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

Transcript, Video 2: Culture-based Methods for Pathogen Detection

Attempting to culture the culprit of an outbreak is usually the first step toward identifying it. Many specialized media are developed for the enrichment, isolation and rapid identification of common bacterial pathogens of humans, animals and even plants. Remember that not all human pathogens can be cultured. Viruses are notoriously difficult to culture and many are not culturable at all. Those viruses that can be cultured, are propagated on tissue cultures of susceptible human or animal cells lines, and not on common laboratory media. Eukaryotic pathogens such as *Cryptosporidium* and *Giardia* are not cultured for the identification. Therefore, genetic or immunological techniques will be important for the detection and identification of viral and eukaryotic pathogens.

Remember that culture-based identification of pathogens requires at least 24 hours, and sometimes as long as a week. Therefore, prior to proceeding to the next step, you must have several reasonable hypotheses that you will test. These hypotheses should be formulated based on the results of the epidemiological survey and based on the symptoms presented by the patients. Keep in mind that many food- and water-borne pathogens have very similar symptoms. Therefore, pay attention to subtle details, such as incubation periods, the ability of the pathogen to be transmitted from human to human, potential food sources with which the pathogen is tentatively associated to narrow down your hypotheses. Having several, but narrow and robust hypotheses, is critical to the success of your investigation, because it will be impossible to test every sample for every possible food- and water-borne pathogen.

For example, if your patients developed diarrhea and vomiting, the list of potential pathogens is quite long. However, if during the epidemiological survey you learned that none of the household members of the patients developed the same symptoms, you can rule out highly contagious pathogens such as Norovirus, Hepatitis A or *Shigella.* If you have also learned that patients who received common antibiotics are recovering rapidly, you can definitely rule out viral and eukaryotic pathogens, against which antibiotics will be ineffective. Based on this information, you can formulate a hypothesis that the outbreak is likely caused by either *Salmonella* or pathogenic *E. coli*. Results of the epidemiological survey should also indicate potential sources of the culprit.

Once you formulated your hypotheses, subject samples from your patients to the culture-based analyses. Remember that a resuscitation, enrichment or a concentration step may be required if your target pathogen is present in low numbers (for example in a water sample) or if your pathogen is likely to be present a in viable or non-culturable state (such as frozen or dehydrated foods). Keep in mind that the selective media often contain antibiotic or bactericidal agents, which will reduce culturability of your target pathogen, necessitating the pre-enrichment or resuscitation steps. Specific liquid media, such as Rappaport-Vassiliadis broth, available from a variety of commercial suppliers, could be used for the pre-enrichment or resuscitation of pathogens. Don't forget that while pre-enrichment may be key for the detection of pathogens, it greatly amplifies numbers of the pathogens in your sample. Therefore, you will <u>not</u> be able to determine how many cells of the pathogen there were in the original sample, following an enrichment step.

Concentration of pathogens from dilute samples (such as a water or clarified juice) could be accomplished using a simple filtration through a 0.2 micron filter. Once your sample is filtered, you may be able to directly place the filter onto a selective medium not only to potentially identify your pathogen, but also to estimate the numbers of the pathogen in your sample.

Alternatively, if a particular pathogen is suspected, immunomagnetic separation could be used. Immunomagnetic separation involves a super-paramagnetic, polystyrene microspheres that are coated with a specific ligand, for example, an antibody against a particular pathogen. When added to a suspension of the environmental sample containing a pathogen of interest, the microspheres bind to the target pathogen. This binding occurs because of the specific interactions between an antibody and the pathogen against which the antibody is effective. Using a powerful magnet, the microsphere-pathogen complex is then removed from the suspension. After rinsing with a weak buffer solution, what remains are the purified target organisms. The specific methodology can vary depending on the kit and the target organism (or toxin) that is being detected. Dense or concentrated samples, such as stool samples, may need to be diluted prior to culture.

