searched in publicly available libraries to determine the genus or species of the organism. These databases although extensive, have been shown to contain inaccuracies[7].

This study aims to streamline fungal identification by introducing automation. The QIAcube®, a robotics platform which can extract DNA from 12 isolates in less than one hour, was evaluated. Robotics platforms have been previously used for the successful extraction of fungal DNA[8]. The study also evaluated the MicroSEQ® D2 Large Subunit (LSU) rDNA identification system which includes reagent kits, analysis software and a DNA sequence library. The kits contain the reagents necessary for amplifying and sequencing the D2 region of the fungal rDNA gene. The software automates the trimming and analysis of sequencing data, employing a proprietary curated fungal DNA library and/or a user developed library. The performance of the MicroSEQ® system has been previously compared to in-house developed protocols and online databases[9].

EXPERIMENTAL

Equipment:

a) 0.5 mm diameter glass beads (BioSpec, Bartlesville, OK)

b) Mini Bead- beater (Biospec)

c) RB tubes (QIAgen, Germantown, MD)

d) QIAcube® (QIAgen)

e) Veriti™ thermal cycler (Applied Biosystems, Foster City, CA)

f) FlashGel™ System (Lonza, Rockland, ME)

g) GelDoc XR+ (Bio-Rad, Hercules, CA)

h) 3500xL Genetic Analyzer (Applied Biosystems)

Reagents:

a) Sabouraud Dextrose Agar (Becton Dickinson, Franklin Lakes, NJ)

b) Molecular biology grade water (Fisher Scientific, Pittsburg, PA)

c) 10 mM Tris-Cl (Fisher Scientific)

d) 1 mM EDTA (Fisher Scientific)

e) 100 mM NaCl (Fisher Scientific)

f) 50 mM Beta-Mercapto-ethanol (Sigma-Aldrich, St. Louis, MO)

g) Proteinase K (Qiagen)

h) DNeasy® Blood & Tissue Kit (QIAgen)

i) MicroSEQ® D2 LSU rDNA PCR Kit (Applied Biosystems)

j) 2x HotStar Taq® Master Mix (Qiagen)

k) Primers (Integrated DNA Technologies, Coralville, IA)

l) FlashGel™ 1.2 % Agarose Gel Cassettes (Lonza)

m) FlashGel™ DNA marker 100 bp – 4.0 kb (Lonza)

n) FlashGel™ loading dye (Lonza)

o) ExoSAP-IT™ (Affymetrix, Cleveland, OH)

p) MicroSeq® D2 LSU rDNA Sequencing Kit (Applied Biosystems)

q) BigDye™ Terminator 3.1 (Applied Biosystems)

r) 5x BigDye™ buffer (Applied Biosystems)

s) Performa® DTR Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD)

t) HiDi™ formamide (Applied Biosystems)

Fungal isolate preparation

Ninety-nine common and clinically relevant fungi (76 molds and 23 yeasts) were included in this study. The cohort included 54 isolates received from the California Orange County Public Health Laboratory; 41 isolates of verified identity obtained from ATCC, CDC, USDA and BEI; 3 isolates recovered at WEAC from medical products; and purified *Sacharomyces cerevisiae* DNA provided with the MicroSEQ® kits. A complete list of the isolates tested is provided in Supplement Tables 1 and 2 at the end of this document. Each fungal isolate was streaked onto Sabouraud Dextrose Agar (SDA) plate in a Biological Safety Cabinet (BSC). The plates were incubated at 25°C for 2 to 7 days. Analysis of *S. cerevisiae* purified DNA was initiated at the Amplicon PCR step below.

DNA Extraction

A loopful of fungal growth from each SDA plate was transferred to a screw capped v-bottom tube containing 540µl of filter sterilized DNA lysis buffer (10mM Tris-Cl, 1mM EDTA, 100mM NaCl, and 50mM Beta-Mercapto-ethanol, pH7), 60µl of Proteinase K and ~0.2g of 0.5 mm diameter glass beads. The tubes were homogenized in a Mini Bead- beater at maximum speed for 1 minute and then centrifuged at 10,000 xg for 5 min to pellet cell debris.

