

Epi-Ready

LABORATORY INVESTIGATION

Module 5

Module Objectives

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By the end of this module, participants will be able to discuss the approaches the laboratory investigator utilizes as a member of the foodborne outbreak response team.

- Describe sample collection methods necessary for identifying agents of foodborne disease.
- Identify approaches for maintaining sample integrity during a foodborne outbreak investigation.
- Discuss the diagnostic testing methods available to the laboratory investigator.

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Performance Objectives

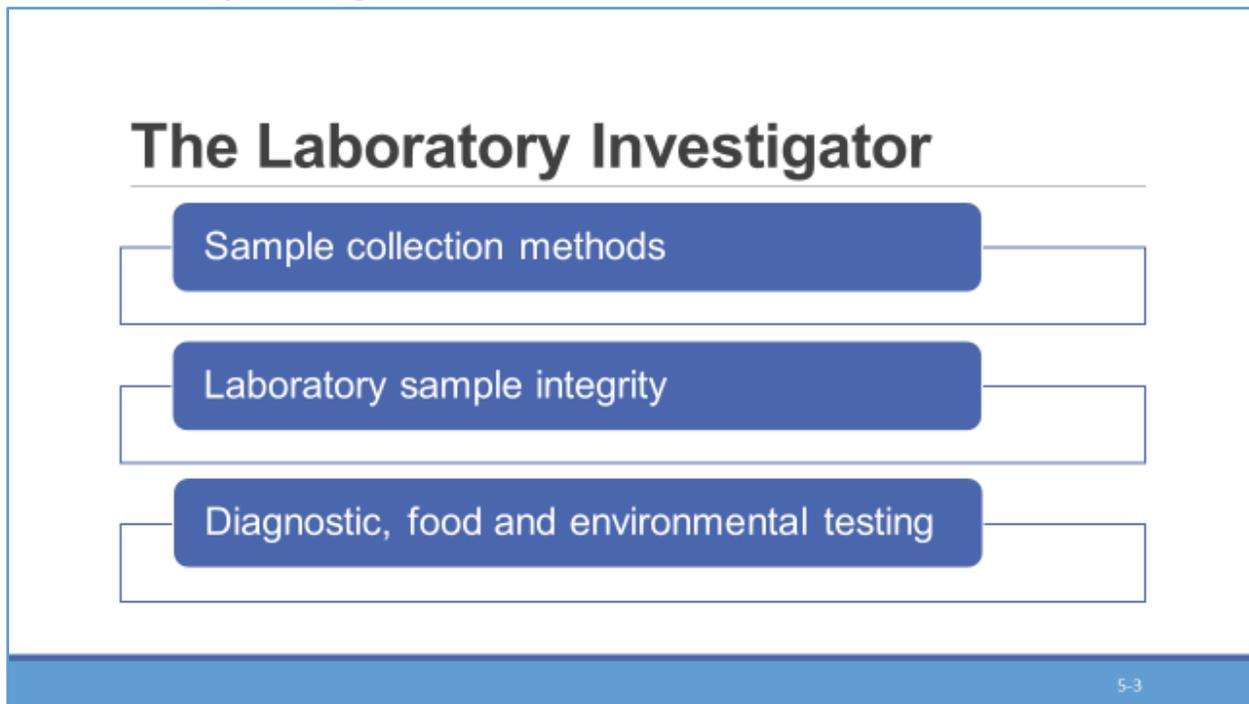
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Enabling Learning Objectives

By the end of this module, the instructor shall accomplish the following learning objectives in support of the performance objective:

- Describe sample collection methods necessary for identifying agents of foodborne disease.
- Identify approaches for maintaining sample integrity during a foodborne outbreak investigation.
- Discuss the diagnostic testing methods available to the laboratory investigator.

The Laboratory Investigator



The laboratorian seeks to identify and characterize agents of disease. This process is necessary for the medical care professional to establish a diagnosis and develop a plan to manage the patient. In the same fashion, they provide valuable information to outbreak response partners, identifying disease causing agents associated with a foodborne illness, possibly linking the cases to others in an outbreak. Laboratory partners can be found in a variety of settings. They may be clinical laboratorians associated with a hospital system or clinical laboratorians in a for-profit diagnostic laboratory. They may be public health laboratorians at the local, state or federal levels. They may be livestock and agricultural laboratorians at the state and federal levels. The laboratorian conducts their activities within the rigors of strict analytic methods. The laboratorian applies these rigors to sample collection methods, diagnostic testing and sample integrity.

Cross-Disciplinary Activities

Epidemiologic Investigator	}	• Focuses on clinical sampling to identify causative agent of disease
Environmental Investigator	}	• Collects appropriate food and environmental samples for analysis

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As part of the investigation team, the responsibilities of the laboratorian is to add their expertise to design of the laboratory study, including types of testing to be performed, the choice of timing of specimens, potential interpretation pitfalls, etc. The laboratory investigator will provide valuable insight into food and environmental sample collection methods to optimize pathogen identification and assure sampling meets the standards of evidence collection. Working closely with the epidemiologic investigator, the laboratory investigator assures that appropriate clinical specimens are collected, preserved and analyzed to rule out/rule in etiologies and optimize pathogen identification.

Standardized Laboratory Methods

The image displays three standardized laboratory methods, each with its respective logo and title in a blue box:

-  Clinical Laboratory Improvement Amendments (CLIA)
-  Bacteriological Analytic Manual
-  Microbiology Laboratory Guidebook

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The laboratorian generally adheres attempts to adhere to strict methods when collecting and analyzing clinical and environmental samples, although there may be circumstances in the course of investigations that require the development of new methods or adaption of older methods to new matrices. The Clinical Laboratory Improvement Amendments of 1988 (CLIA) regulations include federal standards applicable to all U.S. facilities or sites that test human specimens for health assessment or to diagnose, prevent, or treat disease. CLIA is a partnership between the CDC, FDA and the Centers for Medicare and Medicaid Services (CMS). CLIA develops technical standards and laboratory practice guidelines and conducts laboratory quality improvement studies. CLIA regulations establish the quality standards laboratory testing on specimens from humans. FDA's Bacteriological Analytic Manual (BAM) presents the agency's preferred methods for microbiological analysis of foods, drinks and cosmetics. The manual establishes general guidelines and procedures for sampling and identification of microbiological agents and provides methods of detection of specific pathogens. The USDA – FSIS Microbiology Laboratory Guidebook contains current protocols for analytic tests required by FSIS regulatory activities. Microbiological methods are offered for sample preparation, isolation and identification of major foodborne pathogens and their toxins. These materials, as well as others, drive the laboratorian to provide high-quality information for use in foodborne outbreak investigations.

Collecting Specimens and Samples

Proper laboratory analysis begins prior to the specimen or sample arriving at the laboratory. Clinical sampling should be considered when signs, symptoms and history make it likely that the case-patient has contracted a foodborne illness. There are numerous resources that may drive the decision to collect a clinical sample and the appropriate sample collection. Due to the preponderance of diarrheal illness associated with disease, the most common specimen collected for foodborne disease agent identification is stool. Other types of specimens may be

warranted for various presentations. For example, if the cases are febrile, a blood culture may be recommended. If the primary symptom is vomiting, collection of emesis may be recommended. Immediate collection and storage (but not testing) of potentially-implicated food is recommended.

