DNA sequencing of fungi in a microbiology laboratory

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The identification of fungi in a general microbiology laboratory using traditional techniques has always been problematic for several reasons. These include the requirement for trained personnel to identify the fungi, as well as the fact that many fungi take much longer than bacteria to grow, making identification of these organisms in a timely fashion difficult. An additional complication is that some fungi do not show characteristic structures, making identification by traditional techniques difficult, if not impossible. DNA sequencing for the identification of fungal cultures can be easily established in a laboratory that is set up for bacterial DNA sequencing. This technology, when complemented with appropriate levels of training in classical fungal identification, will extend the mycological capabilities of a general microbiology laboratory.

Introduction

The classical approach to the identification of fungi involving biochemical and temperature testing, as well as observance of their microscopic morphology, has always had limitations. A major criticism of this approach is that it depends heavily upon highly skilled laboratory personnel with training that is not readily available to many small laboratories. In addition, a significant number of fungi are not able to be properly identified using these methodologies, mainly because they do not sporulate in most of the commonly used fungal media. Without the formation of structures – other than fungal hyphae in a culture – any attempts at microscopic identification can be compared with a botanist trying to identify a plant from its root structure alone.

It is for these reasons that molecular methods are now used for fungal identifications. Human fungal infections can be detected directly from patients' samples using a panfungal PCR assay (although not from blood fractions or sputum) [1], but accurate identifications of the fungi are made by using DNA sequencing techniques on the isolated fungus.

There are different areas within the fungal genome that can be used for sequencing. The most widely sequenced DNA region in fungi is the ITS region (Internal Transcribed Spacer) in the ribosomal DNA, using standard ITS1 and ITS4 primers. There are two critical steps that markedly improve successful DNA sequencing. They are fungal DNA isolation and clean-up of the DNA after the big dye reaction.

Extraction of fungal DNA

The first step in fungal sequencing is isolation of the DNA. There are many methods and commercial kits available to do this. We have compared several kits for speed and efficiency, and found that the Fungal/Bacterial DNA kit from Zymo Research was suitable for our purpose. This kit yields consistent DNA extractions within 60 minutes and is easy to use. As our laboratory does not have specialised automated equipment to disrupt fungal cell walls, we use a simple vortex mixer and beads supplied with the kit to beat the fungus in order to get DNA release.

PCR inhibition at the initial extraction step

The most difficult fungi to extract DNA are those that have minimal surface mycelium on culture media (difficult to lift fungi from the agar without including some of the agar – for example,

Figure 1. Microscopic and macroscopic features of a basidiomycete. Note the presence of a clamp connection at the septum in the photograph at the left. This is a definitive structure for a basidiomycete, but is not always present. It is normally impossible to further identify such fungi without DNA sequencing, because their fruiting bodies are macrofungi, which are not normally produced on laboratory media. However, on the right is one of the clinically important macrofungi, Schizophyllum commune, that is producing basidiocarps in culture. This is one of the few macrofungi that may do this.

some of the dermatophytes), as well as some of the dematiaceous fungi (for example, non-sporulating variants of the fungi that may cause phaeohyphomycosis) that may have particularly resilient cell walls. Contaminating agar in the DNA extraction mixture can act as an inhibitor, with Sabouraud's agar being particularly inhibitory. Potato dextrose agar has been shown to cause the least PCR inhibition. It is recommended that all fungi are subcultured onto this agar and grown before attempts at DNA extraction to minimise PCR and sequencing inhibition.

The difficulty in extracting DNA from some of the dematiaceous fungi and very occasionally some of the hyaline fungi, is often due to their fungal cell walls being resistant to the lysis buffer used in the extraction process. If the lysis buffer is given a longer reaction time (up to a week), followed by vigorous disruption with beads, the cell walls of the fungi can be sufficiently disrupted to yield their DNA.

