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1. Purpose

The purpose of this document is to provide training guidance to new hire analysts responsible for performing microbiological analysis of food and feed samples and their trainers. This information is both a supplement to local on-the-job training (OJT) and formal classroom training (OTED courses), but also provides the standardized framework and defines the minimal requirements for local OJT.

This training chapter introduces the trainee to four categories of microbiological analysis: Fundamentals, Basic (Tier 1), Intermediate (Tier 2), and Advanced (Tier 3).

The overall purpose of this chapter is to:

- Train the analyst to think as a regulatory microbiologist.
- Introduce typical analytical procedures a regulatory microbiologist should know, understand, and may encounter throughout their career.
- Show where and how the work performed fits into the regulatory framework including: reason for sample collection; procedures of an inspection and sample collection; sample analysis procedures and approaches; and regulatory follow up actions and relationship of items to the FD&C Act.

2. Scope

This document applies to all FDA regulatory microbiologists involved in the analysis of microbiological food and feed samples.

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The FDA microbiologist is well termed a regulatory microbiologist because everything he or she does is related to the regulation of products and manufacturers under the jurisdiction of the Federal Food, Drug, and Cosmetic Act (FD&C Act) and Related Acts. Thus, results of his or her work impacts directly on those products regulated, and therefore on the consumer. The FDA microbiologist can also be called a public health microbiologist as removing contaminated food and feed products from the market directly impacts the health and welfare of consumers.

3. Responsibility

3.1. Trainer

- A. Coordinates training with Supervisor(s) and Trainee(s).
- B. Works with new hire Trainee(s) to ensure completion of all basic training needed to meet their new hire level 1 analyst certification.
- C. Works with Trainee(s) to ensure completion of all intermediate and advanced training needed to meet their regulatory responsibilities.

3.2. Trainee

- A. Completes required training within specified timeframes.
- B. Reports training received and provides documentation of completed training to supervisor.
- C. Reads and complies with standards, regulations, policies, procedures, and work instructions.

3.3. Supervisor and Management

- A. Implements and reviews training records.
- B. Designates local Trainers and ensures that the required and appropriate content is covered.

3.4. Quality Management

- A. Maintains employee competencies matrix records for staff.

4. Background

Food and feed microbiological analyses range from the relatively simple to the most complex. In recent years, microbiologists have transitioned from sole use of conventional methods to a coupling of state-of-the-art rapid methods with the traditional.

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During the first year, there will be several basic training courses offered by OTED. Training will also be supplemented by computer-based modules provided online by ORA U. However, most instruction will come from the laboratory.

Microbiology methodology is an ever-improving science. Training will be a continuing process throughout the microbiologist's career. Future training will reinforce and amplify what the trainee has learned.

5. References

- B. [American Society for Microbiology Laboratory Protocols](#) (background discussion on Gram stain, serial dilutions, ELISA, PCR)
- C. [Center for Disease Control and Prevention Microbiology Series](#) (free training modules on PPE, basic culture media, microscopy, core microbiology skills, and molecular techniques)
- D. [AOAC Official Methods of Analysis](#)
- E. [FDA Bacteriological Analytical Manual \(BAM\)](#)
- F. [United States Pharmacopeia](#) (USP) *Note: to access USP references throughout this document go to [Account Information \(sharepoint.com\)](#) to create an FDA library account if you have not already done so.*

6. Procedure

6.1. Fundamentals

At the foundation of an FDA regulatory microbiologist are the most fundamental microbiology skills and techniques. This section will focus on the fundamentals of media and reagent preparation, laboratory safety and hazardous waste handling, quality assurance and management, and polymerase chain reaction.

The new hire shall also have a basic understanding of the concepts of aseptic technique, serial dilutions, quadrant streaking, Gram staining, ELISA tests, and other molecular methodology. These topics are covered in more depth as the new hire progresses through the Basic category analyses. However, there are links for supplemental review in the references section from ASM and CDC.

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6.1.1. Media, Reagent, and Supply Preparation

6.1.1.1. Objective

- A. Familiarize the trainee with preparing, dispensing, and sterilizing microbiological media, reagents, and supplies.
- B. Familiarize the trainee with media quality assurance procedures and documentation processes.
- C. Familiarize the trainee with safety concerns, such as autoclave safety and weighing of powders.

6.1.1.2. Assignment

- A. The trainer will discuss and demonstrate the equipment used in media preparation such as balances, stirrers, dispensers, pH meters, and autoclaves.
- B. The trainer will discuss proper personal protective equipment (PPE) to be used during each step of media preparation.
- C. The trainee will prepare several kinds of media and buffer solutions, representative of the types generally used. These include, but are not limited to, selective and non-selective enrichment broths, plating media, and phosphate buffer. Examples include Lauryl sulfate tryptose broth, Brilliant green lactose broth, EC broth, Lactose broth, Tetrathionate broth, Plate count agar, Butterfields Phosphate diluent, Triple sugar iron agar, EMB agar, Hektoen enteric agar, Bismuth sulfite agar.
- D. The trainee will prepare and sterilize supplies such as empty bottles and tubes, polyvinylchloride tubing, spoons etc. as dictated by individual lab use.
- E. The trainee will demonstrate the ability to dispense media, either using a manual pipettor, automated pipettor/dispensing system, or both.
- F. The trainer will discuss the function of the components that comprise the commonly used media.
- G. The trainer will discuss storage and shelf life of prepared and dehydrated media/reagents.

6.1.1.3. Questions

- A. Which media should not be steam sterilized and why?
- B. Where is agar derived from? What are the special properties of agar that make it well suited as a solidifying agent in culture media? Once

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melted, what temperature should agar be kept at to prevent it from solidifying?

- C. In which media and buffer preparations is volume particularly critical?
- D. Why is pH important in media and buffer preparation?
- E. What are some safety concerns when autoclaving? What PPE should be utilized when preparing, transporting, and storing media?

6.1.1.4. References for Media, Reagent, and Supply Preparation

- A. [Bacteriological analytical manual \(BAM\) \(current ed.\). Center for Food Safety and Applied Nutrition, U.S. Food & Drug Administration](#)
- B. [AOAC official methods of analysis. \(current ed.\) Arlington, VA: Association of Official Analytical Chemists.](#)
- C. [U.S. Pharmacopeia/ National Formulary \(current ed.\)](#)
- D. [Difco & BBL Manual: Manual of Microbiological Culture Media \(Second Edition\).](#)
- E. [Biosafety in Microbiological and BioMedical Laboratories \(BMBL\) \(current ed.\). Centers for Disease Control and Prevention.](#)

6.1.2. Safety and Hazardous/Infections Waste

6.1.2.1. Objective

- A. Introduce the trainee to laboratory safety practices and correctly using personal protective equipment (PPE).
- B. Introduce the trainee to the hazards involved in working with pathogens and/or their toxins such as Salmonella and Clostridium botulinum toxin.
- C. Develop the trainee's awareness of procedures for the proper segregation and disposal of laboratory waste products.
- D. Identify resources which can assist the employee with the risk assessment process, such as state specific laws and requirements.
- E. Be able to utilize the Globally Harmonized System (GHS) for Hazard Classification, including being able to recognize standard pictograms for hazards, hazard categories, and hazard communication.