Medical care providers may wish to consult the following:

- Diagnosis and Management of Foodborne Illness: A Primer for Physicians (CDC 2004)
- Guidelines for Confirmation of Foodborne-Disease Outbreaks (MMWR March 17, 2000 / 49(SS01);54-62)
- Guidelines from the Infectious Disease Society of America (IDSA)
- Outbreaks of Undetermined Etiology (OUE) Guidelines (CIFOR)

Specimen Collection Guidance

- Diagnosis and Management of Foodborne Illness: A Primer for Physicians (CDC 2004)
- Guidelines for Confirmation of Foodborne-Disease Outbreaks (MMWR March 17, 2000)
- Outbreaks of Undetermined Etiology (OUE) Guidelines (CIFOR)



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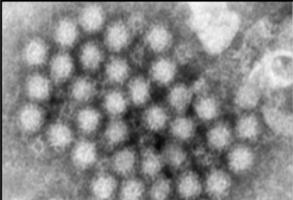
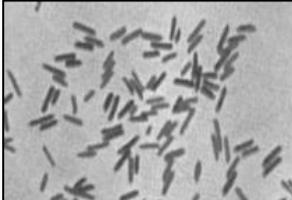
Note to Participants

The CIFOR tool: Outbreaks of Undetermined Etiology (OUE) Guidelines is provided as an Appendix. The OUE Guidelines is a recently-developed tool that supports the identification of an agent of foodborne disease through signs and symptoms coupled with geographic and exposure information (history). It then provides information on primary specimen collection and storage media. Participants are encouraged to review the CIFOR OUE Agent List and download the entire tool for use. This tool should be shared with others on response teams as well as medical care providers.

Collecting Stool Specimens for Submission to the Laboratory

A stool specimen should be collected as soon as signs and symptoms warrant and before antimicrobics are administered. When stool is collected as part of an ongoing outbreak investigation, collecting a representative sample from 20%, or at least from 5 to 6 case-patients is optimal. Good luck!

The following table, replicated on Slide 5-7 provides a useful guide to collecting stool samples by suspected agent. It should be noted that information to guide an etiology hypothesis may be limited in an active outbreak setting, and there is considerable overlap between the different type of agents. Therefore, use of uniform syndrome-based specimen collection guidelines is recommended as is specified in the CIFOR OUE guidelines.

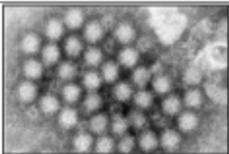
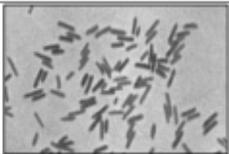
Suspected Agent			
	Virus	Bacteria	Parasites
			
Amount to Collect	10 to 25 Grams	10 to 25 Grams/Swab	10 to 25 Grams
Number of Specimens	One	One	Up to Three Every Other Day
Preservative	None*	Cary Blair	10% Formalin, Polyvinyl Alcohol
Transport Recommendations	Cold or Freeze**	Ambient, Cold or Freeze***	Ambient

* Some laboratories will accept samples to be tested for virus in Cary Blair

**Freezing only acceptable if testing with RT-PCR

*** Freezing will cause *Campylobacter spp.* to die off

Specimen Collection Methodology

	Suspected Agent		
	Virus	Bacteria	Parasites
			
Amount to Collect	10 to 25 Grams	10 to 25 Grams/Swab	10 to 25 Grams
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Transport Recommendations	Cold or Freeze**	Ambient, Cold or Freeze***	Ambient

5-7

Activity

Activity

- Work in your table groups.
- 10 minute working in table groups and 5 minutes to report.
- Use the CIFOR – OUE guidelines.
- Given the scenarios, identify the most probable disease agent, the specimen to be collected and how it should be prepared.

1. Several people have been showing up in the emergency department of the local hospital. All have diarrhea, some bloody. They all have a mild to severe fever and most have had nausea and a few stated they have vomited. They recall eating at a local restaurant 3 or 4 days ago but do not recall what they ate.
2. A husband and wife seek care at an urgent care facility. The husband is complaining of abdominal cramps, diarrhea and gas. He states his stools seem greasy. He states his symptoms started a few days ago. His wife is complaining of severe abdominal cramps and gas. They love to hike.
3. A person presents at the emergency department of the local hospital with sudden onset of nausea and vomiting. He states he had a meal approximately 3 hours ago of rice and beans.

5-7

Work in your table groups to determine the most probable disease agent, what specimen(s) to collect and how to preserve and or prepare for submission to the laboratory for analysis. Record answers in the space provided.

1. Several people have been showing up in the emergency department of the local hospital. All have diarrhea, some bloody. They all have a mild to severe fever and most have had nausea and a few stated they have vomited. They recall eating at a local restaurant 3 or 4 days ago but do not recall what they ate.

Probable disease agent:

Collect:

2. A husband and wife seek care at an urgent care facility. The husband is complaining of abdominal cramps, diarrhea and gas. He states his stools seem greasy. He states his symptoms started a few days ago. His wife is complaining of severe abdominal cramps and gas. They love to hike.

Probable disease agent:

Collect:

3. A person presents at the emergency department of the local hospital with sudden onset of nausea and vomiting. He states he had a meal approximately 3 hours ago of rice and beans.

Probable disease agent:

Collect:

Collecting Stool Specimens

Collecting Stool Specimens

The following video is a product of the Northwest Center for Foodborne Outbreak Management, Epidemiology and Surveillance (FOMES)– the Oregon Food Safety Center of Excellence

Imbed Video

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This video is a product of the Northwest Center for Foodborne Outbreak Management, Epidemiology and Surveillance (FOMES)– the Oregon Food Safety Center of Excellence. It demonstrates the most appropriate way to collect a stool sample.

Food and Environmental Sampling

The testing of food and environmental surfaces should generally only be considered when epidemiologic evidence, environmental investigation results or clinical laboratory results strongly suggest a pathogen and a food vehicle. Evidence from each discipline would be optimal but generally evidence from at least two of the disciplines is adequate to proceed with food collection and laboratory analysis. There may be certain circumstances when a broader approach to sampling and analysis may be necessary but not without the concurrence of the outbreak response team. These exceptions may be associated with life-threatening conditions with public health implications such as botulism poisoning where there may not be a single specific suspect item.

Food Sampling

- Must be supported by epidemiologic, environmental and laboratory information
- May have to look at ingredients
- Containers and sampling equipment must be sterile
- Sampler must adhere to aseptic techniques
- Shipped at temperature at which it was collected – except TCS foods
- Make sure all containers are labeled



7-10

Food sampling should be conducted as soon as possible. Time is generally a critical factor as a suspected food may have been consumed, discarded or combined with other foods. If an establishment has been identified and several foods continue to be investigated as a likely source, it may be necessary to sample several foods and store as directed by the laboratory investigator to maintain its original integrity.