Final isolation and purification step

Isopropanol precipitation and product clean-up are commonly used in DNA extraction procedures. A potential pitfall during this step is the inadvertent loss of the DNA pellet during the washing steps between centrifugations. Despite the use of glycogen as a co-precipitating agent, the DNA pellet is often difficult to see. It has been found that the use of transparent centrifuge tubes with a groove enables easier location of the DNA pellet. The groove collects the DNA pellet in a position distal to the centre of spin, minimising the need for an 'eye of faith' search for the DNA deposit. These tubes (named 'Maxyclear centrifuge tubes') are available from Axygen Scientific.

Interpreting the results

Fungal cultures that are not pure, yield DNA sequencing traces that have a mix of peaks with various heights in the chromatogram. A sequence chromatogram from DNA that is not contaminated tends to have similar sized peaks and troughs. However, mixed peaks sometimes occur in DNA from cultures that appear to be pure. Figure 2 shows two chromatograph traces of DNA extracted from separate subcultures of a fungus taken a week apart. Similar areas of the genomes have been highlighted in Figure 2 to show that the seeming mix of peaks is not random. It can be seen that base position 164 in the top trace corresponds to position 169 in the bottom trace and from then on the traces are very similar. These chromatograms were shown to microbiologists from various disciplines at a National ASM conference. The consensus was that the traces show a mix of viral and fungal DNA, likely due to infection of the latter.

Fungal identifications by DNA sequencing

DNA sequencing has enabled the identification of fungi that would be difficult, if not impossible, to identify by conventional methods alone. For example, yeasts are usually identified in medical laboratories by biochemical tests incorporated within strips (for example, bioMérieux ID32C). These strips work well, but there will always be yeasts that cannot be identified by this method and these are often flagged by the API database itself with the disclaimer, "yeast has an unacceptable profile". One such yeast was identified by DNA sequencing as

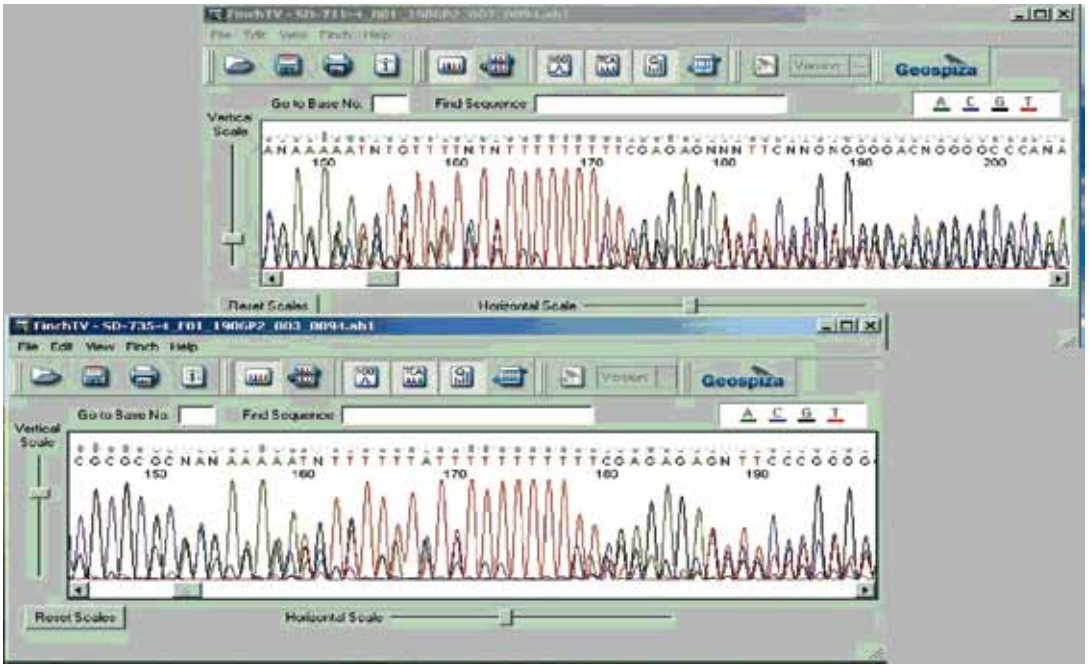

Figure 2. Two chromatograph traces of DNA demonstrated using FinchTV, a free DNA sequencing chromatogram trace viewer downloadable from the internet.