MicroSEQ® method - A total of 200µl of the supernatant from each tube was transferred to an RB tube. The DNA extraction was then continued in the QIAcube® following the "DNeasy® Blood & Tissue - Animal tissues and rodent tails - Standard Protocol" preloaded onto the instrument.

ORA-LAB.017 method – Added 400µl AL lysis buffer to the remaining supernatant and continued extraction using the DNeasy® Blood & Tissue Kit as described in ORA-LAB.017.

Extraction controls were prepared as described above, except that no fungal culture was added. The controls were subjected to extraction and amplicon PCR by both the MicroSEQ® and ORA-LAB.017 methods followed by agarose gel electrophoresis. Analysis of these controls was stopped in the absence of visible bands on the agarose gels.

Positive culture controls consisting of *Aspergillus brasiliensis* ATCC 16404 were prepared as described above. Analysis of these controls was continued throughout capillary electrophoresis and data analysis.

Amplicon PCR

MicroSEQ® method – Prepared 1:100 dilutions of the extracted DNA using sterile molecular grade water. The D2 region of the rRNA gene was then amplified on a Veriti™ thermal cycler using the MicroSEQ® D2 LSU rDNA PCR Kit. The reaction volumes were reduced to one half of that recommended by the manufacturer's protocol. Briefly, combined 7.5 µl of diluted extracted DNA with 7.5 µl of PCR reaction mix. The reactions were run on a Veriti™ thermal cycler with the following conditions: 1) 10 min at 95^0C, 2) 35 cycles of [30 sec at 95^0C, 30 sec at 53^0C, 1 min at 72^0C] and 3) 10 min at 72^0C.

Purified *Sacharomyces cerevisiae* DNA and sterile water provided with the MicroSEQ® kit were used as positive and negative PCR controls, respectively. These controls were continued throughout capillary electrophoresis and data analysis.

ORA-LAB.017 method –The D1D2 region was used as the primary target. The ITS region was only sequenced when D1D2 failed to assign the isolate to one single species. Both regions of the rRNA gene were amplified using the primers listed in Table I. Each PCR reaction was performed in a 15µL volume containing the following: 1µL of DNA template, 7.5µL of 2x HotStar Taq® Master Mix, 0.03µL of a 100µM solution of each primer and 6.44µL of PCR grade water. The reactions were run on a Veriti™ thermal cycler with the following conditions: 1) 15 min at 95^0C, 2) 35 cycles of [30 sec at 95^0C, 30 sec at 58^0C, 1 min at 72^0C] and 3) 5 min at 72^0C.

Purified *Aspergillus brasiliensis* ATCC 16404 previously extracted in-house and sterile water were used as positive and negative PCR controls, respectively. These controls were continued throughout capillary electrophoresis and data analysis.

Table 1. Primers used for amplicon PCR and cycle sequencing by the ORA-LAB.017 Method.

Primer	Nucleotide sequence (5' → 3')	Target region
D1-forward	GCATATCAATAAGCGGAGGA	D1-D2
D2- reverse	TTGGTCCGTGTTTCAAGACG	D1-D2
ITS-5 forward	GGAAGTAAAAGTCGTAACAAGG	ITS
IT-4 reverse	TCCTCCGCTTATTGATATGC	ITS

Agarose Gel Electrophoresis

The size of the PCR products was confirmed by agarose gel electrophoresis. FlashGel™ 1.2 % agarose gel cassettes were loaded with 4 µl of PCR amplification product and 1 µl of FlashGel™ loading dye. Five µl of FlashGel™ DNA marker (100 bp – 4.0 kb) were also loaded onto each cassette and used to assess the size of the fragments. The cassettes were run on a FlashGel™ System at 200V for approximately 5 minutes. Digital images of the gels were captured using a GelDoc XR+ digital imaging system.

PCR Product Clean Up

Unused dNTPs and primers were removed from the remaining PCR product mixture using ExoSAP-IT™. Added 2.2 µl of ExoSAP-IT™ to the remaining 11 µl of PCR amplification product. Incubated reactions in a Veriti™ thermal cycler at 37°C for 15 minutes followed by 80°C for 15 minutes.