If the investigation points towards a complex food, review of the recipe for ingredients for sample collection may be necessary. Attribution data (Module 4) regarding the foods on the menu may focus the sampling to a few of the ingredients.

Food sampling procedures are established so that the growth of non-critical organisms is minimized and the introduction of environmental contaminants is prevented. The following must be followed to ensure product integrity:

- The surfaces where food sampling is to be conducted must be clean and sanitized.
- Containers for storing food must be clean, dry and leak-proof. The containers must be sterile and be of sufficient size to hold the food product.
- Food are to be collected with sterilized sampling equipment.
- The sampler must adhere to aseptic technique.
- Food samples should remain in their original containers whenever possible. Unopened foods are preferable. Opened containers/packaging should be placed in sterile containers prior to shipping. Note: the laboratorian with perform an organoleptic inspection of the shipped food. If there is significant destruction of the packaging and deterioration of the food, it is likely that analysis will not be performed.
- Foods must be shipped at the same temperature where they were identified. The exception to this is when a food is a TCS and should be refrigerated to minimize growth of pathogenic organisms. NOTE: TCS is an acronym established in the 2013 Food Code

and has the meaning “Time, Temperature Control for Safety”. It replaces the term “potentially hazardous food”.

Environmental Surface Sampling

- Daunting task – 100’s of samples required
- Deliberative process – takes a team
- Sample by zone
- May be beneficial for isolation of *Salmonella spp.* and *Listeria monocytogenes* in processing operations
- Contact laboratory pre and post sampling



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Environmental sampling for suspected foodborne pathogens is a daunting task and should not be commenced without strong evidence that a foodborne illness pathogen may be present in a facility and is the cause of an outbreak. Finding the foodborne illness pathogen in a food or facility is probably the highest level of confirmation of the source of illness that can be achieved. With this in mind, environmental sampling must be a deliberative process and not be initiated haphazardly. The sampling team must be in contact with the laboratory pre and post sampling.

Sampling of this nature should be conducted by a team – usually three or four investigators. A facility map should be utilized and sampling conducted based upon the best available information regarding the pathogen and likely places to sample based on the characteristics of the pathogen. This zone concept of sampling identifies and prioritizes processing areas with the highest probability for contamination by the pathogen and where the pathogen may either grow or be located. The FDA breaks down environmental sampling zones into four zones.

- Zone 1 is food contact surfaces such as slicers, mixers, utensils and work tables. If *Salmonella spp.* is the pathogen of concern, zone 1 is not generally sampled. If *Listeria monocytogenes* is the pathogen of concern, sampling should focus in zone 1.
- Zone 2 is the area immediately adjacent to food contact surfaces. If the environmental sampling is focusing on *Salmonella spp.*, this is the focus area for sampling.
- Zone 3 is the area immediately surrounding zone 3 and would generally be comprised of floors, walls and ceilings and larger equipment not usually associated with cross-contamination. Pathogens in these zones are either re-entrained and introduced via the air or on a fomite and introduced into the food.
- Zone 4 is considered remote areas and not directly in processing areas. This area is generally not sampled. This area on a few occasions has been implicated in foodborne

illness investigations. You may recall from module 2 that the source of the cookie dough outbreak of *E. coli* was due to the pathogen being harbored in a walled off area in the processing facility.

Of the three major foodborne illness bacterial pathogens, environmental sampling should be limited to sampling to identify *Salmonella spp.* and *Listeria monocytogenes*. Sampling for Shiga toxin-producing *E. coli* has proven to be ineffective as it generally is considered a contaminated ingredient issue and not one of cross-contamination. If cross-contamination is considered as a source and *E. coli* is the presumed pathogen, focus sampling in zone 1 and only with concurrence of the foodborne outbreak team.

Specimen and Sample Integrity

Specimen and Sample Integrity

- Contact laboratory
- Seal the container!
- Clean and sanitize the container
- Properly label container
- Use tamper tape or tamper-resistant bags
- Chain of Custody



Make sure the laboratory that you plan to ship specimens to will conduct the analysis requested. Not all laboratories perform or are certified to perform all food and environmental sampling analysis. Additionally, samplers should be in contact with the receiving lab to confirm collection/transport conditions and discuss sample volumes (number of samples that will be collected). Some testing requires special media/broth that will need to be prepared prior to receiving samples. Additional routine media may need to be prepared for large sample volumes. Give your receiving lab a heads up!

Once samples are collected, important steps must be taken to maintain the integrity of the sample. Specimen containers must be properly sealed and labeled with the following (as applicable):

- Use parafilm or similar sealer on specimen container with lids.
- The outside of the specimen container must be clean and sanitized.
- Use a sealable, leak-proof bag with absorbent material to store the specimen.
- Identify specimen container with pertinent information.

- Include specimen submission forms.
- Use tamper tape or tamper resistant bags.
- Proper labeling of specimen to include: Patient name, DOB, Patient ID – Specimen source – Date of Collection – Location – Time of Collection – Lot#, Expiration date of transport medium – Person performing collection – Pertinent information useful for the laboratory such as outbreak or investigation name and number.

Check with the laboratory for any pre-shipping instructions. If cold chain maintenance is necessary, be sure that the proper cold maintenance equipment and temperature indicators are available prior to sampling. It is also important to contact the shipping company to ensure their ability to ship, if there are any regulatory requirements, latest receiving time at the shipping company and shipping time to destination.

Shipping containers must be sealed and properly labeled. A Chain of Custody form must accompany the container. Oftentimes, chain of custody is the shipping form. At the time of shipping, the form will be signed over to the shipping company and the on-scene investigation team should notify the laboratory that the shipping container has been turned over to the shipping company. The chain of custody form is an NCR form and the shipping company, once they have filled in the receiving information, will provide a copy of the form to the on-scene sampling team. Investigators must develop a familiarity with the use of chain of custody forms and their use become routine. If sampling conducted becomes evidence in a legal proceeding, an improperly fill out form could result in the sampling results (evidence) not allowed in the proceeding.

Sampling should be considered an extension of the laboratory. Failure to comply with your laboratory investigators can lead to a devastating sampling result.

Laboratory Analysis of Clinical Specimens and Environmental Samples

Once a specimen or sample reaches the laboratory, the goal of the laboratorian is to identify the pathogen causing disease. There are three generally-recognized categories of laboratory testing that are used, to varying extent, to identify pathogens of foodborne illness.

