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## Phenotype Analysis May Rival PCR in Labs' Future

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Fungi are ubiquitous throughout nature and exist fundamentally as saprophytes. Their primary role is to act as decomposers. They are in essence the garbage men of the world. Of the several hundred thousand species, less than 200 are known to be regularly involved in human and animal mycoses. But the list of clinically relevant fungi continues to grow at an alarming rate. This can be attributed mainly to the increasing number of immunocompromised patients, including cancer chemotherapy patients, organ transplant patients, those with AIDS and those who are hospitalized. Organisms once thought to be common contaminants are now being confirmed as true pathogens in the immunocompromised patient.

One can begin to appreciate the mycology laboratorian needing a system that goes beyond the culture-based method if you consider the following phenomenon: A convergence of the ever growing list of true and potential pathogens with the explosive growth of the IAQ marketplace. This

convergence plus the potential litigious aspect of the IAQ marketplace has placed a premium on the need to speciate more fungal isolates.

Until recently, identification of filamentous fungi has been performed by macroscopic and microscopic techniques. This approach has often been referred to as the classical approach to mycological identification.

A well trained laboratorian can couple such macroscopic features as color of colony growth, texture of the aerial mycelium, rates of growth on respective media, temperature differentials and topography of colony with microscopic features such as segmented or non-segmented hyphae, length and shape of conidiophores, conidial size, color and many other microscopic features considered unique to both the genus and species level.

With these two subsets of data, which can comprise a large number, protocols have been developed with fairly modest objectivity to rule out all but a handful of fungal candidates. Subjectivity has and always will play a role in the traditional methods of fungal identification. This is why the field of mycology has often been considered as much an art as it has been a science.

Without question, the diagnostic mycology laboratory, must supplement the more traditional approaches with the emerging technologies that are often much quicker, more specific and in some instances more cost-effective. Of all the emergent technologies, molecular methods are receiving the most fanfare. Undoubtedly, molecular microbiology has arrived and is here to stay. In many clinical microbiology laboratories, molecular diagnostics has become the new gold standard for the diagnosis of several infectious diseases.

In 1985, Kary Mullis and his colleagues reached a milestone in biotechnology by the development of polymerase chain reaction. Other nucleic acid amplification and non-amplification methods are being developed and are having a positive impact on the clinical microbiology laboratory. But it is with PCR that many are placing their future

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goals. But is PCR the inevitable path for all future fungal identification?

Quite simply put, PCR is a chemical reaction allowing for the synthesis of limitless quantities of a targeted DNA sequence. Under proper conditions, a copy of the original targeted sequence can be made. Each copy or amplification constitutes a cycle. In theory, at the end of each cycle, the targeted DNA is doubled. At the end of two cycles, the original sequence has now quadrupled. Consequently, in a matter of a few hours, the original targeted DNA sequence has now increased a billion times to attain analytical quantities.

The DNA sequencing approach to microbial identification does have many obvious benefits:

1. Results can be obtained rapidly.
2. It can be very sensitive and specific.
3. You are able to work with small quantities of material.
4. In some instances, it can be easy to perform.
5. It is not an antigen-based test.
6. It appears to have unlimited prospects for future growth and applications.

However, at the moment, there are some serious obstacles that PCR-based tests will have to overcome before they can become more widely used in the mycology laboratory:

1. The initial startup cost can be prohibitively expensive, particularly for smaller laboratories. Only a high volume of work can justify the initial cost.

2. Currently, there are only a few FDA-approved PCR-based diagnostic assays available. Like most governmental agencies, waiting for FDA approvals can and will continue to be lengthy.

3. PCR is a patented technology as well as many of the primers used. Like all patents, if there is any financial gain, an arrangement must be made between the patent holder and the end user. The royalties and up-front cost have seriously restricted the development and introduction of new PCR-based diagnostics.

4. PCR technologies have the potential to be very easily contaminated. As little as one molecule can have deleterious effects.

5. In order to develop new PCR-based assays for fungal identification, sequencing information must be made available. However, in the case of fungal identity sequences, the data is limited and may remain so for some time. If the market does not provide an impetus to provide incentives for additional fungal sequences for the more esoteric fungal isolates, who or what will?