Cycle Sequencing

MicroSEQ® method - The MicroSeq® D2 LSU rDNA Sequencing Kit was used to prepare labelled fragments of the amplified DNA on a Veriti™ thermal cycler. The reaction volumes were reduced to one half of that recommended by the manufacturer's protocol. Briefly, combined 3.5 µl of cleaned up PCR product with 6.5 µl of each cycle sequencing reaction mix. The reactions were run on a Veriti™ thermal cycler with the following conditions: 1) 1 min at 96^0C, 2) 25 cycles of [10 sec at 96^0C, 30 sec at 50^0C, 4 min at 60^0C].

ORA-LAB.017 method – Prepared labelled fragments of the amplified DNA using the primers listed in Table I. Separate forward and reverse PCR reactions were performed in a 10µL volume. Each reaction contained the following: 3.5µL of cleaned up DNA template, 0.75µL of BigDye™ Terminator 3.1, 1.75 µL 5x BigDye™ buffer, 3.95µL PCR grade water and 0.05µL of a 100µM solution of primer. The reactions were run on a Veriti™ thermal cycler with the conditions were as follows: 1) 2 min at 95^0C, 2) 30 cycles of [15 sec at 95^0C, 2 min 30 sec at 58^0C], and 3) 4 min at 72^0C.

Cycle Sequencing Product Clean Up

Excess dye terminators and primers were removed from the cycle sequencing reactions using Performa® DTR Gel Filtration Cartridges following manufacturer's instructions. Briefly, centrifuge columns at 750 xg for 2 minutes to dry the gel matrix. Add 10 µl of sterile water to each cycle sequencing product. Transfer mixture to the dried gel matrix and centrifuge at 750 xg for 2 minutes.

Capillary Electrophoresis

After purification, the extension products were mixed with HiDi™ formamide at a 1:1 (v/v) ratio, loaded onto 96-well plates and ran on a 3500xL Genetic Analyzer.

Data Analysis

MicroSEQ® method - The sequencing data was automatically analyzed by the MicroSEQ® software and searched in the MicroSEQ® Fungal Library v2013. The report generated by the software was evaluated using the following acceptance criteria: 1) the % of Consensus Length must be ≥ 80%, 2) the Specimen Score must be ≥ 37 and 3) the % Match was interpreted as described in Table 2. For isolates with a % Match below 99 or % of Consensus Length below 80, the consensus sequence was searched on the NCBI GenBank and MycoBank databases applying the criteria outlined in Table 2.

ORA-LAB.017 method – The sequencing data was trimmed and assembled using Geneious R7 (Biomatters, Newark, NJ) as described in ORA-LAB.017. The resulting consensus sequences were compared to sequences in 1) the GenBank (NCBI) database using the basic local alignment search tool (BLAST) algorithm and 2) the MycoBank database using the MycoID pairwise sequence alignment tool. The % Identity was used as the criteria for isolate identification as described in Table 2.

Table 2. Identity (Match) criteria used to identify fungal isolates by both methods.

% Identity (%Match)	Identification
One single species at 100 %	Identify isolate by species
Multiple species at 100 %	Isolate most closely related to all species
One single species at ≥ 99 %	Identify isolate by species
Multiple species at ≥ 99 %	Isolate most closely related to all species
Single or multiple species at <99 to ≥ 98 %	Identify isolate by genera or genus
Single or multiple species below 98 %	Unable to identify with the data available

RESULTS

Comparison of DNA extraction using the QIAcube® versus manual

The DNA extracted by the QIAcube® was amplified using the MicroSEQ® D2 LSU rDNA PCR Kit and as per ORA.LAB.017. The resulting amplicons were visualized on agarose gel and found to be of the expected length. These were then successfully sequenced using the MicroSEQ® D2 LSU rDNA Sequencing Kit and as per ORA.LAB.017.

Figure 1. Image of 1.2 % agarose gel containing D2 amplicons prepared with DNA extracted by the QIAcube®. The DNA marker fragment sizes are 100, 200, 300, 500, 800 and 1250, 2000 and 4000 bp. The resulting amplicons were of the expected length (300 to 500 bp).