Laboratory Analysis

Direct
Microscopy

Immunoassays

Nucleic Acid
Amplification
Tests

Culture-based
Assays

5-13

Direct Microscopy

Direct Microscopy

- Common method for diagnosis of many pathogenic intestinal parasites.
- Labor-intensive
- Requires highly-skilled laboratorians



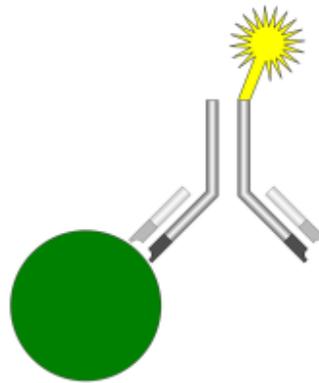
5-14

Microscopic examination of stool specimens continues to be a common method for the diagnosis of most pathogenic intestinal protozoa. The microscopic ova and parasite examination (O&P) is the traditional method for stool parasite testing. O&P is labor-intensive and requires a high level of skill for optimal interpretation.

Immunoassay

Immunoassay

- Detects surface antigens or other molecules
- Relatively fast and easy to read results
- Accurate and reliable
- Can be used in clinical settings
- ELISA and EIA are common



5-15

An immunoassay is a test that can be used in the clinical setting or in the laboratory to detect specific molecules. These molecules, or analytes, may be an antibody produced by a lymphocyte in response to infection (to confirm active infection or previous infection in a patient), or an antigen associated with the pathogen (to confirm the presence of the pathogen of interest). Immunoassays rely on specific antibody-antigen interaction for detection and quantification of a chemical compound. Immunoassays are relatively fast tests, yielding accurate and easy to read results. Immunologic assays can be used to detect bacterial cells, spores, viruses and toxins. This is a rapid test method that can test for toxins associated with foodborne illness. The most common and recognized immunoassays are the Enzyme-linked immunosorbent assay (ELISA) and the enzyme immunoassay (EIA).

Commercially-available Immunoassays - Food

Bacillus cereus
toxin

Campylobacter
spp.

Clostridium
perfringens
enterotoxin

E. coli O157:H7

Listeria
monocytogenes

Salmonella *spp.*

Shigella *spp.*

Vibrio cholera

At best, immunoassays can differentiate to the serotype level and these tests are useless in linking food to cases based on subtyping.

5-16

Immunoassays are commercially available for the detection of the following more commonly-associated foodborne pathogens and toxins in food:

- *Bacillus cereus* toxin
- *Campylobacter* *spp.*
- *Clostridium perfringens* enterotoxin
- *E. coli* O157:H7
- *Listeria monocytogenes*
- *Salmonella* *spp.*
- *Shigella* *spp.*
- *Vibrio cholera*

Due to their low cost, reduced time to identification, accuracy and reliability, immunoassays provide an excellent screening tool to detect contaminant in foods. Several immunoassays have been employed in the food safety industry due to their analytic accuracy and rapid results. From a clinical perspective, immunoassay is the generally-accepted method to detect toxins associated with foodborne illness. FDA-cleared immunoassays are available for detection of selected pathogens directly from samples, specimens, enrichment broths, or isolates. For example, latex agglutination is commonly used to differentiate *E. coli* O157:H7 strains from other strains of *E. coli*. The greatest challenge associated with the use of immunoassays in a foodborne outbreak disease investigation is linking subtype data from human clinical specimens to a food or environmental sample differentiated only, at best, to the serotype level with immunoassay.

Nucleic Acid Amplification Tests

Nucleic Acid Amplification Tests

- Culture-independent Diagnostic Testing (CIDT)
 - Fast
 - Syndrome-based
 - Not dependent on viability of organism
 - Polymerase Chain Reaction (PCR) Test developed in the 1980's



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Nucleic acid amplification tests (NAATs) is the most appropriate name for this analysis method but may also be referred to as Molecular Detection Assays or culture-independent diagnostic testing or CIDT. This type of testing is syndrome-based; ordered by healthcare providers to rule in or rule out certain infections associated with the syndrome(s) exhibited by the case-patient. The sequential blocks show the evolution of the NAAT from its origins in the 1980s to identify DNA segments of genetic material (PCR), to the ability to identify RNA segments of genetic material (RT-PCR), to quantitative detection methods (RT-qPCR), and, finally to the multiplex PCR panels that are now in use.

These testing methodologies do not require the isolation of the pathogen through the culture process. Although immunoassay would be considered a culture-independent diagnostic test, this section will focus on the genetic material (nucleic acid) of pathogens. Culture-independent diagnostic testing (CIDT) was created out the inability to routinely culture many of the microorganisms responsible for human disease.

Developed in the early 1980's, the polymerase chain reaction (PCR) test is a method of analyzing short (target) sequences of DNA or RNA by the amplification of the genetic material sufficient for identification. The sequence of genetic material in a cell to be identified is matched to a specific primer. Amplification is a process of breaking the primer-extracted DNA strand into two single strands by denaturing, usually with heat, and then building a new DNA strand by annealing each strand with a DNA polymerase enzyme.

Originally created to identify DNA segments, PCR had very little utility to identify virus and other foodborne illness pathogens such as bacteria, parasites, and fungus due to a predominance of RNA within their cells. Reverse transcription PCR (RT-PCR) converts the RNA into a complimentary DNA strand (cDNA) by the enzyme reverse transcriptase. The process then follows the PCR process to amplify genetic material for identification.

Conventional PCR methods have limitations when attempting to quantify pathogen load. A major development in PCR technology was the advent of real-time or quantitative RT-PCR (RT-qPCR). This technology added to the strength of PCR as a diagnostic tool as the amplification of the gene is detected in real time by the use of a fluorescent maker. This step in the evolution of PCR has made it possible to use the technology to detect genetic material to a sensitivity that viral pathogens can be identified prior to disease onset.

A significant limitation to the use of PCR is the lack of an isolate necessary to compare by PFGE or WGS but there are other challenges with PCR. PCR may produce false positive results as PCR does not distinguish between viable and non-viable organisms. Additionally, PCR in clinical settings has been hampered largely by background contamination from exogenous sources of DNA. As powerful as PCR is as a detection tool, it may provide false negative results as the relatively small sample volumes for testing and low concentrations of infectious material may lead false negative findings.

CaliciNet serves a vital purpose during foodborne disease investigation. The CaliciNet database includes the genetic sequences of norovirus strains associated with acute gastroenteritis (AGE) outbreaks in the United States. Sequencing norovirus requires gene amplification with PCR technologies. Norovirus is subject to genetic modification on a regular basis. CaliciNet monitors these emerging strains and is able to link a norovirus strain to a foodborne illness, assisting the foodborne outbreak response team in their efforts to identify the source of disease and establish controls strategies to mitigate the impact of the outbreak.

Of particular importance in the evolution of PCR-based technology and its impact of the public health surveillance system is the multiplex PCR. Multiplex PCR is an analytic method where more than one target genetic sequence is amplified using more than one pair of primers. This multiplex-based use of PCR is the technology that has brought on the use of multi-analyte CIDTs, commonly referred to as CIDTs. CIDT is changing the way that many clinical laboratories diagnose patients with foodborne illness. These tests are generally syndrome-based, meaning that if someone seeks medical attention for AGE, a specific “panel” will be used to identify the pathogen associated with causing illness. The panel is able to rapidly identify the more common pathogens associated with AGE and will be able to identify viral, bacterial and/or parasitic agent of disease on one panel. The utility for clinical use of CIDT is well understood as medical care providers only wish to identify the disease-causing agent for treatment purposes with little regard for the pathogen’s association to a possible outbreak. Commercially-available CIDTs can detect 4 to 23 enteric pathogens on one panel (Janda 2014).