PCR technologies could actually be considered a genetic profiler. This means that the entire identification process is based on the genetic information obtained from a selected specimen. The exclusively genetic approach has provided enormous advances in molecular taxonomy and new fungal classifications. However, must we inevitably rely exclusively on genotyping (a genetic portrait)? Is there not room for a phenotypic (physical portrait) approach, which could integrate the advances of science, mathematics and computers with the ever-growing list of discovered fungal phenotypes?

Of the chemical-based tests on the market, Biolog, with its state-of-the-art phenotype of testing system, is by far the leader. In 2000, Biolog introduced the FF (filamentous fungi) MicroPlate. The FF database currently has identification patterns for over 618 taxa from 120 plus genera. It also provides a macroscopic and microscopic photo library for many of the species. Biolog's identification system is based on the fungal isolates ability to use a specific carbon source. A fungal isolate, in pure culture, is suspended into an inoculation fluid attaining an inoculum turbidity of 75 percent transmittance.

The suspension is then pipetted into a 96 well microtitre plate, which contains 95 different carbon sources, as well as a negative control. Each well contains a different carbon source.

The microtitre plate is then incubated at 26 degrees Celsius and has the capability to read the plate at 24, 48, 72 and 96 hours and at seven days. Once the desired time period

is selected, the plate is subsequently read by an automated plate reader. When a particular carbon source is utilized, either or both of the following changes occur: increased mitochondrial activity, leading to a reddish-orange color change; or increased growth, leading to turbidity. Turbidity is an important indicator of fungal growth. The microtitre plates are read at both 490 and 750 nanometers in order to detect and quantify both the color and the turbidity reactions. There are two sets of data points generated: 96 color data points and 96 turbidity data points, for a total of 192 points of data per microtitre plate per reading. The resulting data, a series of positive and negative reactions, is interpreted by the biology software. If an adequate match is found, an identification is called.

The software will display the species identification in ranked order of the closest match. Identification scores of percent probabilities, similarity index value and the distance between the microtitre plate results and the database pattern for that species are listed. You can then verify the match with the fungal isolate tested by comparing the macroscopic and microscopic morphologies against the Biolog photo library. It is important to look at the top three choices displayed. There are a few restrictions that should be noted:

1. A pure culture also must be used – there is no room for a mixed culture. Fungal isolation from an originally mixed culture can be tedious and time-consuming.

2. Startup cost can be significant but are not as high the start up cost for PCR.

3. Because Biolog's system is based on a pure and viable culture, the total identification process can take up to two weeks, if not a little longer depending on laboratory work flow.

All existing methods have both pros and cons; Biolog is no exception. However, its positive attributes far outweigh its negatives:

1. Biolog's fungal database is considerably larger than PCR and continues to grow.

2. Biolog's photo library is a true asset, displaying both macroscopic and microscopic features at the touch of a finger.

3. The ability to compare visually your well reactions with known values from a Bergey's manual is an additional plus. When your PCR-based system fails to give you identification, you do not have the luxury of double-checking the data. You have no choice but to start over or rely on a culture based method.

4. Biolog's microtitre plate generates 192 points of data. Just think of how many profile numbers that can be potentially created. Currently, PCR is restricted by the primer(s) used.

Biolog's advance system by phenotypic testing is a natural progression from the traditional approaches to mycological identification. In the mycology laboratory, there is no reason why phenotype testing should trail behind genotype testing. Both systems can and should complement one another. Both have found their niche, and their futures remain bright. Advances in molecular biology and automation will eventually make it more and more feasible for the diagnostic mycology laboratory to incorporate more advanced approaches to fungal identification. And in many instances, nucleic acid amplification tests will be used in the future as primary tools for the diagnosis of fungal infections.

But if and when these events occur, I would like to defer to a highly respected mycologist who quipped, "It will be some time before molecular methods allow the differentiation between *Exophiala jeanselmei* and *Exophiala jeanselmei* variety *lecanii-corni*."

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