PCR for Fungal Infections

DR SUJATA DHANUKA
Associate Vice President- Medico-Marketing

METROPOLIS HEALTHCARE LTD
Superficial & Invasive Fungal Infections

While superficial and subcutaneous fungal infections often produce characteristic lesions that suggest the diagnosis, a thorough knowledge of potential causative organisms is yet required to aid the diagnostic process, mainly in situations where systemic fungal infection is suspected but the clinical presentation is nonspecific and then ascribable to a wide range of infections, underlying illnesses, or complication of treatments.
Invasive Fungal Infections

- The epidemiology of systemic fungal infections has evolved rapidly over the last 2 decades.
- The global human immunodeficiency virus (HIV) pandemic has led to unprecedented numbers of opportunistic fungal infections.
- Certain groups of patients are at an increased risk of systemic fungal infections:
  - Patients with indwelling catheters
  - Patients in intensive care
  - Patients on immunosuppressive therapies
  - Patients undergoing organ or stem cell transplantation

Early, rapid, and accurate identification of pathogenic fungi is important in order to guide the selection of appropriate antifungal therapy and thus improve patient outcomes.
Conventional Methods of Diagnosis

1. Culture based detection methods
   - Direct microscopic examination of etiologic agent in clinical specimens - Identification of the fungus is based on cellular morphology and staining properties
   - Isolation and identification of the pathogen in culture
     - A variety of culture media and incubation conditions are required for the recovery of fungal agents
     - Filamentous fungi need identification by visualization of macroscopic (colonial form, surface colour, and pigmentation) or microscopic (spore-bearing structures) morphologic characteristics, following to sub-cultivation of a mould isolate to encourage sporulation, a process that takes days to weeks
Conventional Methods of Diagnosis

2. Antigen-based detection methods

• Based on the detection of markers such as –

  Galactomannan (GM)

  1,3-beta-d-glucan (BG)

• GM, a component of fungal cell wall that can be detected by a sandwich type enzyme-linked immunosorbent assay (ELISA) in serum or plasma, bronchoalveolar lavage (BAL) fluid, and cerebrospinal fluid, has recently been approved by the US Food and Drug Administration.

• In contrast to GM, BG is a cell wall constituent of several fungi, including Aspergillus, Candida, and Fusarium. Measurement of serum BG has been shown to be an aid in the diagnosis of fungaemia and deep-seated mycoses, including IA (Invasive Aspergillosis).
Disadvantages of Conventional Methods

- **Microscopic examination** allows for rapid detection; however it lacks sensitivity and specificity and allows for primary identification only.

- **Culture** - Recovery of the organism in culture is still considered gold standard method for diagnosis of fungal infections. Cultures demonstrate excellent sensitivity and specificity, but their growth may take days (e.g., Aspergillus spp) to weeks (e.g., Histoplasma capsulatum), thereby delaying the diagnosis and the initiation of appropriate therapy. In addition, the recovery and accurate identification of fungi in the clinical laboratory requires considerable expertise and can often represent a significant safety hazard to laboratory personnel.

- **Non-culture based methods** for diagnosis of fungal infections are of limited value because the levels of circulating antigens are low and the transient nature of the antigenaemia requires sensitive assays and frequent sampling of at-risk patients.
PCR for fungal infections
PCR for fungal infections

- The PCR method for DNA amplification was developed by Kary Mullis and colleagues in 1984 and was rapidly adapted to detect a variety of infectious agents, particularly viruses.

- Recently PCR has also been developed and widely adopted to diagnose fungal pathogens in human infections.

- Procedure
  The detection and identification of fungi in clinical specimens involves a broad-range polymerase chain reaction (PCR) followed by nucleic acid sequencing, after which the nucleic acid sequence is compared with known sequence database and identification is based on DNA homology.
Understanding PCR and DNA Sequencing
How PCR works:

1. Begins with DNA containing a sequence to be amplified and a pair of synthetic oligonucleotide primers that flank the sequence.

2. Next, denature the DNA to single strands at 94°C.

3. Rapidly cool the DNA (37-65°C) and anneal primers to complementary single-stranded sequences flanking the target DNA.

4. Extend primers at 70-75°C using a heat-resistant DNA polymerase such as Taq polymerase derived from Thermus aquaticus.

5. Repeat the cycle of denaturing, annealing, and extension 20-45 times to produce 1 million \((2^{20})\) to 35 trillion copies \((2^{45})\) of the target DNA.

6. Extend the primers at 70-75°C once more to allow incomplete extension products in the reaction mixture to extend completely.

7. Cool to 4°C and store or use amplified PCR product for analysis.
1. Denature to single strands and anneal primers

2. Extend the primers with Taq DNA polymerase

3. Repeat the denaturation and annealing of primers

4. Extend the primers with Taq DNA polymerase

5. Repeat the denaturation and annealing of primers

6. Extend the primers with Taq DNA polymerase

Continued cycles to amplify the DNA
DNA Sequencing

✓ DNA sequencing = determining the nucleotide sequence of DNA.