CIDT’s Impact on the Public Health System

CIDT has impacted the public health system in several ways. It has changed the pattern of reporting through the surveillance systems in the United States. The FoodNet system has seen changes in reportable disease. In 2015, FoodNet reported 20,107 confirmed cases of reportable enteric disease through traditional, culture-based methods. In the same reporting period, FoodNet also received 3,112 positive, culture-independent diagnostic test with no follow-up with culture confirmation. This increase in the use of culture-independent diagnostic tests has markedly increased over the 2012 year reported disease to FoodNet (Huang 2015). Patterns of CIDT-confirmed cases varies by pathogen with very little change observed with O157 STEC as it is generally identified with an antigen-based CIDT as well as *Campylobacter*. Significant

changes in detection of *Shigella* and *Salmonella* by CIDT was observed when compared to the same reporting period.

As demonstrated above, increase in multi-analyte nucleic acid amplification test with CIDT use and changes in reporting may have the following effects on the public health system.

Benefits of CIDT to Public Health

The Benefits of CIDT for Public Health

- Surveillance of pathogens for which there were previously no practical test
 - enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC)
- Faster information for local public health action
- Potential application in developing countries
- Multi-analyte approach yields
 - Improved data for some pathogens
 - Information about polymicrobial (mixed) infections

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- CIDT allows for the identification and surveillance of pathogens for which there were previously no practical test. Enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC) have been identified by CIDT.
- CIDT tests are fast, yielding results to providers that may guide treatment of the case-patient. This rapid detection also provides surveillance information to public health for action. Coupled with epidemiologic information, local clusters may be identified for response action in a timelier manner.
- Due to their lower cost as compared multiple testing for pathogens, CIDT may have application for use in developing countries.
- A multi-analyte approach may provide improved data on some pathogens not easily detected by other methods and may pick up polymicrobial (mixed) infections that otherwise would go undetected by other methods. CIDTs may pick up viral infection that would go undetected by targeted laboratory methods such as with a single-analyte PCR test. The detection of common viral causes of enteric disease may help curb the use of antimicrobial therapies.

Challenges of CIDT to Public Health

- Difficult to monitor disease burden and associated trends
- No ability to conduct antimicrobial susceptibility
- Interpretation issues for public health action such as screening potential carriers and test-of-cure
- No isolates produced
- Loss of trained microbiologist

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- Reduction in the number of laboratory isolates for cluster identification and epidemiologic investigations. Without subtyping of isolates by advanced molecular detection methods, our PulseNet system will be significantly impacted. Our surveillance systems will lose the ability to detect emerging and novel causes of foodborne illness.
- Many foodborne illnesses may go unidentified as a result of no culture confirmation by CIDT, allowing disease to go unreported to the public health system. This may result in the lack of epidemiologic investigations that may have identified associated cases and could lead to the identification of the source of illness and the lack of short-term controls and long-term institutional controls being implemented.
- Without culture identified isolates, there is no method to generate an antimicrobial susceptibility profile on the foodborne illness pathogen. Some foodborne illness pathogens require antimicrobial therapy for treatment under selected circumstances. Susceptibility patterns of enteric pathogens change and without antimicrobial resistance testing, medical care providers may rely on outdated treatment guidance leading to less-than-optimal outcomes for their patients. Lack of susceptibility data will impact our global system of understanding antimicrobial resistance patterns.
- The use of CIDTs does not require the same level of “microbial expertise” to isolate and identify by culture methods. In the long-term, the loss of microbiologists may impact the ability of public health laboratories to properly staff for new and emerging microbiological analysis methods.

Several agencies and association have made recommendations regarding the use of CIDTs and the potential loss of culture identified isolates. The APHL have published interim recommendations regarding CIDT (February 2016). The recommendations follow:

1. Prior to implementing a CIDT assay, clinical laboratories should contact their local and/or state public health laboratory to discuss specific regulations regarding culture-independent diagnostic tests, required organisms for public health submission and specimen type(s) preferred by their public health partner.
2. When a pathogen that requires public health submission is detected, clinical laboratories should continue to obtain isolates, as much as possible, and submit them to the local or state PHL to facilitate timely detection and investigation of outbreaks. PHLs will perform pulsed-field gel electrophoresis (PFGE) and/or whole genome sequencing (WGS) along with virulence testing and susceptibility testing as warranted.
3. If clinical laboratories are unable to culture the isolates for public health surveillance, the CIDT-positive specimens should be submitted to the PHL within 24 hours of the CIDT result. Ideally, stools should be submitted in Modified Cary-Blair transport medium at ambient temperature even though viability will be compromised due to the elapsed time from collection to submission. If submission of stool specimens in Modified Cary-Blair transport medium is not feasible, specimens should be transported in Gram Negative - GN broth (Hanja) and submitted to the PHL. Clinical laboratories should contact the public health laboratory for other organism-specific guidance.

Culture-based Identification of Foodborne Illness Pathogens

Culture-based Identification

- Classic method to identify bacteria to species level
- Produces an isolate to identify a pathogen
- Isolates are necessary to differentiate bacteria to the sub-species level



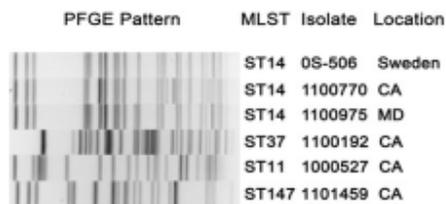
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Identification of foodborne illness pathogens has long required the isolation of a single species of microorganism. Classic methods of identifying microorganisms to species requires an understanding of the morphological and metabolic characteristics of the microorganism. In general, a clinical specimen is prepared and inoculated onto a media that either grows multiple species of microorganisms in colonies or selectively grows a microorganism of interest. These bacterial isolates are necessary for subtyping bacteria – differentiating bacterial isolates past the species and subspecies level.

Molecular subtyping, also referred to as DNA analysis had its first broad application with the development of Pulsed-field Gel Electrophoresis (PFGE) in the mid-1980's. It gained prominence when it was used to identify the strain of an *E. coli* O157:H7 associated with the consumption of undercooked hamburger in 1993. In support of this of this detection method for foodborne illness cluster identification, PulseNet was launched in 1996.