6.1.2.2. Assignment

- A. Read ORA Lab Manual, Volume III, Section 1 for safety issues.
- B. Read Laboratory Chemical Hygiene Plan.

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- C. Read Laboratory Hazardous Waste Plan.
- D. Read the Biosafety in Microbiological and Biomedical Laboratories (BMBL), current edition.
- E. Read the Laboratory Biosafety Plan for local site.
- F. Read the Safety Data Sheets (SDS) for each chemical used in the analytical procedure.
- G. Discuss with your trainer or supervisor how etiologic isolates are shipped.
- H. Discuss with your trainer, supervisor, and/or industrial hygienist how hazardous waste is managed at the local site.

6.1.2.3. Questions

- A. What are the items of personal protective equipment (PPE), minimally needed, in a Biological Safety Level (BSL)-2 biological laboratory?
- B. Describe and give examples of food borne disease microorganisms that cause infections, disease, or other health hazards and identify their biosafety levels. Describe proper procedures for disposal of used media. Describe how to use and operate an autoclave safely.
- C. What is a SDS (Safety Data Sheet)? Where are the SDSs located? What information can be found on a chemical reagent hazard label?
- D. Does the state regulate medical waste? What laboratory wastes are permitted to enter the sewer? What laboratory wastes are incinerated?
- E. What are the guidelines for handling food microorganisms (mostly bacteria or their toxins) in the BMBL?
- F. What are engineering controls? Describe the proper use of these engineering controls in a food microbiology laboratory.
- G. What are administrative controls? Describe administrative controls designed to minimize the risks of hazardous agent exposure to those personnel who are not directly involved with their manipulation. List those administrative controls that assist in the maintenance of quality control.
- H. What safety equipment is normally found within a microbiological laboratory?
- I. What types of laboratory procedures have the potential to generate aerosols? How can these procedures be contained? How can the generation of aerosols be minimized?

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- J. What decontamination procedures are in place and when are they performed?
- K. Describe how spills are handled. Are the cleanups following a spill documented and the cleanup verified?
- L. What kind of signage is to be in place in a microbiological BSL-2 laboratory?
- M. What are the potential routes of exposure when working with infectious organisms?
- N. Why is it necessary for a minimum of two people to be working in the laboratory at any given time?
- O. Has enough training been received to perform assigned tasks and has this training been documented?
- P. Is the facility designed to prevent infectious organisms from being accidentally released to other areas in the building?
- Q. Is there a sharps safety program in place to reduce hazards when handling syringes or pipettes or other sharps?
- R. Are animals used to test infectious agents? Identify what program and laws address the use of animals in research studies?

6.1.3. Quality Assurance

6.1.3.1. Objective

- A. Introduce the concept of quality assurance and quality control.
- B. Emphasize the importance of ensuring analytical results and written or electronic worksheets are of the highest quality.
- C. Introduce the Quality Management Information System (QMIS).
- D. Discuss the basics and importance of audits.

6.1.3.2. Assignment

- A. Read the following:
 - 1) Laboratory Quality Management Plan and International Standard ISO/IEC 17025:2017(E).
 - 2) ORA Lab Manual Volume II, Section 2, and laboratory's corresponding SOPs.
 - 3) ORA Lab Manual Volume III, Section 3, Records of Results – Analyst's Worksheet

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4) SOP-001064 Microbiological Controls for Food and Feed Product Sample Analysis

- B. Trainer will discuss with trainee the various techniques used such as positive, negative and system controls, environmental sampling, media growth promotion testing, correct media volume, pH and formulation, equipment controls, etc.
- C. Trainer should review forms used to report oral and written QA evaluations of worksheets.
- D. Trainer should review forms used to report oral and written QA evaluations of worksheets.
- E. Trainer should discuss the purpose and importance of internal and external audits.

6.1.3.3. Questions

- A. What culture controls are used for an *Escherichia coli* enumeration analysis? For a *Staphylococcus aureus* analysis?
- B. What is the significance of environmental monitoring within an accredited microbiology laboratory? How does your laboratory perform environmental monitoring?
- C. Why are temperatures recorded for incubators, water baths etc.?
- D. Why are worksheets reviewed and signed by an analyst who has not worked on the sample?
- E. Why are validated methods used?
- F. What is the purpose of QMIS? What are some of the actions/uses of QMIS?
- G. What is your laboratory's current external auditing body?

6.1.4. Polymerase Chain Reaction (PCR)

6.1.4.1. Objective

- A. Introduce the basic principles and applications of the polymerase chain reaction.
- B. Introduce the trainee to proper the techniques, procedures, and equipment used in PCR analysis.

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6.1.4.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of laboratory demonstrations.
- B. Read [American Society for Microbiology Laboratory Protocols, Polymerase Chain Reaction Protocol](#)
- C. Read [Center for Disease Control and Prevention Microbiology Series, Basic Molecular Biology Module 4: PCR and Real-Time PCR](#)
- D. Practice using a micropipette to dispense small liquid quantities.
- E. Practice using proper molecular biology techniques while opening and closing small reaction tubes and dispensing reagents.
- F. Learn how to calculate correct reagent quantities needed for master mix preparation.
- G. Trainer will demonstrate how to use a thermal cycler.
- H. Trainer will discuss which organisms can be analyzed using PCR.

6.1.4.3. Questions

- A. What are some of the benefits to using PCR to detect microbial pathogens in foods? What are some of the potential problems associated with using PCR to detect microbial pathogens in foods?
- B. What are the three major steps (processes) involved in the PCR cycle?
- C. Why would using an enrichment procedure before PCR analysis be useful?
- D. What components are needed in the PCR reaction mixture and what function does each element serve?
- E. Why is selection of the primers so important to the success of the reaction?
- F. Why are there forward and reverse primers?
- G. Why does a microbiologist need an excess quantity of primers in the reaction mixture?
- H. What is real-time PCR? How does it differ from conventional PCR?
- I. Why always run a reagent control?
- J. List three ways to prevent contamination when performing PCR.
- K. In what ways can PCR be inhibited?

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6.1.5. Establishment Inspections

6.1.5.1. Introduction

Microbiologists are routinely requested to accompany investigators on inspection of establishments that produce/store foods or feed. The trainee will accompany an investigator and/or an experienced microbiologist. The investigator has the basic responsibility for an establishment inspection and the microbiologist is a technical advisor in matters pertaining to microbiology, but should participate fully in the inspection, noting items for the inspection report and final discussion with management. The investigator oversees the inspection.

There are various types of inspections that a microbiologist may be requested to assist with such as: seafood HACCP; frozen, chilled, or prepared foods, nutmeats; canned food manufacturing; fresh produce processing facility; produce farm traceback investigation. Additionally, your role as an analyst on inspection may vary from microbiologist subject matter expert reviewing firm records and or environmental conditions both inside or outside of the facility, to collecting aseptic raw ingredient, in-line, water, environmental swab, or finish product samples.

6.1.5.2. Discussion

It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to establishment inspections.

Prior to inspection, the trainee should read and discuss the following with a microbiologist experienced in performing establishment inspections to gain a fundamental understanding of the various roles you may assume during an inspection.