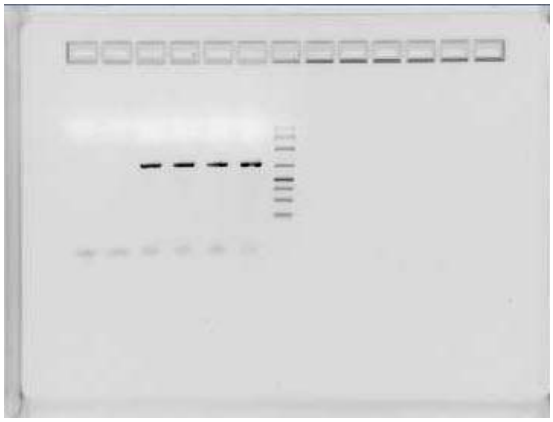


Figure 2. Image of 1.2 % agarose gel containing DID2 amplicons prepared with DNA extracted by the QIAcube. The DNA marker fragment sizes are 100, 200, 300, 500, 800, 1250, 2000 and 4000 bp. The resulting amplicons were of the expected length (400 to 800 bp).

Comparison of the level of identification attained by the two methods

ORA-LAB.017 identified 99/99 isolates to the species level. Eighty-four isolates (84/99) were assigned to a single species and 15/99 were assigned to multiple species of the same genus.

MicroSEQ® identified 98/99 isolates to the species level. Eighty-one isolates (81/99) were assigned to a single species and 17/99 were assigned to multiple species of the same genus. One isolate (1/99) was identified as *Phialophora americana* with a % Match below 98. A search of the consensus sequence in the public databases yielded Identities below 98% to multiple species, including *P. americana*. The isolate could not be identified using MicroSEQ® as the established % Match threshold was not reached.

Table 3. Comparison of the level of identification attained by the two methods.

Level of Identification	**MicroSEQ®**	**ORA-LAB.017**
one single species	81	84
multiple species of the same genus	17	15
unable to identify	1	0

Comparison of the agreement between the identifications

- Seventy-one (71/99) isolates were identified by both methods as the same single species.

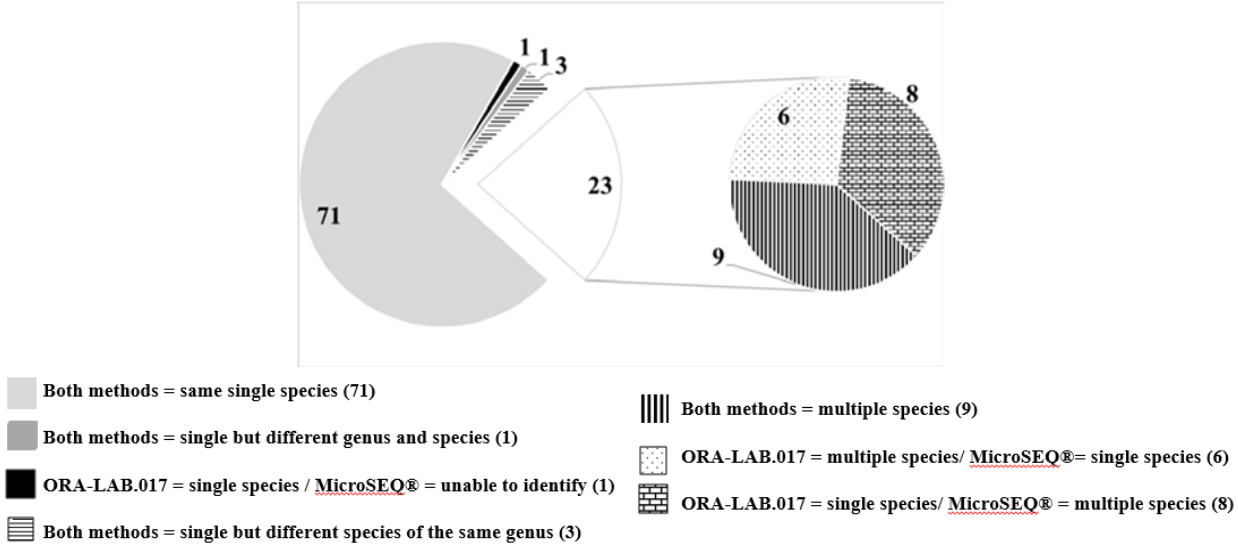
- Three (3/99) isolates were identified by both methods as a single but different species of the same genus. ORA-LAB.017 identified one isolate as *Trichophyton verruscosum* while MicroSEQ® identified it as *Trichophyton erinacei*. Similarly, ORA-LAB.017 identified one isolate as *Exophialla dermatitidis*, while MicroSEQ® identified it as *Capronia munkii*. Capronia is an anamorph synonym of genus Exophialla. Finally, *Fusarium incarnatum equiseti* was identified by MicroSEQ® as *Fusarium chamydosporum.*