Pulsed-field Gel Electrophoresis (PFGE)

- DNA is separated from the isolate by lysing the bacterial cell
- DNA is placed in agarose and loaded on a gel
- A pulsing electric field is applied to the gel and DNA separates based on size



Source: CDC

5-21

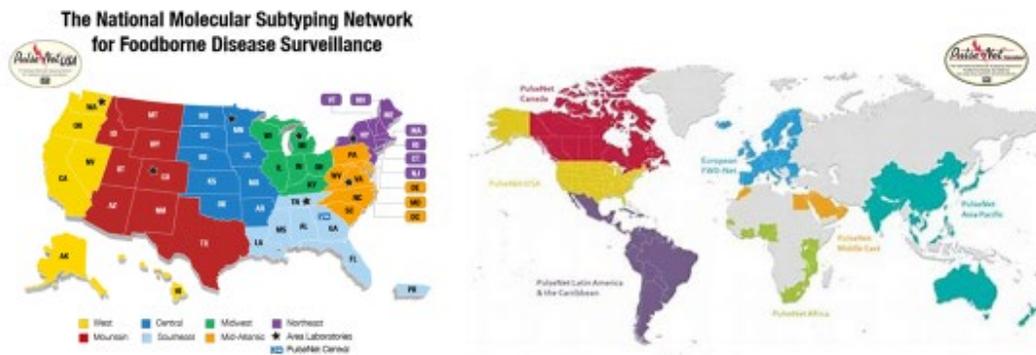
The PFGE process starts by suspending isolated bacterial cells in agarose (a gelatinous substance) “plugs” and lysing the cells with a biochemical (or enzyme) to free the genetic material from the interior of the cell. The free DNA is then cut into smaller fragments using restriction enzymes. The plugs are loaded into a gel, with one isolate per lane, and a pulsed electric field is applied to the gel. This electric field separates the DNA fragments into “bands” based on their size. The pattern of “bands” can be visualized by staining the gel and viewing it under an ultraviolet light. These band patterns, or “fingerprints”, are specific to a subtype of bacterial pathogen and can be compared to other patterns in a database maintained by PulseNet known as BioNumerics. Patterns are compared by both state and CDC staff to determine if the isolated subtype is the part of a cluster of illness possibly caused by the same pathogen. Isolates with similar or identical DNA fingerprints are usually genetically related.

This relationship of local and state public health laboratories and the CDC, FDA and USDA-FSIS laboratories - sharing information on the genetic subtyping of eight bacteria make up the system of PulseNet. The eight pathogens covered by PulseNet are:

- *E. coli* O157 and other Shiga toxin-producing *E. coli*
- *Campylobacter*
- *Cronobacter*
- *Listeria monocytogenes*
- *Salmonella*
- *Vibrio cholera*

- *Vibrio parahaemolyticus*
- *Shigella*

PulseNet USA and PulseNet International



5-22

PulseNet USA is a network of 83 laboratories in seven regions of the United States. Due to the utility of PulseNet as a cluster detection laboratory method, it is used globally as PulseNet International.

Evolving PulseNet

Evolving PulseNet



PFGE ►►► WGS

Pulsed-Field Gel Electrophoresis || Whole Genome Sequencing

Source: CDC

5-23

PulseNet is a powerful cluster detection tool and has made a significant positive impact on the US food safety system. During the first 20 years, PulseNet has attributed to the following:

- Billions of pounds of food have been recalled due to PulseNet-triggered outbreak investigations
- \$507 million saved each year in medical costs and lost productivity due to quick cluster detection and outbreak response, and identification and remediation of problems in the food supply that might not otherwise been detected
- One million DNA fingerprints of foodborne illness bacteria in the US PulseNet database
- 89,000 DNA fingerprints uploaded to the database in 2015 – a record number
- 1500 local clusters and 250 multi-state or national clusters of foodborne illness identified annually by PulseNet

The PFGE technology made the PulseNet system a success but it has its limitations. The process is time consuming and it does not discriminate between all unrelated isolates. Since the system is based on the size of the chromosomal material, bands of the same size may not have come from the same part of the chromosome. PFGE is not a true phylogenetic measure and therefore cannot be used to determine the genetic relatedness of subtypes.

Whole Genome Sequencing

Whole Genome Sequencing (WGS)

- An isolate is needed
- This is a laboratory and computational process
- WGS first used as a surveillance and response technique in 2013 – *Listeria* initiative
- WGS is a process of genotyping - understanding the genetic makeup of an organism

The Whole Genome Sequencing (WGS) Process
WGS is a laboratory procedure that determines the order of bases in the genome of an organism. In this process, WGS provides a very precise DNA fingerprint that can help scientists track and identify outbreaks, and respond to the outbreak and control source.

Bacterial Culture
1. Isolate the bacteria from the sample and grow them in a culture. The DNA is then extracted.

DNA Sequencing
2. The DNA library is created using a sequencing machine. The DNA is broken into small fragments and sequenced. The resulting data is then analyzed to determine the order of bases in the genome.

DNA Library Preparation
3. The DNA is broken into small fragments and sequenced. The resulting data is then analyzed to determine the order of bases in the genome.

Data Analysis
4. The resulting data is analyzed using computer programs to identify the order of bases in the genome. The resulting data is then analyzed to determine the order of bases in the genome.

Source: CDC

5-24

The ability to more fully understand the genetic nature of a pathogen and be able to make nuanced distinctions between pathogens is accomplished by whole genome sequencing (WGS). The uniqueness of any organism is produced by the sequence of the four “building block” bases of DNA (adenine, thymine, guanine, cytosine). WGS is a laboratory and computational method that sequences the order of bases in the genome in one process. The efforts to sequence a genome commenced in the in the 1950’s but the first major breakthrough in DNA sequencing occurred in 1977 when Frederick Sanger sequenced the first genome – a bacteriophage. The field of DNA sequencing has since eclipsed Sanger’s first generation

technology to now a third generation technology that does not require amplification of genetic material (PCR) and can read extremely long DNA chains utilizing commercially-available sequencing machines. It must be noted that an isolate is needed to perform WGS.

In 2013, the CDC, in collaboration with the FDA and the USDA-FSIS, began using whole genome sequencing (WGS) as the main method to detect outbreaks caused by *Listeria*. NGS, referred to as next-generation sequencing will greatly improve the efficiency of how PulseNet conducts surveillance. “Instead of only having the ability to compare bacterial genomes using 15-30 bands that appear in a PFGE pattern, we now have millions of bases to compare. That is like comparing all of the words in a book (WGS), instead of just the number of chapters (PFGE), to see if the books are the same or different.” (CDC 2016)

Multilocus Sequence Typing (MLST)

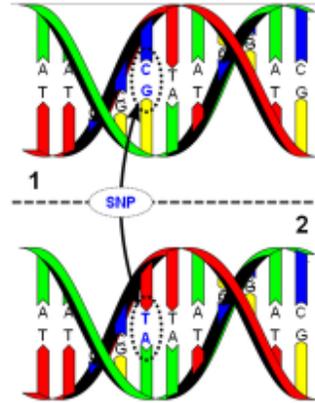
- Process of sequencing to characterize isolates of bacterial species
- Primarily focuses on the 7 housekeeping genes
 - Present in all isolates within a species and their rate of genetic variability is relatively low

5-25

As mentioned above, the concept of next generation sequencing (NGS) is a laboratory and computational method that occurs in one process and provides an understanding of the phylogenetic nature or genetic relatedness of the pathogen. WGS is a process of genotyping – of genetically understanding the nuanced differences or similarities between strains or subtypes of microorganisms. Two general approaches can be used with WGS of bacteria species. One approach is to sequence specific core genetic material, referred to as the housekeeping genes. Housekeeping genes are a smaller number of constitutive genes responsible for maintaining basic cellular function. Constitutive genes transcribe at a fairly constant level regardless cell environmental conditions. These core, housekeeping genes are sequenced because they are present in all isolates within a species and their rate of genetic variability is relatively low. This latter approach, the multilocus sequence typing or MLST, is the approach that has been used to characterize most isolates of bacteria species. The core genome MLST (cgMLST) approach identifies a set of core genomes that generally include the housekeeping genes as well other consistently-identified genomes for the strain of the bacterial species under consideration.

