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Molecular genetics in clinical practice for the rapid identification of pathogenic organisms

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A 50 year old male presents to the clinic with a history of fever and cough lasting a week. He has recently immigrated to Canada, and cannot communicate well. Upon examination, he is found to be hypotensive with an elevated heart rate. A chest x-ray is ordered, and it indicates pulmonary infiltrates. This finding, along with the clinical presentation, suggests that the patient has community-acquired pneumonia. He needs treatment, but what is the infection that brought about this episode? What should the clinician do when there is a limited amount of time to find the pathogen?

It is established that timely administration of antimicrobial therapy is critical for good patient outcome. Unless the clinical features and history are very suggestive of a particular causative agent, the therapy must be empiric. The recommendations in this case are to begin with broad-spectrum antibiotic therapy, and to adjust the treatment regimen once laboratory cultures identify the best antibiotics to use. This approach, although logical given the current potential of identifying the causative agent, is fraught with problems. For one, inappropriate initial empiric therapy is an independent risk factor for increased patient mortality and duration of hospitalization. Furthermore, the use of a variety of antibiotics may promote the development of antibiotic resistance among pathogenic organisms. Ideally, once the clinical picture is suggestive of an infectious crisis, rapid identification of the causative agent would allow for targeted therapy that optimizes outcome while minimizing the risk for the development of antibiotic resistance. While current medical technology does not have the rapid identification capacity in all cases of infections, it is progressing steadily towards this goal with the application of molecular genetics in the clinical environment.

**GENOTYPING**

The distinguishing characteristic of every organism must lie in its genes. Two pathogenic bacterial organisms may appear alike in presenting clinical symptoms, on gram stain, and in their growth characteristics, yet be distinctly different and require different treatment. The differences between these organisms that cannot be seen based on culturing and staining alone will be reflected in their genetic makeup. Gene sequencing, therefore, is an excellent approach for the identification of a particular infectious organism from a slew of possibilities. As sequencing technology becomes more efficient, the approach will not only be highly sensitive and specific, it will be rapid and inexpensive. Before this can happen, however, hurdles must be overcome. One is specificity – there must be an identifying genetic sequence for each pathogenic organism. This is a new task given the large variety of known pathogenic species as well as newly evolving ones. But the automation of gene sequencing coupled with computer processing power allows for the alignment of whole genomes from different pathogenic species in order to identify key differences that can be used to distinguish one infection from another. As these genetic differences are identified, it remains for highly sensitive tools to be developed that can assay for the differences in clinical specimens.

Let us return to the patient with pneumonia symptoms. He is recently immigrated, meaning the infection could have been picked up at his place of origin. One of the most common global human infections is with members of the family Mycobacteriaceae. They can be missed as causes of community-acquired pneumonia because of the absence of readily available rapid diagnostic tests. Recent technological advances have made progress in rectifying this problem. In identifying a mycobacterial causative agent, it would be important to distinguish tuberculous mycobacteria from non-tuberculous mycobacteria (NTM). Furthermore, it would be important to determine the particular subspecies as this can be critical in identifying the source of the infection. Taking advantage of known genomic data for the mycobacterial organisms, multiplex PCR is one assay that can be used.

**MULTIPLEX PCR**

The principle of the polymerase chain reaction (PCR) is to specifically amplify a target DNA sequence using thermostable enzymes such as Taq polymerase or Pfu. The sequence to be amplified is localized between two DNA primers that hybridize to the target DNA. The major advantage of this technique in clinical diagnosis is that it requires very little starting material for amplification. For infections with mycobacteria, this could allow for identification without the culturing step that is so time-consuming. Unlike traditional PCR techniques whereby a single target sequence is amplified, multiplex PCR is able to specifically amplify several target sequences in one reaction. This poses several technical challenges because every sequence in the reaction requires an optimal temperature and buffer for amplification. Furthermore, once the reaction conditions are optimized it is necessary to distinguish one amplified sequence from another. When these challenges are overcome, it is possible to amplify several species-specific genetic sequences, including testing for the presence of antibiotic-resistance genes.

