Mycology with molecular probes

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Sensitivity, specificity, simplicity, speed, and economy fairly well describe the desirable attributes of any clinical diagnostic test. In the mycology laboratory, these conditions unevenly apply to detection and identification of various organisms. They are adequate for the routinely encountered pathogens, but for the opportunistic and emerging pathogens the situation is not optimal. This occurs for a variety of reasons not the least of which is the large number of potential species involved. It also occurs because the morphological and physiological characteristics used for analysis are complex, frequently slow to appear, sometimes variable within a species, and usually require significant experience to evaluate. In contrast, molecular tests, once established, avoid these problems by focusing on a single parameter. This parameter is the detection of a nucleotide sequence that is known to be unique for a given species of organism.

Prior work by Carl Woese [1] and his colleagues has established that ribosomal DNA sequences have evolved slowly enough that species specific sequences appear to exist for every living creature. Since all cellular organisms use ribosomes for protein synthesis, one can detect and identify organisms by analysis of ribosomal genes. Many have used this logic to find probes for limited sets of fungi using a wide spectrum of approaches. However, what is needed for the clinical laboratory is a common approach to greatly simplify execution and validation of assays.

Sandhu *et al.* [2] recently described a combination of molecular methods which allow one to detect and identify a large spectrum of fungal species. The combination of methods consists of treating samples with a broad spectrum lytic reagent to release DNA. Next, a uniquely hypervariable region of the 28S ribosomal gene is amplified by the polymerase chain reaction (PCR) using DNA primers that are universally specific for fungi. The amplified DNA is characterized to a species level by hybridization with a species specific nucleotide probe using a single assay condition for all probes, or it may be characterized by direct DNA sequencing. The entire procedure can be done in one to three days depending on the method chosen for characterizing the amplicon. The important points are that a common set of processing and assay conditions are used for all samples irrespective of their source and the method works equally well with culture and clinical specimens.

The sensitivity of our molecular method is as low as one organism. However, in practice it must be one organism in a volume of about 25 µl, a condition that could be difficult with some clinical samples. On the other hand, the organisms need not be viable which is a major advantage over non-culturable organisms or with material prepared for thin sectioning, including archival material.

Pure cultures of organisms yield PCR amplicons whose entire sequence can be compared to a database for identification. Mixed infections which are the norm in certain samples such as sputum and material from other non-sterile sites, invariably yield mixed PCR amplicons. If the presence of a particular organism in a clinical sample producing a mixed amplicon population is questioned, then an answer is easily obtained using a species specific hybridization probe. On the other hand, if identification of all fungal amplicons in a similar situation is desired, one must do several probings with no assurance that the probe choices are appropriate. Clearly, multiple, simultaneous probings could provide answers economically and rapidly, but this technology is not well developed. We can anticipate, however, that it soon will be better developed given its obvious importance and utility to molecular biology in general.

Matsumoto *et al.* [3] and Hazen [4] list a total of well over 100 established, opportunistic, and emerging fungal organisms associated with human disease. The usual pathogens are relatively easy to detect and identify. However, they represent a numerically small fraction of this total group. To acquire expertise in identifying fungi by classic means is a major accomplishment. It is an easier accomplishment using the molecular probe strategy we have discussed. But we caution that the current database of fungal ribosomal sequences, while encompassing the major human pathogens, falls far short of covering the several hundred thousand species of fungi present in nature. Probes designed to be specific for one species of *Aspergillus,* may potentially prove erroneous if the same sequence appears in the genome of a yet uncharacterized species. While this is not a major concern given the relatively limited number of human pathogens, it is well worth considering while trying to identify emerging pathogens based on probe hybridization assays alone. At a time when fungal infections are increasing worldwide and classically trained mycologists are shrinking, the importation of molecular tools into the clinical laboratory may be a godsend not only for the accuracy the tools bring but also for the ease with which the tools can be mastered.

Species identification sometimes is an advantage for drug therapy. In fungi, there are several known correlates between genus and species and drug resistances (see reference 4). Therefore, if a species identification can be had in a day, appropriate therapy can be initiated and ineffective therapy avoided with more certitude and speed.

Molecular tests will likely be even more useful if and when we can identify mutations that convert normal fungal genes into drug resistance genes This is already a feasible strategy for some drugs active against the bacterium, *Mycobacterium tuberculosis*. Resistances to isoniazide (INH) and rifampicin have been characterized to a limited set of mutations in the *katG* and *rpoB* genes, respectively. Since inhibition of ergosterol biosynthesis in fungal cell wall growth is the mechanism of azole sensitivity, we can hope that mutations for azole resistance will

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be found at limited sites in genes for ergosterol biosynthesis.

The immunocompromised patient is especially vulnerable to fungal infections. It would not be unreasonable to suspect that a major nidus for infection is endogenous flora. To scan endogenous fungal flora (and their drug resistances) in a patient by classical means would be quite expensive, but one day it might be economically feasible by molecular means. Such information would be of significant value to a patient whose physician was so forewarned.

Molecular tests are also much better suited to epidemiological studies than classic metabolic and serological methods. Molecular hybridization probes can show rapidly if an outbreak is due to single species, but probes cannot show if all species are the same strain. Strain identification requires one to analyze much larger fractions of genomic DNA. Such examinations are done by molecular techniques collectively known as "DNA fingerprinting."

While the future prospects for molecular mycology are bright, in the foreseeable future this technology will be limited to major institutions with access to instrumentation and trained personnel. Another limitation is certainly the extremely large number of fungi that exist. To develop and use a probe for each one is not practical. However, given the obvious importance of DNA sequence information in medicine and science, we can expect that within a decade or two technological advances will largely solve these problems.

In summary, molecular tests can be rapid, very specific, and economical in clinical laboratory situations where this potential has been lacking. They are especially useful for non-culturable, slow growing, pleomorphic, opportunistic, or unusual organisms. Interpretation of test results is usually straightforward in the form of a sequence match achieved by probe hybridization or database sequence comparison. The time required to achieve the molecular skills is minimal compared to the time required to be trained as a classical mycologist. However, it bears repeating that this promising situation is based on solid correlations of DNA sequence information with species identification. These correlations are only possible through a strong partnership between classical mycology and molecular biology.

References

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