SNP-Based Approach to WGS

- Single Nucleotide Polymorphism
- Variation in a single nucleotide – A, T, C, G within a genomic sequence
- Presence of SNPs does not indicate bacterial strains are unrelated
- Number of SNPs as a measure of relatedness varies by species



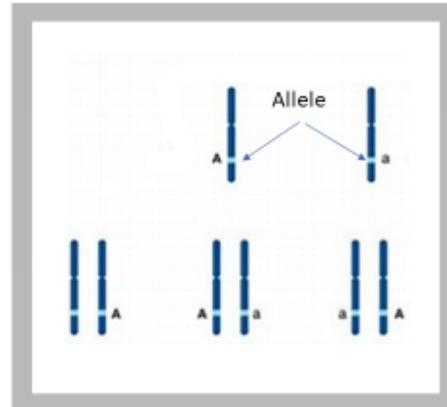
5-26

The genetic variability (diversity) within a bacterial strain can be observed using a gene-by-gene approach (allele-based) or measured by determining the number of single nucleotide polymorphisms (SNP) in the genome based against a reference. A SNP (pronounced “snip”) is a variation in a single nucleotide within a genomic segment. A perfect match of core genetic material between two bacterial strains would have no SNPs or a “no SNP difference”. The presence of a SNP does not indicate that the bacterial strains are unrelated. Depending on the bacterial species, SNPs can be expected and the presence of up to a certain number identified through the sequencing process would not mean they are unrelated. As an example, the relatedness threshold for *Listeria monocytogenes* is 3 or less SNPs whereas the relatedness threshold for *Campylobacter jejuni* is 15 or less SNPs (Schurch 2018).

The FDA has created the first distributed network of laboratories to utilize whole genome sequencing to identify foodborne pathogens. GenomeTrakr is a network of public health and university laboratories in the US and around the world that share genomic and geographic data on foodborne pathogens. The data is housed at the National Center for Biotechnology Information (NCBI). GenomeTrakr is a SNP-based WGS platform.

Allele-based Approach to WGS

- Alleles (Allelomorphs) are pairs of genes at a specific place on a chromosome that occur as an alternate form.
- The presence of an allele(s) does not indicate that a bacterial strain is unrelated
- Number of alleles as a measure of relatedness varies by species



5-27

PulseNet is focusing on allele-based cgMLST for surveillance and investigation of foodborne illness clusters, with on-demand SNP analyses to address specific epidemiological questions. This approach sequences the core genome using a gene-by-gene approach. Alleles, pairs of genes at a specific place or locus on a chromosome that may occur as an alternative form are identified in this process. An allelomorph is synonymous with allele and may be more descriptive of this allele-based approach as these phenotypic changes in the sequencing process help to understand the genetic relatedness of the bacterial strain. As with the SNP-based approach, the presence of alleles does not indicate that bacterial strains are unrelated. For example, the rule of thumb established by the CDC for *Salmonella* spp. and STEC with a suspected foodborne illness is less than a 10 allele difference (CDC 2017).

It is important to stress that all thresholds of relatedness (for SNPs or alleles) are rule-of-thumb. This is because the ecology of outbreaks varies widely. Some outbreaks are true single agent introductions. This is the cases with a single strain that contaminated food or a food preparer has contaminated food and causing illness. Many outbreaks are polymicrobial. That is, they are caused by more than one microorganism. An example of this is chronic contamination in a food processing facility, where the microorganism has been around for a while. Another example is produce that has been irrigated with water contaminated with raw sewage. It would be atypical for only one pathogen to be found in raw sewage.

It is important to understand that WGS does not by itself prove association (the molecular subtype doesn't tell you anything about the route of transmission) and the lack of identity doesn't disprove association. Epidemiologic investigators absolutely need to be aware of the limitations of molecular subtyping. It provides a hypothesis of association, not proof of association, and only one hypothesis out of many possible.

Case Study



Source: CDC

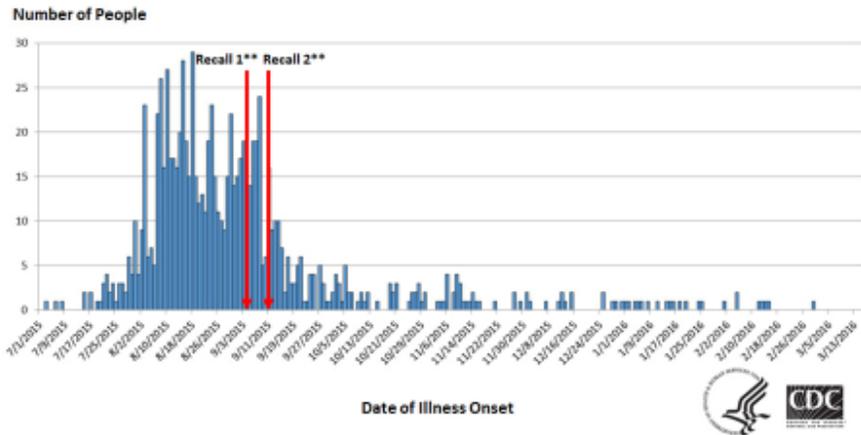
Compare results of PFGE and WGS in a multi-state outbreak of *Salmonella* Poona associated with the consumption of cucumbers.

5-28

This case study will compare the use of PFGE and WGS in a multistate outbreak investigation of *Salmonella enterica* serovar Poona associated with consumption of cucumbers. The PFGE-WGS study was conducted by the California Department of Public Health on isolates associated with the California cases.

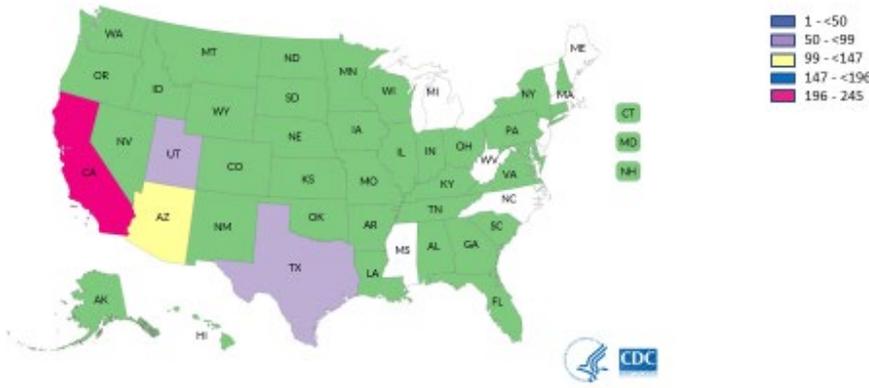
Illnesses associated with this outbreak ranged from July 3, 2015 to February 29, 2016.