Choose a suitable media for testing your hypotheses. Selective media typically contain an antibiotic or a biocidal agent to reduce growth of non-target organisms and select for the target pathogen, which is typically resistant to the biocide. These media also contain specific carbon and nitrogen sources that are selectively utilized by the pathogen that these media target. Selective media are also commonly brightly colored due to the incorporation of a dye, which is sensitive to changes in the medium's pH. Once your pathogen of interest utilizes a specific carbon or nitrogen source, it produces metabolites that change pH in the vicinity of the colony, making the medium more acidic or alkaline. These changes in pH cause the indicator dye to change color, which is an easy way to detect a potential pathogen. It is important to understand how these media work to anticipate potential false-negatives and false-positives. Take a look at the supporting information provided by the manufacturer of the medium: which non-target organisms can also grow on these media? Can these non-target organisms cause the same symptoms in your patients?

While you are conducting culture-based analyses, go ahead and test your food, water and environmental samples. If an outbreak is associated with foods, you may have access to the production and retail facilities. In production and retail facilities, pathogens often reside in biofilms. Biofilms are multi-cellular, multi-organismal microbial communities. They form on any moist surface. The bulk of biofilms in production and retail facilities is typically made up of benign environmental microbes. They typically make copious amounts of extracellular polymers that give biofilms their slimy appearance and feel. Human pathogens, such as *Salmonella*, *Listeria* and pathogenic *E.coli* are commonly found in these biofilms. Within them, protected by the extracellular polymers of the biofilms, these human pathogens avoid drying out and can escape sanitation and disinfection treatments. Therefore, whenever surveying production and retail facilities, attempt to locate and sample biofilms that could have come in contact with the foods that were linked to the outbreak.

Once you've plated the samples on appropriate media and have given them at least 24 hours to incubate, it is time to analyze your plates. Don't forget that in addition to the presumed pathogen, all your samples are likely to contain other microorganisms. Interpretation of the results of culturing of the stool samples may be particularly tricky. Many members of the normal gut microbiota on selective media form colonies that look essentially identical to those formed by closely-related pathogens.

Let's take a look at this XLD plate. This medium contains desoxycholate – it is a bile salt, which discourages growth of gram positive bacteria and some environmental organisms. Gram-negative human pathogens, such as *Salmonella*, *Shigella* and pathogenic *E.coli* are resistant to bile salts. XLD plates contain lysine, xylose and lactose. Utilization of the sugars (xylose and lactose) by organisms such as *E.coli* leads to the acidification of the medium, and utilization of the amino acid lysine by an organism such as *Salmonella* causes the medium to become alkaline. These changes in pH are detected because an indicator dye, phenol red is added to the medium. The medium also contains sodium thiosulfate and ferric ammonium citrate, utilization of which by *Salmonella* results in the production of hydrogen sulfide, which reacts with iron to give a black precipitate, which results in the characteristic appearance of black colonies.

If a stool sample from a patient suffering from gastrointestinal illness was plated on this medium, you will likely detect at least 3 colony types: black colonies, yellow colonies and colorless colonies that take on the color of the medium and appear pink. When you consult the guide for interpreting results of the culture-based detection using XLD medium, you will learn that black colonies are characteristic of *Salmonella*, yellow colonies are likely *E. coli* or other coliforms, while transparent or pink colonies may belong to *Shigella*. Keep in mind that intestinal microbiota of all humans and other animals contains *E. coli*, therefore there is no surprise that yellow colonies appeared on this medium. Note that organisms from all three of these genera may be responsible for the gastrointestinal diseases.

How does one move forward and identifies the culprit? Earlier in this investigation, we highlighted the importance of obtaining control samples, environmental samples and also samples of foods and water that the patients have come in contact with. We also discussed how important it is to obtain control samples (such as stool, or blood) from people (typically family or household members) who ate the same food, drank the same water and participated in the same recreational activities as the patients. Regardless of whether household members remained healthy or developed symptoms, samples obtained from them will be crucial for identifying the culprit of the outbreak. Having bacterial pathogens in culture will also allow you to determine which antibiotics they are resistant to in order to streamline the treatment process. A pure culture of the pathogen will also be critical for the follow-up experiments on fingerprinting.

However, even with all the culture-based data, it will be impossible to conclusively identify the culprit of the outbreak. You will need to conduct nucleic acid testing to determine whether organisms that you detected using culture-based approaches are pathogenic. You will need to carry out immunological assays to establish that your patients are indeed suffering from the illness linked to the pathogen that you tentatively identified, and lastly, you will need to carry out fingerprinting analyses to confirm that the pathogen recovered from patients and from environmental samples and/or water and food is the same organism.