- One isolate (1/99) was identified by both methods as single species of different genus. The isolate was identified as *Sarocladium killiense* by ORA-LAB.017 but as *Nectria mauriticola* by MicroSEQ®. Genus Sarocladium and Nectria are closely related[10]. MycoBank identifies *N. mauritiicola* as the teleomorphic synonym to *Sarocladium kashiense*.

- One (1/99) isolate was identified by ORA-LAB.017 as *Phialophora americana* but could not be identified by MicroSEQ®.

- Twenty-three (23/99) isolates were identified as multiple species by one or both methods. Of these, 9/23 were identified by both methods as multiple species of the same genus. Eight (8/23) were identified as single species by ORA-LAB.017 but as multiple species by MicroSEQ®. Six (6/23) were identified by ORA-LAB.017 as multiple species but as single species by MicroSEQ®. It is worth noting that the single species identified in one method was usually present in the list of multiple species provided by the other method.

Figure 3. Comparison of the agreement between the identifications obtained from the two methods.



Comparison of the accuracy of the identification of known species

Forty-two (42/99) of the isolates analyzed were species of verified identity. Forty-one (41) were obtained from CDC, ATCC, USDA and BEI and one was obtained as purified DNA included in the MicroSEQ® kit. ORA-LAB.017 identified 33/42 isolates as the correct single species. While 9/42 isolates were identified as multiple species, including the correct species. MicroSEQ® properly identified 32/42 isolates as the correct single species. While 8/42 isolates were identified as multiple species, including the correct species.

MicroSEQ® misidentified 3/42 isolates. *Rhizomucor variabilis* was identified as *Mucor circinelloides*. These two species a very closely related[11]. *R. variabilis* is absent from the MicroSEQ® library. ORA-LAB.017 identified this isolate as multiple species including *R. variabilis* and *M. circinelloides*. *Fusarium incarnatum-equiseti* received a 100% Match to *Fusarium chlamydosporum* and a 99.31% Match to *Fusarium equiseti.* Finally, *Penicillium commune* was given a 100% Match to *Penicillium chrysogenum* and a 99.61% Match to *Penicillium commune*.

Table 4. Comparison of the accuracy of the identification of known species.

Isolate identification	MicroSEQ®	ORA-LAB.017
Correct species	32	33
Multiple species including the correct species	7	9
Incorrect species	3	0