Cases Identified by Date of Onset

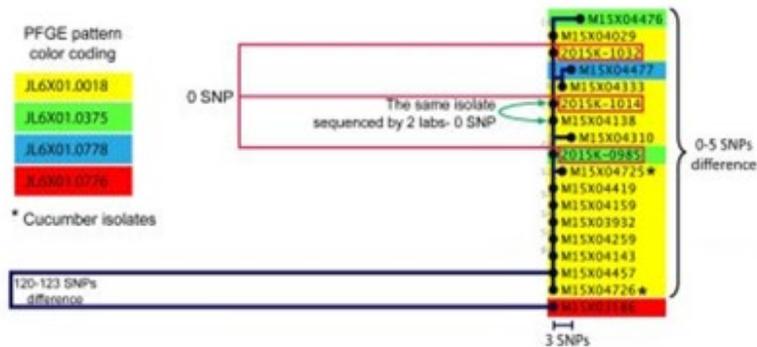


There were 907 cases in 49 states associated with the outbreak. Ill people ranged in age from less than 1 year to 99, with a median age of 18. Forty-nine percent of ill people were children younger than 18 years. Fifty-six percent of ill people were female. Among 720 people with available information, 204 (28%) were hospitalized and there were 6 deaths. The case count map is provided below. California had 245 cases associated with this outbreak.

Case Count Map



PFGE – WGS Comparative Results



Source: Kozyreva, PLoS Curr. 2016 November 22, 8:

5-31

This slide groups the PFGE and the WGS results. The yellow identified isolates are 100% indistinguishable, the yellow and green identified isolates are 96% indistinguishable, and the yellow, green and blue isolates are 88% indistinguishable. WGS results provide a different picture of the laboratory investigation. All isolates coded yellow, green and blue had only a 0 – 5 SNP differences and confirmed their genetic relatedness was in concordance with the epidemiologic investigation. In many foodborne disease investigations, isolates that are not indistinguishable may not have been considered part of the outbreak. Interesting to note that the blue-identified PFGE pattern had a three-band difference but exhibited genetic relatedness to the other strains except the red-identified strain. If PFGE results were solely used, that isolate would not have been considered as part of the outbreak.

Whole genome sequencing has significant advantages over other laboratory methods utilized to identify foodborne illness pathogens. It is a fast and affordable method of identifying bacteria in one process. Without WGS, four or more steps are necessary to fully characterize bacteria, including PFGE. As demonstrated by the case study, WGS provides high resolution results based on the phylogenetic nature or genetic relatedness of the pathogen. PFGE is based on the size of the chromosomal material and bands of the same size may not have come from the same part of the chromosome. PFGE is not a true phylogenetic measure and therefore cannot be used to determine the genetic relatedness of subtypes.

Metagenomic Analysis

Metagenomics

- A process to identify all of the DNA present in a sample
- Identification of pathogen and subtyping in as little as 1.5 hours
- University of Georgia and CDC have identified pathogen with quasi-metagenomics

A portable sequencer in a laboratory in the University of Georgia Center for Food Safety in Griffin, Georgia

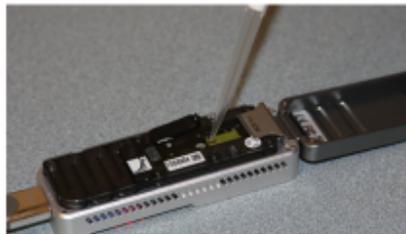


PHOTO COURTESY OF
UNIVERSITY OF GEORGIA

5-32

With the eventual absence of cultured isolates from clinical laboratories, innovative technologies are in the pipeline for the subtyping of pathogens utilizing culture-independent methods. These methods have the capability to reduce the identification and subtyping process from over a week to a few days – at most. Metagenomic analysis is the future of pathogen identification.

In the early stages of development, metagenomics identifies all of the DNA present in a sample and compares the genomic data to a database of genomic material of known pathogens. Researchers at CDC and Georgia Tech applied metagenomics successfully to identify the causative agent in two *Salmonella* Heidelberg outbreaks in Alabama and Colorado. This study was conducted in parallel with classic culture-based methodology, yielding similar results on the *Salmonella* infection but the metagenomic investigation revealed a co-infection with *Staphylococcus aureus* (Huang 2017).

Researchers at the University of Georgia Center for Food Safety are using magnetic beads with antibodies (immunomagnetic separation - IMS) to pull genetic material out of pathogens within a food sample for metagenomic testing. Once the pathogen's genetic material is concentrated, amplification of genetic material using a specialized approach (multiple displacement amplification – MDA) creates a sufficient amount of DNA so that it can be sequenced. Identification of pathogen and subtyping have occurred in as short a time as an hour and a half. This process, referred to as quasi-metagenomics by the researchers, has significant potential to reduce the time necessary to subtype a pathogenic microorganism (Deng 2017).

Summary

Summary

Describe sample collection methods necessary to identify agents of foodborne disease

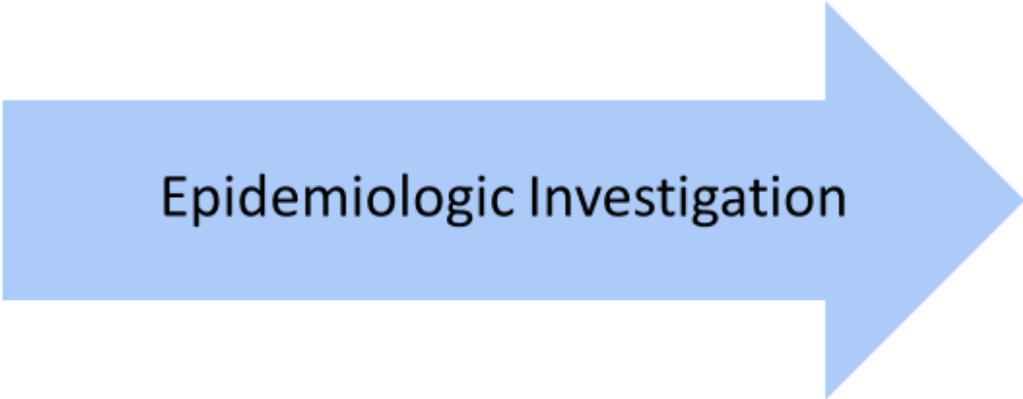
Identify approaches to maintain sample integrity during a foodborne outbreak investigation

Discuss the diagnostic testing methods available to the laboratory investigator

7-33

Coming up Next

Coming Up Next



Epidemiologic Investigation

5-34