- 1) [Investigations Operations Manual \(IOM\)](#), current edition, especially Chapter 1 Administration, Subchapter 1.6 -Public Relations, Ethics and Conduct; Chapter 4 Sampling, and Chapter 5 Establishment Inspection: Food.
- 2) ORA Laboratory Manual Volume III, Section 5, Analysts on Inspection

Prior to inspection, the trainee should talk with the investigator, read the previous Establishment Investigations report (EIR), firm history, Safety report and read any Compliance Programs or assignment that applies. The trainee should also work closely with the senior microbiologist on preparation of sample collection materials and on proper protective clothing for the establishment.

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6.1.5.3. Exercise

While on the inspection, the trainee should:

- A. Aseptically collect or assist in collection of raw materials, in line materials, finished product, water, or environmental swab samples.
- B. Document in a regulatory note book all observations (483 items), conversations, questions, equipment, production specific details, cleaning procedures, filth and insect control, storage, and all specific sample collection information.
- C. Assist with preparing the Sample collection report (CR).
- D. Help prepare and edit the list of observations (Form FD 483) for presentation and discussion with firm management.
- E. Contribute to the Establishment Inspection Report and evaluate if the report contained enough detailed evidence to support a recommendation for legal action.
- F. Evaluate if the analytical results substantiated observations of insanitation, OOS (out of specification), or other deficiencies.
- G. Evaluate and discuss if in line sub samples were sufficient to "point out" where bacterial contamination could enter and/or proliferate. Evaluate and discuss what control measures are in place to control/detect any microbiological contamination.
- H. Evaluate the firm's environmental sampling plan, review the methodology and reported findings.
- I. Discuss with the team what aspects of the inspection the trainee would like to improve or learn before the next inspection.

6.2. Basic (Tier 1, i.e. analyses required for analyst level 1 certification)

The microorganisms listed in this section represent the basic microbiology analyses performed in the ORA Food and Feed Microbiology labs. On-the-job training and successful mastery of these analyses are key for a new hire Microbiologist. This Basic tier of analyses are the minimum set of analyses required to become eligible for their New Hire Level 1 Certification.

A new hire regulatory microbiologist should expect to be issued a minimum of two training samples per microorganism type. The second training sample for each analyte can be a proficiency or competency sample that demonstrates the new hire's ability to independently work through the primary method with minimal assistance. However, the quantity of samples for each analyte may be determined by laboratory procedure and or adjusted to the Trainee's individual situation and/or prior experience. Following the successful

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demonstration of competency of each analyte, laboratory management may authorize a new hire to begin being assigned regulatory samples as determined by individual lab procedure.

The training sample ideally should be a food or feed sample which is representative of some of the common matrices (spices, frozen seafood, cheese, produce, etc.) for the lab and for each analysis. Training samples shall simulate a regulatory sample in that it helps the new hire understand the care and tact that a regulatory microbiologist needs bring to the sample analysis. The trainee should have the opportunity to demonstrate appropriate chain of custody and aseptic subsampling all throughout the analysis. However, the overall assessment criteria should be based upon the successful mastery of the analysis.

Ideally the trainee shall have the opportunity to successfully recover and identify the target organism from a blind training sample. Training samples shall consist of at least three blind subsamples: one positive or spiked subsample (reference SOP-001064 for general guidance on spiking levels and spike can occur after sample setup directly into the pre-enrichment); one negative subsample spiked with a competitor microorganism (the purpose of the negative competitor spike is that it may mimic the target organism culturally); and one negative un-spiked subsample.

6.2.1. Salmonella

6.2.1.1. Objective

- A. Learn about *Salmonella*, Food Categories, sample compositing, and sample analysis.
- B. Recover *Salmonella* by using pre-enrichment and enrichment techniques.
- C. Screen samples for *Salmonella* utilizing current rapid methods.
- D. Identify *Salmonella* using biochemical and rapid methods
- E. Describe cases and symptoms of *Salmonella* food borne disease.

6.2.1.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.

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- B. Read [Bacteriological Analytical Manual \(BAM\) Chapter 1, Food Sampling and Preparation of Sample Homogenate](#)
- C. Read [Bacteriological Analytical Manual \(BAM\) Chapter 5, Salmonella](#)
- D. Read [AOAC Official Method](#) (2004.03, 2011.03, or other current methods utilized in the laboratory)
- E. Using BAM/AOAC methods, analyze the minimum number of training samples for *Salmonella* utilizing various sampling techniques and pre-enrichment broths.
- F. The new hire (either through training sample or individual demonstration) shall have the opportunity to prepare at least one 15-sub composite.
- G. The trainee shall screen samples using VIDAS AOAC method (or other routinely used screening methods) for *Salmonella*.
- H. The trainee (through either training sample or individual demonstration) shall inoculate both typical and atypical *Salmonella* strains onto selective agar plates and TSI & LIA slants.
- I. The trainer shall ensure that the trainee understands the biochemical reactions for both typical and atypical *Salmonella* and understands which can be ruled out and which must continue through confirmatory testing.
- J. Identify isolates using conventional biochemicals and become familiar with the various rapid methods referenced in the BAM and utilized within the local laboratory (API 20E, VITEK 2 GN, MICRO ID, VITEK MS, LAMP confirmation, and or Real-time PCR).
- K. Perform *Salmonella* serological testing including polyvalent flagellar (H) and polyvalent somatic (O) on various serovars as available locally.
- L. The trainer should ensure that the trainee understands the basic concepts of *Salmonella* O & H serotyping and how to perform the testing according to local practice.

6.2.1.3. Questions

- A. Why are different pre-enrichment media used for different foods? Give examples. Why is a pre-enrichment step needed for the recovery of *Salmonella*?
- B. Why is more than one type of selective enrichment (Rappaport-Vassilades, selenite cystine and tetrathionate broths) used instead of just one?

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- C. Describe sugar reactions in triple sugar iron agar (TSI) tubes.
- D. Why use three primary plating media instead of one?
- E. Which *Salmonella* serovars do not typically produce hydrogen sulfide?
- F. What is indole?
- G. Can we identify all serovars of *Salmonella*? Give a reason.
- H. Describe symptoms and onset time of *Salmonella* food borne disease.
- I. Describe and give examples of foods in Food Category I, II and III. (See BAM Chapter 1 Food Sampling)
- J. Why is the pH of the enrichment broth adjusted after addition of sample?
- K. What does the VIDAS assay detect?
- L. Why do we need to boil the sample before performing the VIDAS assay?
- M. Which organism is most likely to cause a VIDAS false positive result?

6.2.2. *Listeria monocytogenes*

6.2.2.1. Objective

- A. Evaluate what types of samples may be candidates to be analyzed for *Listeria monocytogenes*.
- B. Examine sample matrices and determine appropriate enrichment procedures.
- C. Choose appropriate methodology involved in the detection of *Listeria monocytogenes*.
- D. Differentiate biochemical characteristics between *Listeria monocytogenes* and other *Listeria spp.*
- E. Develop skills in the use of rapid method test kits for the detection and differentiation of *Listeria* species including but not limited to ABI-3500, VIDAS, VITEK, RT-PCR, and MICRO-ID.
- F. Be able to setup and interpret a MPN and direct plate method for *Listeria monocytogenes* enumeration.