Use of GenBank and MycoBank databases to supplement MicroSEQ® library

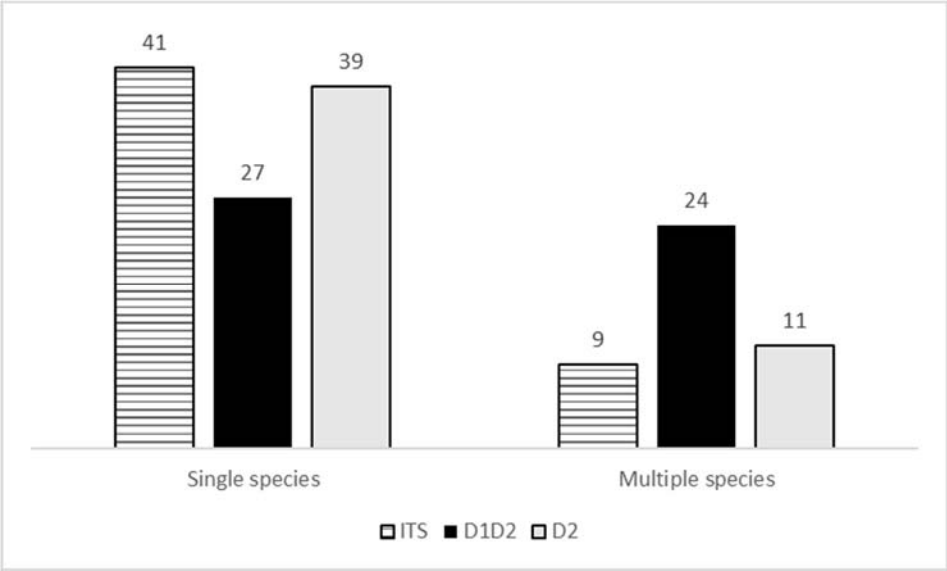
The NCBI GenBank and MycoBank databases were used to supplement the identification of 27/99 isolates which were assigned a low % Match or a low % of Consensus Length by MicroSEQ®. In 11/27 instances, the species identified by ORA-LAB.017 for that isolate was not present in the MicroSEQ® library. In another 3/27 instances the genus identified by ORA-LAB.017 for that isolate was not present in the MicroSEQ® library. MicroSEQ® assigned these isolates low percent matches to other species of the same genus or to another genus. In 13/27 instances, the species identified by ORA-LAB.017 was present in the MicroSEQ® library. However, MicroSEQ® assigned these isolates low percent matches to the same species identified by ORA-LAB.017.

It is important to note that in 26/27 instances a search of the D2 sequence on the online databases provided the same identification as ORA-LAB.017. One isolate was identified as *P. americana* by ORA-LAB.017 and could not be identified using either the MicroSEQ® library or the public databases due to a low % identification.

Comparison of the identification attained by using ITS, D1D2 and D2 as targets

The ITS and D1D2 regions of 51/99 isolates were sequenced using ORA-LAB.017. For the remaining 48/99 isolates, only one target was sequenced as that provided an adequate identification. These results were compared to sequencing of the D2 region with MicroSEQ®. Sequencing of ITS allowed the identification of more isolates as single species than sequencing of D1D2 or D2. D2 was unable to identify one isolate of *Phialophora americana* due to low percent Match.

Figure 4. Comparison of the identification attained by ORA-LAB.017 using ITS and D1D2 and MicroSEQ® using D2 as targets.



Controls

All controls performed satisfactorily. No bands were observed on agarose gels for any of the extraction controls. The positive culture and PCR controls were identified as the appropriate organism. The negative PCR controls failed to generate sequencing data.

DISCUSSION

Recent incidents of fungal contamination in medical products have led to an increase in the number of isolates ORA laboratories must identify. A fast and accurate identification method is essential to FDA's ability to respond to incidents of fungal contamination. This study compared a streamlined protocol incorporating automation to the current ORA method for fungal identification.

Both methods agreed on 97/99 of the genus identifications and 71/99 of the species identifications. Twenty-seven (27/99) isolates were identified as multiple species by one or both methods. In these instances, the single species identified by one method was usually listed by the other method as one of multiple species. However, the methods disagreed in the genus identification of *Sarocladium killiense* and MicroSEQ® was unable to identify *Phialophora americana*.

The FDA method was more accurate than MicroSEQ® on the identification of isolates of verified identity. ORA-LAB.017 identified the correct species of 42/42 isolates. While MicroSEQ® identified 38/42 isolates. An isolate of *Rhizopus variabilis* was identified as the closely related *Mucor circinelloides*. *Fusarium incarnatum-equiseti* was identified as *Fusarium chlamydosporum* and *Penicillium commune* as *Penicillium chrysogenum*.