✓ Dideoxy sequencing developed by Frederick Sanger in the 1970s.

1980: Walter Gilbert (Biol. Labs) & Frederick Sanger (MRC Labs)
Dideoxy DNA sequencing relies on chain termination:

1. DNA template is denatured to single strands.

2. Single DNA primer (3’ end near sequence of interest) is annealed to template DNA and extended with DNA polymerase.

3. Four reactions are set up, each containing:
   1. DNA template
   2. Primer annealed to template DNA
   3. DNA polymerase
   4. dNTPS (dATP, dTTP, dCTP, and dGTP)

4. Next, a different labeled dideoxynucleotide (ddATP, ddTTP, ddCTP, or ddGTP) is added to each of the four reaction tubes at 1/100th the concentration of normal dNTPs.

5. ddNTPs possess a 3’-H instead of 3’-OH, compete in the reaction with normal dNTPS, and produce no phosphodiester bond.
Dideoxy DNA sequencing (cont.):

7. Whenever the labeled ddNTPs are incorporated in the chain, DNA synthesis terminates.

8. Dideoxy DNA sequencing also called dye terminator sequencing.

9. Each of the four reaction mixtures produces a population of DNA molecules with DNA chains terminating at all possible positions.

10. Extension products in each of the four reaction mixtures also end with a different labeled ddNTP (depending on the base).

11. Next, each reaction mixture is electrophoresed in a separate lane (4 lanes) at high voltage on a polyacrylamide gel.

12. Polyacrylamide gels can be thinner $\rightarrow$ higher voltage $\rightarrow$ faster.

13. Pattern of bands in each of the four lanes is visualized on X-ray film or automated sequencer.

14. Location of “bands” in each of the four lanes indicate the size of the fragment terminating with a respective labeled ddNTP.

15. DNA sequence is deduced from the pattern of bands in the 4 lanes.
Automated Dye-Terminator dideoxy DNA Sequencing:

1. Original dideoxy DNA sequencing methods were time consuming, radioactive using P$^{32}$ labels and throughput was low, typically ~300 bp per run.

2. Automated DNA sequencing employs the same general procedure, but uses ddNTPs labeled with fluorescent dyes.

3. Combine 4 dyes fluorescing at different wavelengths in one reaction tube and electrophoreses in one lane on a capillary containing polyacrylamide.

4. Capillary is thinner than gel → higher voltage → even faster.

5. UV laser detects dyes and reads the sequence.

6. Sequence data is displayed as colored peaks (chromatograms) that correspond to the position of each nucleotide in the sequence.

7. Throughput is high, up to 1,200 bp per reaction and 96 reactions every 3 hours with capillary sequencers.

8. Most automated DNA sequencers can load robotically and operate around the clock for weeks with minimal labor.
Fig. 8.11, Chromatogram of about 250 bp
Sequence Based Identification of Fungi

- The general structure of the fungal eukaryotic rRNA gene region consists of four ribosomal genes (the 18S small subunit, the 5.8S subunit, the 25–28S large subunit, and the 5S subunit genes) separated by ITS regions.
- The targets most commonly used for fungi are the ITS1 and ITS2 regions between the 18S and 28S ribosomal subunits and an w600 base-pair region of the D1-D2 region of the 25–28S large ribosomal subunit.

Fig. 1. Structural organization of the fungal ribosomal genes. SSU, small ribosomal subunit; LSU, large ribosomal subunit.
Ciardo and colleagues used sequencing of the ITS region to correctly identify 98% of 113 medically important yeast strains to the species level. These 113 yeast strains were selected because they failed to be identified by traditional methods.

Sequencing of the D1-D2 region of the 25–28S rRNA gene has also been extensively investigated regarding the identification of yeast. Linton and colleagues examined 3,033 clinical isolates of yeast submitted to the United Kingdom Mycology Reference Laboratory over a two-year period. The majority (>90%) could be identified using traditional methods, but sequencing of the D1-D2 region was useful for unambiguous identification of 153 isolates (5%) that exhibited atypical biochemical and phenotypic profiles using other methods.
Advantage of PCR for over conventional methods

- PCR assays can have detection limits of a few gene copies per reaction, providing the ability to detect a fraction of an organism.
- Primers and probes can be designed such that the target can be refined to a specific phylogenetic/taxonomic level; for example, species or genus, or broadened to include most fungi using a consensus sequence PCR approach.
- Sequence variation within amplified product can enable accurate species-level identification.
- Improved turn-around times compared to conventional culture.
- Offers sensitive and specific diagnosis of viable and nonviable fungal pathogens in a variety of clinical specimens.
Fungal PCR at Metropolis

1. Panfungal PCR – MRP 2250
2. PCR for Aspergillus – MRP 6000
3. PCR for Candida – MRP 6000
4. PCR for Cryptococcus – MRP 6600
Sample requirement for fungal PCR

PCR for fungal infections can be done from the following specimen types:

- Sputum
- Urine
- CSF
- BAL
- Body Fluids
- EDTA Whole Blood
- Tissues
- Wound Specimens
THANK YOU