6.2.2.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once

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the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.

- B. Read Bacteriological Analytical Manual (BAM) Chapter 1, [Food Sampling and Preparation of Sample Homogenate](#)
- C. Read Bacteriological Analytical Manual (BAM) Chapter 10, [Listeria](#).
- D. Read Rapid method AOAC Official Methods for *Listeria* in Foods
- E. Using BAM/AOAC methods, analyze the minimum number of training samples for *Listeria* (Basic/Tier 1). Use foods or feed matrices that require different subsampling techniques.
- F. The new hire (either through training sample or individual demonstration) shall have the opportunity to prepare at least one 5-sub composite.
- G. Screen sample using VIDAS AOAC method (or other routinely used screening methods) for *Listeria*.
- H. The new hire (through either training samples or individual demonstration) shall inoculate or at a minimum discuss typical *Listeria* species including at least 4 different types
- I. The trainer shall ensure that the trainee understands the biochemical reactions for typical *Listeria* species and understands which can be ruled out and which must continue through confirmatory testing.
- J. Identify isolates using conventional biochemicals and become familiar with the various rapid methods referenced in the BAM and utilized within the local laboratory (VITEK 2, MICRO ID, API listeria, and or Real-time PCR).
- K. Perform *Listeria* serological testing including type 1 and 4.
- L. The trainer shall ensure that the trainee understands the basic concepts and demonstrates how to set up and perform MPN testing of confirmed positive samples.

6.2.2.3. Questions

- A. What is unusual about the appearance of the motility of *Listeria*?
- B. At what high and low temperatures will *Listeria* grow?
- C. Describe the CAMP reactions of different *Listeria* species. What do the letters "CAMP" stand for in the CAMP test?

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- D. Why is *Rhodococcus equi*, and not another *Rhodococcus* species used in the CAMP test?
- E. Describe the symptoms and onset time of *Listeria* food borne disease.
- F. Describe the appearance of typical *Listeria monocytogenes* colonies on selective plating media including but not limited to Oxford, Palcam and chromogenic agars?
- G. Describe how acid production from various carbohydrates (mannitol, rhamnose, xylose) typically differs between *Listeria monocytogenes* and *Listeria innocua*.
- H. What role does the addition of selective agents in pre-enrichment and enrichment media play?
- I. Name two β -hemolytic *Listeria* spp and two non β -hemolytic *Listeria* spp.

6.2.3. Escherichia coli

6.2.3.1. Objective

- A. Enumerate and identify *E. coli* in foods.
- B. Familiarize the trainee with the different pathogenic strains of *E. coli*.
- C. Understand the MPN technique and how it is calculated.

6.2.3.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) Chapter 1, [Food Sampling and Preparation of Sample Homogenate](#)
- C. Read Bacteriological Analytical Manual (BAM) Chapter 4, [E.coli](#).
- D. Using BAM/AOAC methods, analyze the minimum number of training samples for *E.coli* (Basic/Tier 1). Use foods or feed matrices that require different subsampling techniques.
- E. The new hire (either through training sample or individual demonstration) shall have the opportunity to prepare at least one 10-sub sample.

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- F. Screen sample using BAM method and coli complete for *E. coli*.
- G. The new hire (through either training sample or individual sample demonstration) shall inoculate media with typical *E. coli*.
- H. The trainer shall ensure that the trainee understands the biochemical reactions for both typical and atypical *E. coli* and understands which can be ruled out and which must continue through confirmatory testing.
- I. Identify isolates using conventional biochemicals and become familiar with the various rapid methods referenced in the BAM and utilized within the local laboratory (VITEK 2, VITEK MS, and or IMViC).
- J. The trainer shall ensure that the trainee understands the basic concepts and demonstrates how to set up MPN testing of confirmed positive samples.
- K. Analyze or discuss analysis of samples of oysters or clams using the "Shellfish Method".
- L. Analyze or discuss analysis of finished product shelled walnuts or pecans using the "Tree Nut Method".
- M. The trainer will discuss the methods used to determine *E. coli* pathogenicity.
- N. Discuss and provide examples for MPN chart exercises.
- O. Review and discuss [BAM Appendix 2: Most Probable Number from Serial Dilutions](#)

6.2.3.3. Questions

- A. Describe different types of *E. coli* that cause food borne disease. List methods used for their determination.
- B. Is there an acceptable way to minimize foaming when shellfish are homogenized?
- C. What percent foam is aspirated when pipetting sample from homogenate? Would this affect the results?
- D. Describe the bottled water definition and how one would initiate analysis.
- E. Describe how a Gram stain is performed and the meaning of the results.
- F. Describe the quadrant streaking technique to obtain isolated colonies.
- G. What is the IMViC pattern for *E. coli*?
- H. What does a typical *E. coli* isolate look like on L-EMB?

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- I. When is it appropriate to use LST-MUG? How does LST-MUG work?
- J. What time period is allowable between sample shaking and inoculation of tubes?

6.2.4. Enterohemorrhagic *E.coli*

6.2.4.1. Objective

- A. Isolate and identify *E.coli* O157:H7 and other non-O157 Shiga-toxin producing *E. coli* (STEC) from food samples.
- B. Introduce PCR assay for detection of Shiga-like toxin genes (stx1 and stx2) and the WZY gene and applications for food enrichment screening and as part of the isolate confirmation.
- C. Examine the challenges of recovering *E.coli* O157:H7 and non-O157 STEC from foods.
- D. Understand the need for complete STEC isolate molecular characterization to determine potential health risk.
- E. Familiarize the trainee with the different pathogenic strains of *E. coli*.

6.2.4.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) chapter 4A, [Diarrheagenic *Escherichia Coli*](#) online.
- C. Using BAM methods, analyze the minimum number of training samples for *E.coli*, *E.coli* O157:H7 and if available non-O157 STEC (Basic/Tier 1). Use foods or feed matrices that require different subsampling techniques and pre-enrichment broths.

6.2.4.3. Questions

- A. Are all STEC also Enterohemorrhagic *E.coli* (EHEC)?
- B. Do all *E.coli* strains ferment sorbitol?
- C. Why is tellurite cefixime sorbitol MacConkey agar (TCSMAC) particularly useful for the isolation of *E. coli* O157:H7?
- D. Are all STEC hemolytic?

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- E. Why is it important to use multiple differential and selective agars to recover *E. coli* O157:H7 and other non-O157 STEC?
- F. Explain why the PCR to confirm the presence of shiga-toxin genes must be done as part of the isolate confirmation process even if the PCR was used during the initial screening of the food enrichment.
- G. Why is it important to have confirmed STEC isolates further characterized for complete virulence profile and serotype by methods such as microarray or whole genome sequencing (WGS)?

6.2.5. Staphylococcus aureus

6.2.5.1. Objective

- A. Analyze foods for *S. aureus* using both the MPN and direct plating techniques.
- B. Identify and differentiate coagulase positive versus coagulase negative *staphylococci*.
- C. Differentiate biochemical characteristics between *Staphylococcus spp.*
- D. Introduce ELISA techniques for toxin detection.

