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14. ABSTRACT
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Report Title

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June 12, 2014

Study Unveils New Method for Universal Extraction and PCR Amplification of Fungal DNA

By Madeleine Johnson

NEW YORK (GenomeWeb) — A team led by researchers at the University of Texas Health Science Center in San Antonio has developed a new method of extraction and amplification for fungal DNA, creating a tool that could be crucial for clinicians treating patients with rare or hard to identify fungal infections.

The new extraction and amplification method can be universally applied to fungi, according to the researchers, which could be important in battling a growing incidence of pathogenic fungal infections, including hospital-acquired, or nosocomial infections.

Pathogenic fungal infections attack vulnerable people and can be fatal, and correctly detecting the species of fungus is critical for determining the best treatments. In addition, rare fungi, or species with phenotypic doppelgangers, can stump medical mycologists, so molecular methods are critical. However, isolating DNA from fungi can be problematic, and an inexpensive method to isolate and amplify nucleic acids from all types of pathogenic fungi has been lacking.

A team of scientists led by Brian Wickes, a microbiologist at the UTHSCSA, have aimed to solve this problem, though. In a study appearing in the journal *Mycoses* the researchers recently described a universal DNA extraction and PCR amplification method that enables sequence-based identification from fungal ribosomal DNA.

Wickes told *PCR Insider*, "Anybody can get DNA out of a fungus, there's 20 ways to do it. We were going for something that was reliable, quick, easy, and didn't require a lot of manipulations."

Wickes' lab now uses the method in their work with the Fungus Testing Laboratory at UTHSCSA, a reference lab for clinical mycology that is CLIA and CAP certified. The lab is staffed by classically trained mycologists, Wickes said. "In cases where they can't identify the organism, or the organism is so closely related to another species that they can't distinguish

them morphologically, they send them up to us, and we use a sequence-based method," he said.

"In hospitals that have large oncology populations, at centers that deal with trauma or burns, or transplant centers, you can have infections by just about any type of fungus," Wickes added. "Any fungus in the wrong patient can be a pathogen; there are people that get infected with baker's yeast."

He said that hospital technicians are often able to identify the "easy stuff," or the most common 30 to 50 pathogenic fungi. The standard method is to grow the suspected infectious agent in a Petri dish, then examine it by eye or under the microscope.

However, "the number of fungi that have shown up in people at one time or another, at least once, is probably well over 1,000," Wickes said.

"If you look in a textbook, they'll tell you there's about 300 that are pathogenic, or maybe 500, but there's many more," he said. "You may have a fungus that shows up once in a technician's lifetime, they may have never seen it, and they may never see it again," Wickes said.

It is important for clinicians to be able to discriminate fungi at a very fine-grained level because fungal species can differ in their responses to common therapies.

For example, Candida species, a type of yeast, are the leading cause of mycosis-associated mortality in the US, and the fourth most common nosocomial infection, according to a review in *PLoS Pathogens*. *Candida albicans* responds readily to antifungals, but non-*albicans* species are on the rise, and each has a different profile of response to the three main classes of antifungal drugs.

Another example is the genus *Aspergillus*, which has some 400 members, but not all are easy to distinguish by standard culture- and microscopy-based methods.
A. fumagatus is considered a ubiquitous human fungal pathogen. It is the most commonly seen *Aspergillus* infection, and it responds to the usual antifungal treatment, amphotericin B. *A. terreus*, however, is resistant.

A recent case report in *The Journal of Clinical Microbiology* illustrates the potentially fatal consequences of being unable to quickly distinguish between these two. In the report, after chemotherapy for acute myelogenous leukemia, a patient acquired pneumonia, followed by brain abscesses. She died 37 days later, after intensive diagnostic and treatment efforts failed. Ultimately, culturing of her brain abscesses showed that the fatal infection was *A. terreus*.

In the report, the authors make the case for better fungal diagnostics: "Our patient was treated with extended courses of empirical antifungal therapy while fungal cultures were incubating. Even after mold was apparent on culture media, species identification of the fungus required several more days. During this time, our patient was treated empirically with intrathecal amphotericin B. Our case highlights the need for more rapid methods of fungal identification."

Interestingly, this group ultimately used Abbott's Plex-ID for post-mortem identification of the fungus infecting their patient. They argued that commonly adopting this fungal identification technology might save lives. The Plex-ID system, which uses PCR-electrospray ionization mass spectrometry, was recently vetted by researchers from the Mayo Clinic for broad fungal identification directly from clinical specimens. However, the system currently costs about $500,000, and some consider it not user friendly.

Wickes' method, on the other hand, is quite inexpensive and fast; it takes approximately 30 minutes to yield DNA suitable for sequencing, according to the researchers. After that step, "you don't even really need to be a mycologist, you just need to know how to use the Internet and understand how to do a BLAST search," Wickes said.

The method employs a combination of physical and chemical extraction methods, but no organic solvents or ethanol precipitation. The PCR primers are ones commonly used for fungal ribosomal DNA. Once the researchers obtained good template DNA, they sequenced the samples using a BigDye Terminator cycle sequencing kit from Applied Biosystems.

In the *Mycoses* study, using the described extraction and amplification of rDNA, Wickes and his group were able to correctly identify 519 different strains of fungi. These numbered 129 yeasts and 390 molds, including 95 different genera and 172 species.

"In every organism that we've tried it on, the method works," Wickes said.

Sample prep was key to making the method universal, Wickes said. The protocol needed to be able to crack open all fungi, but in as few processing steps as possible, generating the least amount of toxic waste.

"We've found over the years that getting the DNA out of some organisms is not easy. Fungi are very tough, very resilient. They almost have a structure that is analogous to plants because they have a cell wall," Wickes said.

The group's solution coupled a chemical extraction step utilizing Prepman Ultra lysis reagents from Applied Biosystems and a physical extraction method using bead beating.

In bead beating, a sample is agitated with beads made of glass or other hard material. Wickes' protocol used a mini bead beater and 0.5mm glass beads from Biospec. This company's large-format beater processes 192 samples simultaneously and costs about $6,000, according to their website, while their smallest format bead-beater, the micro-mini, costs about $600.

Besides the tough exterior, some fungi also have melanin in their cell walls, and may contain carbohydrates and other substances that inhibit PCR reactions. However, Wickes' method managed to overcome many common obstacles. "You don't need to do a phenol chloroform extraction, you just pellet the debris, take the supernatant, and our extraction method does not inhibit the PCR reaction," Wickes said.

The *Mycoses* study also draws comparisons between the new method and several commercially available kits appropriate for fungi, including ones from Qbiogene, Zymo Research, and Qiagen.

Wickes pointed out that these all use spin columns. "We've used just about every column out there and they have high rates of failure," he said. They have a higher likelihood of fungal contamination, either occurring during manufacture or during numerous centrifuge steps and column changes, Wickes said.

Column-based kits are also much more expensive. They cost between two to six dollars per sample, according to Wickes' study. The PrepMan Ultra reagent, however, costs about 50 cents per sample.

In addition to clinical applications, the universal method could be useful for environmental surveillance. Wickes noted that there are methods to extract fungi from soil, for example, and "once you get down to pure DNA, everything else is the same," he said.

Wickes said his group has no plans to commercialize their method, but would be happy to collaborate with any entities that might like to adopt the technique.

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