The ability to identify isolates as single rather than multiple species was greater with ORA-LAB.017 than for MicroSEQ®. ORA-LAB.017 identified 84/99 isolates as a single species while MicroSEQ® identified 81/99. The availability of two targets in ORA-LAB.017 increased the likelihood of discerning a single rather than multiple species. Sequencing of the ITS target was particularly helpful in enhancing the identification of isolates as single species. Furthermore, the targets used in ORA.LAB.017 are longer than that used in the MicroSEQ® methods. This provides more data to search on the database which may assist on species identification

Public databases were used to supplement the MicroSEQ® library in instances where the % Match or the % Consensus Length was below the established threshold. In most instances, a search of the D2 sequence in the public libraries yielded the same species identification as the corresponding search of the D1D2 or ITS regions. Thus, the overall result was an agreement in the identification of these isolates. An examination of the MicroSEQ® library revealed that, in
52 % of the cases the species or the genus identified by ORA-LAB.017 was not present in their library.

The QIAcube® was comparable to the manual extraction in ORA-LAB.017 and suitable for sequencing. Use of an automated platform reduces analyst hands-on time while minimizing the probability of user error.

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Supplement Table 1. List of mold isolates used in the study

Isolate name	Number of isolates	Isolate name	Number of isolates
Aspergillus candidus	1	*Cladophialophora carrionii*	2
Aspergillus fumigatus	2	*Cladosporium ramotenellum*	1
Aspergillus terreus	2	*Cladosporium cladosporioides*	2
Aspergillus flavipes	1	*Cladosporium herbarum*	1
Aspergillus flavus	2	*Curvularia lunata*	1
Aspergillus nidulans	1	*Bipolaris spicifera*	2
*Multiple Aspergillus species**	1	*Epicoccum sp./ Phoma sp.**	1
*Multiple Aspergillus species **	1	*Exophiala dermatitidis*	1
Aspergillus ustus	1	*Exophiala mesophilla*	1
*Multiple Aspergillus species **	1	*Fusarium oxysporium*	1
Aspergillus hiratsukae	1	*Fusarium solani*	1
Aspergillus fischeri	1	*Fusarium incarnatum-equiseti*	1
Arthroderma tuberculatum	1	*Lecythophora sp**	1
Arthrodema quadrifidum	1	*Trametes flavida*	1
Aureobasidium pullulans	1	*Lichtheimia ramosa*	2
Beauveria bassiana	1	*Microsporum audouinii*	3
Byssochlamys spectabilis	1	*Microsporum gypseum*	3
Cladophialophora bantiana	2	*Mucor circinelloides*	1

*Identification could not be narrowed to one single species using either method.

Isolate name	Number of isolates	Isolate name	Number of isolates
Ochroconis gallopava	1	*Rhizomucor variabilis*	1
Penicillium brevicompactum	2	*Rhizopus microsporus*	1
Penicillium camembertii	1	*Rhizopus oryzae*	2
Penicillium chrysogenum	1	*Rhizopus stonolifer*	1
Penicillium comune	1	*Sarocladium kiliense*	1
Penicillium crustosum	1	*Scedosporium prolificans*	1
Penicillium olsonii	2	*Scopulariopsis brevicaulis*	1
Phanerochaete chyrsosporium	1	*Sporothrix schenckii*	1
Phialemonium atrogiseum	1	*Syncephalastrum racemosum*	1
Phialophora americana	1	*Thamnostylum piriforme*	1
Pseudallescheria boydii	1	*Tricophyton mentagrophytes*	2
Purpureocillium lilacinum	1	*Tricophyton verruscosum*	1

Supplement Table 2. List of yeast isolates used in the study

Isolate name	Number of isolates	Isolate name	Number of isolates
Candida albicans	1	*Candida parapsilosis*	1
Candida auris	1	*Cryptococcus albidus*	1
Candida boidinii	1	*Cryptococcus neoformans*	1
Candida catenulate	1	*Cryptococcus gatti*	1
Candida fermenticarens	1	*Cryptococcus magnus*	2
Candida galli	1	*Papiliotrema laurentii*	1
Candida glabrata	1	*Rhodotorula mucilaginosa*	1
Candida intermedia	1	*Rhodotorula sphaerocarpa*	1
Candida krusei	1	*Sacharomyces cerevisiae*	1
Candida metapsilosis	1	*Saccharomycopsis fibuligera*	1
Candida orthopsilosis	1	*Saprochaete clavate*	1