6.2.5.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) Chapter 1, [Food Sampling and Preparation of Sample Homogenate](#)
- C. Using BAM methods, analyze the minimum number of training samples for *Staphylococcus aureus* (Basic/Tier 1). Use foods or feed matrices that require different subsampling techniques.
- D. Identify isolates using conventional biochemicals and become familiar with BAM specific chapter requirements
- E. The trainer shall ensure that the trainee understands the basic concepts and demonstrates how to set up MPN testing of confirmed positive samples.
- F. Read Bacteriological Analytical Manual (BAM) chapter 12, online at: [Staphylococcus](#)

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- G. Test or discuss one spiked food for staphylococcal enterotoxin using an ELISA based assay (Vidas SET). If a test kit is not found, the trainer is responsible for explaining the theory and principals involved.

6.2.5.3. Questions

- A. Describe the symptoms and onset time of staphylococcal food borne disease.
- B. Describe the difference between intoxication and infection. Which one is associated with *S. aureus*?
- C. Which ingredient/s of Baird Parker medium help injured organisms grow?
- D. What are typical observations of coagulase positive *Staphylococcus aureus* when plated on Baird Parker medium? What is indicated by the presence or absence of a halo around an isolated colony on Baird Parker medium?
- E. Name the staphylococcal enterotoxin types and tell which the most common cause of food borne disease is.

6.2.6. Coliforms

6.2.6.1. Objective

Analyze food and water for coliforms and fecal coliforms

6.2.6.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) Chapter 1, [Food Sampling and Preparation of Sample Homogenate](#)
- C. Read Bacteriological Analytical Manual (BAM) Chapter 4, [Coliforms](#).
- D. Using BAM method, analyze the minimum number of training samples for Coliform and fecal coliforms (Basic/Tier 1). Use foods or feed matrices that require different subsampling techniques.
- E. The trainer shall ensure that the trainee understands the biochemical reactions for both typical and atypical and understands which can be ruled out and which must continue through confirmatory testing.

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F. The trainer shall ensure that the trainee understands the basic concepts and demonstrates how to set up MPN testing of confirmed positive samples.

G. Use foods prepared for *E. coli* analysis above for the coliform analysis.

H. Read and discuss methodology for water test specific to interstate travel program using Standard Methods for Water and Wastewater, current edition.

6.2.6.3. Questions

A. Why is sodium thiosulfate added to jars used to collect water samples? (See Investigations Operations Manual (IOM), current edition.)

B. Explain the difference between coliforms and fecal coliforms.

C. What organisms are considered coliforms?

6.2.7. Aerobic Plate Count

6.2.7.1. Objectives

A. Training should be consistent with methodology utilized in the home laboratory.

B. To analyze foods for number of aerobic organisms that grow at 35°C.

C. Demonstrate effective preparation of decimal dilutions from food homogenate, milk, cosmetic or product rinse.

D. Apply conventional pour plate technique.

E. Apply spiral plate count (SPLC) method.

F. Distinguish significant figures when calculating APCs or SPLCs.

G. Calculate reporting results for APCs in common and uncommon cases.

H. Utilize proper aseptic technique and quality control principles for conventional or SPLC methodology.

I. Apply proper plating procedure.

J. Implement proper sterility controls.

6.2.7.2. Assignment

A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and

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independently perform the analysis, the trainee will be issued a series of training samples.

- B. Read Bacteriological Analytical Manual (BAM) Chapter 1, [Food Sampling and Preparation of Sample Homogenate](#)
- C. Using BAM method, analyze the minimum number of training samples for plate count agar (Basic/Tier 1). Use foods or feed matrices that require different subsampling techniques.
- D. The trainer shall ensure that the trainee understands the growth parameters and common techniques used to establish proper counting skill.
- E. The trainer shall ensure that the trainee understands the basic concepts and demonstrates how to set up an APC samples.
- F. Read Bacteriological Analytical Manual (BAM) chapter 3, online: [APC](#) .
- G. Analyze or discuss at least four foods for APC/g or APC/ml. The trainee will report results in correct significant figures.

6.2.7.3. Questions

- A. Name foods that may have natural high counts. Name foods that should have low counts.
- B. Why are we interested in an APC count?
- C. Why do "spreaders" sometimes form when doing an APC? At the air-agar interface? At the agar-glass interface?
- D. How long and at what temperature can one "thaw" a frozen food? Discuss.
- E. Will there be a change to APC results with repeated freezing and thawing of product?
- F. What is the proper procedure for manually mixing dilution blanks?
- G. At what temperature should pour plates be dispensed?
- H. How should the analyst interpret plates with more than 250 colony-forming units (CFUs)?
- I. How should the analyst interpret plates with less than 25 CFUs?

6.2.8. Yeast and Mold Count

6.2.8.1. Objective

- A. Enumerate colonies of yeasts and molds in foods.

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B. Introduce dilution and spread plate techniques.

C. Revisit reporting in significant figures.

6.2.8.2. Assignment

A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.

B. Read Bacteriological Analytical Manual (BAM) Chapter 1, [Food Sampling and Preparation of Sample Homogenate](#)

C. Read Bacteriological Analytical Manual (BAM) Chapter 18, [Yeast and molds](#).

D. Using BAM method, analyze at least a minimum number of training samples for yeast and mold samples. Use foods or feed matrices that require different subsampling techniques.

E. The trainer shall ensure that the trainee understands the biochemical, morphically, or growth differences between yeast and mold.

F. The trainer shall ensure that the trainee understands the basic concepts and demonstrates how to perform Yeast and mold testing of samples.

G. Read ORA Lab Manual Volume III, Section 4.

H. Discuss and/or perform tabletop exercise for calculating APC count. The trainee will report results in correct significant figures.

6.2.8.3. Questions

A. Why are we interested in yeast and mold counts?

B. What is the medium of choice for yeast and mold analysis?

C. What medium is especially useful for analyzing samples containing "spreader" molds?

D. What agent or agents are added to the agar to inhibit bacterial growth?

E. Why should the plates be left undisturbed until the incubation period is complete?

6.3. Intermediate (Tier 2)

The microorganisms listed in this section represent the intermediate microbiology analyses performed in the ORA Food and Feed Microbiology

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labs. The analyses of some of the microorganisms in this section may need to be performed in laboratories that have more experience or specialize in the analysis of such organism. Additionally, analysts performing the analyses of these microorganisms require advanced analytical technique and ability to analyze complex data. At minimum, the analyst should be proficient in analyzing microorganisms under the basic microbiology section but does not necessarily have to be Level 1 Certified.

Although proficiency or competency in analyzing microorganisms under this section is not required to obtain Level 1 Certification, it is recommended for analysts to familiarize themselves with analyzing these complex microorganisms and obtain proficiency or competency. On-the-job training and successful completion of training for this section should be defined by local laboratory procedure. Exercises/Assignments in this tier are intended to give introduction to the trainee of the concepts and methodology. Some sections may provide additional details for sample training.

6.3.1. Vibrio

6.3.1.1. Objective

- A. Recover and identify *Vibrio cholerae*, *V. vulnificus* and *V. parahaemolyticus*.
- B. Introduce polymerase chain reaction (PCR) techniques.

