Outbreak Investigation Video Series
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https://www.youtube.com/playlist?list=PLvgkamPnkczlPMSlXhNfqaW4KcFWyoW9q

Transcript, Video 3: Nucleic Acid-based Methods for the Detection of Pathogens

Culture-based methods are extremely useful for isolating potential bacterial pathogens and obtaining some preliminary information about their identity. However, culture-based methods have inherent limitations: viruses and eukaryotic pathogens cannot be cultured on laboratory media. Many bacterial pathogens are difficult to culture as well. When plates are crowded, in other words there are too many colonies, it is often difficult to detect pathogens in the midst of the colonies formed by the members of the native microbiota (if, for example, stool samples were plated).

Another important limitation of culture-based tests is that some pathogens, such as pathogenic *E. coli* or *Shigella* form colonies that are very similar to those of non-pathogenic organisms. Furthermore, as the media manufacturers will point out, some environmental bacteria or benign members of the gut microbial community can form colonies that are very similar to those formed by the pathogens. Therefore, false positives and false negatives are quite possible. Nucleic acid-based and immunological techniques will be important to work through these uncertainties. Keep in mind that all of these techniques have limitations, and it is not prudent to rely on a single technique for the identification of the pathogen, especially during an outbreak investigation.

There are a number of nucleic acid-based techniques. They can help you determine whether a particular gene is present in your sample, and—sometimes—they can also tell you whether this particular gene of interest is expressed. Note that DNA could be fairly stable even after bacteria die or get inactivated by bacteriostatic treatments (such as tetracycline, for example) or certain disinfectants. Furthermore, some bacterial virulence genes can be carried by phages. As you recall, phages, are not virulent to eukaryotes. Therefore, finding a particular virulence gene in an environmental sample or in a sample from a patient does not unequivocally establish that it belongs to an active pathogen that was the culprit of the outbreak. Therefore, nucleic acid-based testing is only informative in combination with other methods for pathogen detection.

For nucleic acid-based pathogen detection to be effective, keep in mind that some viruses only have RNA, and no DNA. RNA is not a template for the DNA-based DNA polymerases that are used in PCR reactions. Therefore, RNA needs to be first converted to complementary (or cDNA) prior to PCR. An enzyme called reverse transcriptase catalyzes conversion of RNA to cDNA. Such reverse transcription PCR needs not be limited to the identification of viruses. It can be used for the detection of active bacterial and eukaryotic pathogens as well. Recall that unlike DNA, RNA is extremely unstable, and it's half-life within living cells is very short – half an hour or so at most, or even shorter in the environment. Therefore, RNA-based detection could be extremely useful for identifying living and actively metabolizing bacterial or eukaryotic pathogens. It could also be useful to establish whether virulence genes are expressed inside a patient, assuming that you have appropriate samples.

Nucleic acids (DNA and RNA) can be isolated from pure cultures (for example if you managed to isolate a potential pathogen using culture-based steps) or from complex samples, such as stool or blood, soil or foods. A number of kits for the extraction of nucleic acids from various matrices are now commercially available. Typically, the first step in the extraction of nucleic acid

requires lysis of cells – this is achieved either with detergents (such as SDS) or enzymes (such as lysozyme). All kits used for DNA isolation require use of a buffer that contains EDTA, a chelating agent that sequesters divalent cations. Because nucleic acid-degrading enzymes (nucleases) require divalent cations, their removal with EDTA renders them inactive and protects DNA during purification. Different kits include steps for the removal of proteins (following their denaturation with organic solvents or salts) following by centrifugation to precipitate denatured proteins and other debris. Once DNA is in a solution, it is typically applied onto a glass fiber filter to which DNA will bind. DNA is than eluted off the glass fiber filter with a slightly alkaline buffer: DNA is more soluble and stable in slightly alkaline solutions.

Protocols for RNA extraction are quite similar, however, extreme caution has to be exercised to prevent RNA-degrading enzymes (RNAses) that are ubiquitous in the environment and also on our hands from degrading RNA in the sample. Special solutions and RNAse-free gloves and plastic-ware will have to be used during RNA extraction. Once RNA is purified, it will have to be reverse-transcribed using reverse transcriptase enzyme to generate cDNA. Reverse transcriptase uses RNA template to synthesize a copy of complementary DNA (cDNA). Recall that in eukaryotes and bacteria, RNA polymerases typically carry out a different reaction, which uses DNA template to synthesize RNA.

Once you have DNA (or cDNA), you will most likely use it for PCR to detect the presence of gene of interest. When working with pathogens, we are most interested in detecting virulence genes. For example, when you plated a stool sample from a patient on XLD medium and observed yellow colonies, you are faced with two possibilities: these may be pathogenic *E. coli* or benign members of the gut microbial community. Testing for the presence of virulence genes, such as *stx* genes that encode the Shiga toxin will allow to distinguish between the two possibilities. All pathogens carry virulence genes, and it is the detection of these virulence genes that will allow you to distinguish between pathogens and their closely related avirulent relatives.

In recent years, quantitative PCR (or qPCR) became popular for the simultaneous detection and quantification of the virulence genes. Traditional PCR is run for 30-35 cycles, and the product is detected as a band on a gel. Instead of detecting PCR products using a gel, qPCR reaction contains a fluorescent dye (or a probe) that allows you to measure – in real time – how much product has formed during the PCR reaction. The number of PCR cycles is not fixed, and the fewer PCR cycles it took to form a detectable product, the more template was present in the original sample. Therefore, one could infer that more cells of the pathogen were present in the tested sample. In the context of an outbreak investigation, qPCR is least useful when used on DNA extracted from pure cultures of the pathogen, and is more informative when used with samples collected from patients or from water, foods and the environment.

Like all biochemical reactions, PCR reactions sometimes fail. It is also possible to obtain false-positives, therefore, it is important to include positive and negative controls. If you designed primers to amplify a particular gene, a great positive control would be a template that certainly contains this target. You can never have too many negative controls. The tendency is to use a control that contains all the components of the PCR reaction (the primers, nucleotides and the polymerase enzyme), and then withholding a template. However, additional negative controls would also be appropriate. For example, DNA from samples from individuals that do not present with the symptoms or DNA from the environmental samples that are not linked with the outbreak would be informative negative controls.

What if you had a great hypothesis, but your PCR reaction failed? In other words, all controls worked but your sample tested negative for the virulence gene that you thought was associated with the pathogen that caused the outbreak? Another alternative would be to amplify and sequence the genes that are involved in the production of the small subunit of the ribosome. All organisms have ribosomes, and all bacteria have the 16S ribosomal subunit. As you recall, the gene encoding 16S has conserved and variable regions. Conserved regions that flank variable regions are the sites that you will use to design primers. The rationale is that designing primers that universally bind to the conserved regions will allow generating PCR products from a great number of bacteria. Amplified variable genes are typically representative of a particular bacterial species. You will sequence them to identify the organism. Keep in mind that this will work only if you have a pure culture of a potential pathogen. If you use primers that bind to the conserved region of the 16S gene in a PCR reactions in which the template is DNA from a complex sample, you will end up with thousands, if not millions of products. They will be representative of the entire microbial community in a particular sample, not just a pathogen that you are trying to identify.