In the case of mycobacterial infections, multiplex PCR can distinguish between M. tuberculosis complex (MTC) and NTM based on known genetic differences. These have been tested on clinical specimens, yielding high sensitivity and specificity. Other multiplex PCR assays exist that can distinguish between the NTM subspecies, in particular those of the Mycobacterium avium complex. The major advantage of all of these assays is the sensitivity and rapidity of PCR, allowing for very small amounts of DNA sequences to be detected within several hours. However, results must be interpreted with caution, as the possibility of false negatives is considerable when running several PCR amplification reactions at
non-repeating spacer regions in the genomes of these bacteria. The presence of conserved direct repeat (DR) sequences separated by different strains vary in the number of DRs as well as the length and sequence of the spacer regions. By designing oligonucleotide primers that hybridize to conserved regions within the DRs, it is possible to run a PCR reaction that yields a large variety of amplified DNA sequences of different sizes (Figure 1A). The pattern of these amplified DNA sequences is characteristic of a strain of MTC bacterium (Figure 1B).

Figure 1. The basic principles involved in spoligotyping. A Two juxtaposed blue rectangles indicate a conserved direct repeat (DR) in the genome of a particular strain of Mycobacterium tuberculosis complex. Only a subset of DRs are shown for clarity, whereas there would be many more in the actual organism. PCR primers are designed to hybridize at particular locations within the DRs as shown by the arrows. An arrow pointing to the right is a forward primer, while one pointing to the left is a reverse primer. The primers may hybridize at any of the DRs, and the resulting possible PCR products are indicated. The red square indicates a specific sequence in the first spacer region, while the yellow rectangle indicates a specific sequence in the second spacer region. B The PCR products from A are hybridized to an array containing immobilized oligonucleotides whose sequences correspond to a spacer region. Examples of this are demonstrated with the red square and yellow rectangle from A, whereby the sequence in each of the coloured rectangles is recognized by a specific oligonucleotide probe on the array. Hybridization between a PCR product and the array is indicated by a black circle on the spoligotype. Each row on the array corresponds to hybridization signals from a single strain.

Spoligotyping has been applied to clinical specimens. It is a rapid assay because it relies on PCR, and studies have shown that it is sensitive and specific to MTC infections. It has the additional advantage of being able to distinguish strains of Mycobacterium bovis from those of M. tuberculosis, a difficult task with traditional techniques. Much of the use of spoligotyping has involved contact tracing in instances of suspected MTC infections so as to limit the spread of infection. Further studies are undergoing to definitively determine its use in clinical diagnosis and genotyping of MTC infections.

REAL-TIME PCR (rtPCR) WITH HIGH-RESOLUTION MELTING ANALYSIS (HRM)

A spoligotype performed on clinical isolates from the pneumonia patient revealed a pattern consistent with a highly virulent species of Mycobacterium belonging to the Beijing lineage. This lineage is known to be highly transmissible and often drug-resistant, requiring specific treatment regimens that differ from other mycobacterial infections. For the purposes of rapidly confirming an infection with this pathogen, one assay that can be used is rtPCR with HRM analysis. This assay relies on the presence of a single-nucleotide polymorphism (SNP) corresponding specifically to the Beijing lineage that was identified using methods such as spoligotyping and resulted in nearly 100% sensitivity and specificity as long as there was a sufficient load of bacterial organisms in the sputum. The latter method is based on the digestion of mycobacterial DNA with endonucleases, and the resolution of species-specific fragments by comparison of their migration patterns on a gel.

rtPCR is a technique that allows for the rapid, quantitative amplification of DNA using PCR. As the PCR reaction is run, the PCR product generated during each cycle is bound by a dye that fluoresces at a particular wavelength when bound to double-stranded DNA. The resulting fluorescence is quantified during each cycle, yielding a real-time DNA amplification curve. The amplification product is then subjected to HRM analysis, whereby a known amount of DNA is melted by raising the temperature in fixed increments. Since the melting temperature (Tm) of double-stranded DNA is very sensitive to sequence, a significant difference in Tm may be observed between Beijing and non-Beijing strains of Mycobacterium by virtue of a single SNP.

rtPCR with HRM analysis was applied to clinical specimens and resulted in nearly 100% sensitivity and specificity as long as there was a sufficient load of bacterial organisms in the sputum. The latter method is based on the digestion of mycobacterial DNA with endonucleases, and the resolution of species-specific fragments by comparison of their migration patterns on a gel.