6.3.1.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) chapter 9, [Vibrio](#)
- C. Read Bacteriological Analytical Manual (BAM) chapter 28, [Detection of Enterotoxigenic *Vibrio cholerae*](#)
- D. It is suggested to examine at least one oyster sample for *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*. The trainer will spike the sample with various *Vibrio spp.*
- E. It is suggested to examine one food for toxigenic *V. cholerae* using the PCR method.

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6.3.1.3. Questions

- A. Describe the food borne illness and onset times of the *Vibrio spp.* that were studied.
- B. What are the advantages and disadvantages of using a PCR method?
- C. Why is NaCl added to media used for *Vibrio spp.*?
- D. Why do some methods enumerate the organism and other methods only check for the presence of the organism?
- E. What are the characteristics of *V. mimicus*? What are the similarities and differences of this organism compared to *V. cholerae*?
- F. What are the major factors in the pathogenesis of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus*?

6.3.2. Bacillus cereus

6.3.2.1. Objective

- A. Recover and identify *B. cereus* group organisms from foods.
- B. Differentiate biochemical characteristics between various *Bacillus spp.*

6.3.2.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of proficiency samples.
- B. Read Bacteriological Analytical Manual (BAM) chapter 14, online: [B. cereus](#).
- C. The trainer should spike dry foods with *B. cereus*. In addition, the trainer should spike foods with *Bacillus* species that will challenge the student to differentiate between the species.
- D. Using BAM method, analyze training samples for *B. cereus*. Use foods or feed matrices that require different subsampling techniques.
- E. The new hire (either through training sample or individual demonstration) shall have the opportunity to prepare enough sub samples to feel confident about the set-up procedure.
- F. The trainer shall ensure that the trainee understands the biochemical, morphological, or growth differences between the different species.

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6.3.2.3. Questions

- A. Describe the symptoms and onset times for the two types of *B. cereus* food borne diseases. What foods have been implicated in each type?
- B. Can *B. thuringiensis* produce diarrheal antigens? Diarrheal disease?
- C. The symptomatic profile of emetic type of food poisoning produced by some strains of *B. cereus* most closely mimics that of *Clostridium pefringens* or *Staphylococcus aureus*?
- D. The toxins of *B. cereus* most commonly associated with food poisoning are?
- E. Explain the purpose for each ingredient used in MYP agar and how *B. cereus* can be interpreted on MYP agar.
- F. When is the Plate Count Method recommended? When is Most Probable Number (MPN) recommended?
- G. When interpreting test results (motility, hemolytic activity, plating characteristics, and crystal production), what are typical results for each pertaining to *B. cereus*?

6.3.3. Campylobacter

6.3.3.1. Objective

- A. Recover and identify *Campylobacter jejuni* and *C. coli* from foods and water.
- B. Introduce microaerobic culturing techniques.
- C. Introduce the use of rapid methods for presumptive identification of *Campylobacter*.

6.3.3.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) chapter 7, online: [BAM Ch7 Campylobacter](#)

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- C. It is suggested to analyze different products such as water, milk/dairy, and a poultry product for *Campylobacter* spp. Trainer will spike samples with *C. jejuni* and *C. coli*.

6.3.3.3. Questions

- A. Describe the symptoms and onset time of *Campylobacter* food borne disease.
- B. Describe methods used to obtain microaerobic conditions.
- C. What are the oxygen-quenching compounds added to *Campylobacter* media?
- D. Which biochemical test is used to differentiate between *C. jejuni* and *C. coli*?
- E. What are the advantages and disadvantages of using rapid methods such as the Dryspot Campy Test or Alert for *Campylobacter*?

6.3.4. Yersinia enterocolitica

6.3.4.1. Objective

- A. Familiarize the trainee with the public health significance of identifying *Yersinia* species in foods.
- B. Familiarize the trainee with growth characteristics of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*.
- C. Understand how to enrich and identify *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in foods.

6.3.4.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) Chapter 1, [Food Sampling and Preparation of Sample Homogenate](#).
- C. The trainer shall ensure that the trainee understands the biochemical reactions for both typical and atypical and understands which can be ruled out and which must continue through confirmatory testing.

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- D. Read Bacteriological Analytical Manual (BAM) chapter 8, [Yersinia enterocolitica](#)
- E. The trainer will discuss the importance of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* as it relates to public health and food consumption.
- F. The trainee will enrich food samples for the identification of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. The trainee will identify typical *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* on MacConkey agar plates.
- G. The trainee will identify typical *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* on Yersinia Selective Agar (YSA) plates (also referred to as CIN agar plates).
- H. The trainee will identify typical *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* on LAIA slants.
- I. The trainee will perform confirmatory and biochemical testing on typical *Yersinia* colonies.

6.3.4.3. Questions

- A. Describe the symptoms and onset time of *Yersinia enterocolitica* illness.
- B. Does *Yersinia* grow and survive during refrigerated storage?
- C. What enrichment broths and selective media are used to culture *Yersinia enterocolitica*?
- D. What enrichment broths and selective media are used to culture *Yersinia pseudotuberculosis*?
- E. Describe the biochemical characteristics of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*.
- F. What is the relationship of plasmids and *Yersinia*?
- G. What types of tests are used to determine pathogenicity?

6.3.5. Clostridium perfringens

6.3.5.1. Objective

- A. Recover and identify *C. perfringens* from foods.
- B. Display anaerobic culture techniques.
- C. Differentiate biochemical characteristics between various *Clostridium* spp.

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- D. Effectively utilize Reversed Passive Latex Agglutination (RPLA) test kit for the detection of enterotoxin.

6.3.5.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. [Read Bacteriological Analytical Manual \(BAM\) Chapter 16: *Clostridium perfringens*.](#)
- C. The trainer should spike food with *C. perfringens*. The trainee will analyze the samples by plate count method using Tryptose-Sulfite-Cycloserine (TSC) without egg yolk and the alternative plating method using TSC with egg yolk. The trainee should also complete all presumptive confirmation and completed confirmation testing. If possible, the trainee should also be introduced to the RPLA enterotoxin test kit.

6.3.5.3. Questions

- A. Describe the symptoms and onset time for *C. perfringens* food borne disease.
- B. How would a microbiologist test the organism for toxin production?
- C. Tell when a microbiologist would use each enumeration method.
- D. How do anaerobe jar commercial systems produce anaerobic conditions? How can you tell if it worked?
- E. Describe stormy fermentation and how to test *C. perfringens* for stormy fermentation.
- F. Describe key characteristics of *C. perfringens* including motility, gram-reaction, nitrate reduction, and lecithinase activity.

6.3.6. Clostridium botulinum

6.3.6.1. Objective

- A. Present theory and methodology on recovery of *C. botulinum* from food.
- B. Introduce the method for detection of *C. botulinum* using the BAM q-PCR method

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- C. Introduce the theoretical concepts of the mouse bioassay.
- D. Discuss immunologic and other methods published and/or under collaboration to replace the use of animals.