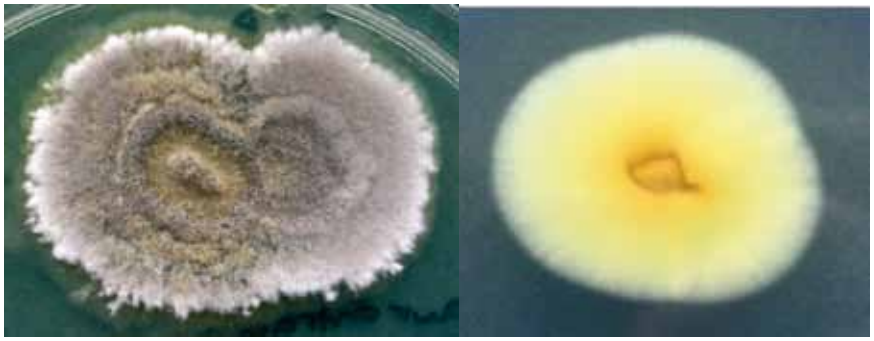

Figure 3. Appearance of *Scedosporium aurantiacum* on potato dextrose agar, showing mouse-grey thallus (left), and yellow reverse pigment (right).

Eremothecium sp., a dimorphic fungus related to *Saccharomyces* spp. that is rarely encountered in medical diagnostic laboratories. Once known, this identification can then be confirmed by traditional methods. Another area that has been fruitful is the identification to genus level of what were previously termed 'non-sporulating hyphomycetes'. These fungi are well-known in mycology laboratories. They are usually white and do not sporulate even on the most nutritionally deficient media. They are often isolated from the lungs of immunosuppressed patients and cannot be identified by conventional methods. Sequencing has identified these fungi in the main as being from the class 'basidiomycetes', a group that consists mainly of macrofungi (commonly called mushrooms and toadstools), as well as some urease-positive yeasts (including *Cryptococcus* spp.).

Examples of basidiomycetous moulds isolated from lungs and identified by DNA sequencing include *Irpex* sp., *Ganoderma* sp. and *Coprinellus* sp. It appears that these fungi are part of a new group of opportunistic pathogens from the basidiomycete class of fungi that are in the process of being described.

Another area where DNA sequencing has been invaluable is identification to species level of medically important fungi within the *Aspergillus fumigatus* complex, as well as fungi within the *Pseudallescheria boydii* complex. Such knowledge is helpful for medical diagnostics, because there may be differences in fungal sensitivities between species within these complexes. For example, it is known that *Aspergillus lentulus*, a member of the *Aspergillus fumigatus* complex, is more resistant to antifungals than are other members of this complex [2]. Prior to DNA sequencing techniques, *Aspergillus lentulus* was believed to be a slow-growing *Aspergillus fumigatus*.

In a similar fashion, the yellow reverse on some *Scedosporium apiospermum* isolates (Figure 3) was regarded simply as strain variation. DNA sequencing, however, has shown that these isolates are a different species, now known as *Scedosporium aurantiacum*. This new species tends to be more resistant to voriconazole than other species within the *Pseudallescheria boydii* complex (including *Scedosporium apiospermum*) [3].

Unfortunately, there are misidentifications within the GenBank fungal database, but the use of traditional mycological methodology is helpful to minimise or resolve these problems [4].

Conclusion

Knowledge of traditional mycological techniques, coupled with molecular sequencing technology, can help to extend and confirm fungal identifications which have traditionally relied on time-consuming and technically demanding classical techniques.

Acknowledgement

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Biography

Stephen Davis is a Senior Medical Scientist for SA Pathology at the Women's and Children's Hospital in Adelaide, and adjunct lecturer at the University of South Australia. He was originally trained in Mycology by Mrs Geraldine Kaminski (aka Miss Brown), a renowned world expert in this discipline. He has been Chair for the SA branch of the ASM for the past two years, and has had several papers published including his research on the antifungal properties of allitridium, a proprietary garlic extract used in China. He has also conducted several well-received workshops on the identification of fungi using classical methods.