6.3.6.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. [Read Bacteriological Analytical Manual \(BAM\) Chapter 17: *Clostridium botulinum*](#)
- C. Analyze samples using the BAM Chapter 17 for the detection of *Clostridium botulinum*.
- D. Trainer should spike food with various *Clostridium* species.

6.3.6.3. Questions

- A. What are the symptoms and onset time of botulism?
- B. What is wound botulism and infant botulism?
- C. Name the different types of toxins and which have been implicated in human botulism.
- D. Describe how the MLD is calculated.
- E. What is the difference between preformed and formed toxin?

6.3.7. Alkaline Phosphatase

6.3.7.1. Objective

- A. Discuss the reason we test for alkaline phosphatase in dairy products and the reasons a dairy product may contain alkaline phosphatase.
- B. Discuss the methods used to test for alkaline phosphatase in dairy products and the chemical reactions occurring at various steps in the method.
- C. Detect levels of alkaline phosphatase in dairy products using both the BAM screening method and the AOAC confirmation method.

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6.3.7.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) chapter 27, online: [BAM CH 27 Alkaline Phosphatase in Cheese](#)
- C. Analyze cheese samples for alkaline phosphatase. The trainer will supply samples with known levels of alkaline phosphatase. Samples with violative alkaline phosphatase levels will be confirmed with the AOAC confirmation method.

6.3.7.3. Questions

- A. Why is the presence of alkaline phosphatase determined in dairy products?
- B. What is the reason for heating the control blank?
- C. Why is a buffer used in the analysis?
- D. In the AOAC confirmation method, why are different buffer and precipitant concentrations used for different cheeses?
- E. What parameters need to be controlled during analysis for the phenol to be liberated from the disodium phenyl phosphate substrate?
- F. To what compound is the alkaline phosphatase enzyme activity proportional?
- G. Describe the reaction that takes place in order for phenol to be measured colorimetrically?

6.3.8. Canned Food and Canned Seam Examination

6.3.8.1. Objective

- A. Learn can classification, progressive decomposition, headspace gas, culturing, pH, water activity, direct smear, odor and appearance, etc.
- B. Distinguish between an Acidified and Low Acid Canned Food.
- C. Familiarize the trainee with other types of hermetically sealed containers, i.e., jars & pouches.

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- D. Discuss 21CFR, Parts 113 and 114 (Low Acid Canned Food and Acidified Food Regulations).

6.3.8.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) [Chapter 21A](#), [Chapter 21B](#), [Chapter 22A](#), [Chapter 22B](#), [Chapter 22C](#), [Chapter 22D](#), online
- C. Read [AOAC Official Method 978.18 Water Activity of Canned Vegetables](#) and [AOAC Official Method 981.12 pH of Acidified Foods](#).
- D. Read [SOP-000620](#) Water Activity of Foods Using AquaLab 4TE
- E. Read [SOP-000621](#) pH Analysis of Food Products
- F. Read related Compliance Programs [7303.003 Import Acidified & Low-Acid Canned Foods](#) and [7303.803\(A\) Domestic Acidified & Low-Acid Canned Foods](#).
- G. Perform a complete canned food analysis, comprising each individual test on a representative number of low acid canned foods.
- H. Perform a pH and water activity analysis.
- I. Perform a complete canned seam tear down analysis.

6.3.8.3. Questions

- A. What is a Flipper, Springer, Hard Swell, and Soft Swell?
- B. What is flat-sour spoilage? What bacteria can cause this?
- C. Does microbial spoilage of a food always result in a swollen can? Explain.
- D. Why are certain canned food media incubated at 55 degrees C.?
- E. Explain the microbiology and physical attributes of each of the following: under processing, leaker spoilage, hydrogen swell, overfill.
- F. Explain the construction of a metal can. Name the components of the double seam.
- G. Explain water activity and its significance in an acidified and low acid canned food.

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6.3.9. *Cronobacter* spp. (NEW)

Cronobacter is a gram-negative rod within the family Enterobacteriaceae. Reports of *Cronobacter* infection usually occurs in infants, however clinical cases in adults have been described as well. While a reservoir for *Cronobacter sakazakii* is unknown, evidence suggests powdered infant formulas as a vehicle for infection. The method for detection and isolation of *Cronobacter* spp. is found in BAM Chapter 29. This method includes a real-time PCR for rapid screening and a cultural method for isolation/confirmation.

6.3.9.1. Objective

- A. Become familiar with *Cronobacter* spp. and understand their significance as a public health concern.
- B. Become familiar with FDA validated methods to detect *Cronobacter* spp. in food.

6.3.9.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Consult the following website for more information: BAM Chapter 29. [Cronobacter](#)
- C. Perform analysis of infant formula samples for detection, isolation, and confirmation of *Cronobacter* spp. using the appropriate FDA method and successfully interpret and report results.

6.3.9.3. Questions

- A. What species belong to genus [Cronobacter](#) and which species have been involved in human illnesses?
- B. What is the morphology of typical colonies on chromogenic agars?
- C. Can the method be performed without running the qPCR assay?

6.3.10. Cosmetic Analysis

6.3.10.1. Objective

- A. Gain theoretical knowledge of cosmetic microbiology.

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- B. Analyze cosmetic products for microorganisms that may cause injury to consumers, including pathogenic bacteria and yeast and mold (e.g. bacterial contamination of eye cosmetics).
- C. Familiarize analysts with the yeast and mold count.
- D. Familiarize analysts with preservative systems employed in multiple use eye area cosmetics.
- E. Familiarize analysts with Gram negative, non-fermenter identification.
- F. Familiarize analysts with opportunistic pathogens present in eye area cosmetics.

6.3.10.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read Bacteriological Analytical Manual (BAM) chapter, online: [Cosmetic](#)
- C. Trainer should spike and discuss different matrices and ensure trainee should receive spiked subs with *Pseudomonas*, other Gram-negative rods, Gram positive rods and cocci, and yeast.

6.3.10.3. Questions

- A. Describe the sample preparation techniques for liquids and semi powders; solids and powders; preparations with petroleum base; aerosols of powders and liquids; and aerosols of soaps and other foamy liquids.
- B. What is the purpose of dilutions and other added ingredients?
- C. How do *Pseudomonas* and *Klebsiella* differ biochemically? What characteristics clearly separate them?
- D. If, biochemically, a culture was indicated to be *Pseudomonas* but it did not produce a fluorescent yellow or blue pigment, would a microbiologist still consider the culture as *Pseudomonas*? Explain.
- E. Which organisms are considered pathogenic in the eye area? On the skin?

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6.3.11. Nutritional and Dietary Supplements (NEW)

6.3.11.1. Objective

- A. Determine the presence of viable aerobic microorganisms and pathogenic microorganisms in pharmaceuticals, from raw materials to finished product.
- B. Practice aseptic technique when handling isolates.
- C. Introduce the use of Official Monographs from the USP: (current edition)
 - 1) <2021> Microbial Enumeration Test-Nutritional and Dietary Supplements
 - 2) <2022> Microbiological Procedure for Absence of specified Microorganisms- Nutritional and Dietary Supplements
 - 3) <2023> Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements
- D. Introduce the following concepts: inhibition, inactivating agents, and neutralization of inhibiting substances.

6.3.11.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. [Read USP](#)<2021, 2022 and 2023> (current edition).
- C. Analyze or discuss a solid, fluid, water-immiscible fluid, and fluid specimen in aerosol form for Total Aerobic Microbial Count, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella*, *Escherichia coli*, Molds, and Yeast Counts or according to specifications of Official Monographs.
- D. Perform preparatory test.

6.3.11.3. Questions

- A. What are the controls used in this experiment?
- B. What are some ways to neutralize inhibitory substances?
- C. What is Purified Water?

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D. How does someone determine what microbial test to run on products?

E. What is the preparatory test?

6.4. Advanced (Tier 3)

The analyses and subsequent identification of microorganisms in this section may require more advanced training and/or understanding of complex FDA methodology. As a result, analyses/organisms in this section are categorized as Tier 3 throughout this document. Analyses/organisms that fall under this category include, but are not limited to the following: Viruses, *Cyclospora*, Select Agents, and Whole Genome Sequencing.

Familiarity with Tier 3 analyses/organisms is recommended for new hire Microbiologists, however the demonstration of competency in these areas are not required to obtain a Level 1 Certification. If an analyst is interested/selected to perform any of these advanced topics, competency will have to be established prior to working on regulatory analysis in these areas. Demonstration and documentation of competency in these areas may be determined by the individual laboratory, however the following should be included as a minimal requirement: completion of official OTED training course (if one is available), comparable on-the-job training by a competent and certified trainer, and/or acceptable outcome on a proficiency/audit examination. Exercises/Assignments in this tier are intended to give introduction to the trainee of the concepts and methodology. Some sections may provide additional details for sample training.

6.4.1. Viruses

6.4.1.1. Objective

A. Become familiar with viral pathogens capable of causing foodborne illness.

- 1) Viruses are infectious microorganisms, much smaller than most bacteria, which cannot grow or reproduce apart from a living cell. They consist of genetic material, either DNA or RNA, encased in a protein coat known as the capsid. Several viruses have been associated with foodborne illness including Rotaviruses, Noroviruses and Hepatitis A. Viruses are difficult to propagate in culture but are readily detected using molecular methods such as PCR and qPCR.
- 2) Human enteric viruses can be transmitted by water, food, fomites, and by human contact. They typically have a low infectious dose which makes them an immediate public health concern. The infectious dose can be a little as one to ten virions

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with a secondary attack rate of 50%. Enteric viruses are the leading cause of foodborne infectious diseases in the United States and of the 9.4 million annual estimated cases of known foodborne illnesses, 58% are attributed to human noroviruses. Due to its severity, hepatitis A virus (HAV) is also a concern.

- 3) Norovirus (NoV) is a single-stranded, polyadenylated RNA virus and can be separated into seven genogroups (GI, GII, GIII, GIV, GV, GVI, and GVII) based on the sequences of the RNA polymerase and capsid regions. Genogroups I, II, and IV are known to infect humans, with norovirus GII.4 being the most common strain identified.
- 4) Hepatitis A is a non-enveloped RNA virus 27 to 32 nm in diameter. It has an icosahedral symmetry and belongs to the genus Hepatovirus of the Picornaviridae family. Infections with HAV can produce effects that range in severity from asymptomatic to death from fulminant hepatitis. Infections with HAV are typically self-limiting and do not result in chronic liver disease, however, outbreaks of HAV require considerable amounts of public health resources due to long incubation and shedding period after infection.

B. Become familiar with methods to detect viral food pathogens.

A. Human noroviruses and wild-type hepatitis A virus detected in foodborne outbreaks are non-culturable, therefore detection is based primarily upon RT-PCR assays. Advancements in RT-PCR technology have allowed the recent development of several RT-qPCR assays for the rapid detection and enumeration of norovirus and hepatitis A virus.

B. Molecular detection methods for RNA viruses depend on converting the RNA to cDNA to detect the cDNA by PCR. These molecular detection methods must be sensitive enough to detect small amounts of extracted RNA. Co-extracted inhibitors from the matrix being tested can often cause difficulty in detecting the low levels of virus present in foods. PCR methods employ an internal control in the PCR reactions to enable the analyst to assess the amount of inhibition is present in the PCR reaction. The analysts should be able to assess raw data according to the methods and interpret and report results accordingly.

C. Understand the approaches to detecting viruses in food products.

- 1) The most important approach to detecting viruses from foods is the concentration step because viruses are usually present in low tier amounts. Removing the virus from the food and

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concentrating the virus for molecular level detection is of primary importance. The approach is based on the mechanism by which virus contamination occurred in the food being tested along with the characteristics of the food matrix itself.

- 2) Surface contaminated foods are rinsed at a pH higher than the isoelectric point (pH at which the virus will adsorb to food particles) of the target virus.
- 3) Internally contaminated food is homogenated prior to lower pH adsorption of the virus to the food particle and centrifugation to concentrate the adsorbed virus particles. The sample is treated at a higher pH to elute the virus from the matrix prior to physical separation of the food particles from the elution. Eluted viruses are then concentrated at Ultra high speeds to concentrate the virus. RNA is then extracted from the sample concentrate and tested for virus target RNA.
- 4) FDA enteric virus methods rely on successful detection of a surrogate virus (Murine Norovirus) which is spiked into the sample prior to analysis. Detection of this virus demonstrates that the concentration and extraction of virus was successful.

6.4.1.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Consult the following resources for more information on how these approaches are used for various products:
- C. [BAM Chapter 26: Concentration, Extraction and Detection of Enteric Viruses in Foods](#)
- D. FDA ORA Field Lab Virus Analysis Standard Operating Procedures [SOP-000578](#)
- C. Complete analysis of samples of frozen strawberries, scallops and molluscan shellfish using the appropriate FDA method and successfully interpret and report results. MNV should be successfully detected, PCR data interpretations properly performed, and sample results accurately reported.

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6.4.1.3. Questions

- A. List two ways that viruses differ from bacteria.
- B. What is the genetic material found in viruses?
- C. What process is required before RNA viruses can be detected by qPCR?
- D. How are viruses removed and concentrated from fruit, finfish and molluscan shellfish?
- E. What is the importance of pH in removing viruses from foods and what characteristic of the target virus is important in this approach?
- F. How do you determine if the amount of inhibition is too high to be able to rely on negative PCR results for a given virus target?

6.4.2. Cyclospora (NEW)

Cyclospora cayetanensis is a protozoan parasite responsible for a waterborne and foodborne diarrheal illness called cyclosporiasis. *C. cayetanensis* oocysts are spherical measuring 7.5-10 µm in diameter and are unsporulated and not infective when freshly passed in stools by infected individuals. Therefore, direct fecal-oral transmission cannot occur, and this differentiates *Cyclospora* from another important coccidian parasite, *Cryptosporidium*. After a week or longer in the environment at optimal temperatures, *C. cayetanensis* oocysts sporulate and become infective. *C. cayetanensis* oocysts are not culturable, therefore the application of viability assays is impractical. Methods for the identification of oocysts in food matrices and water by microscopy are available, however these methods are challenging and lack specificity and sensitivity to detect low concentrations of oocysts or differentiate between morphologically identical species of *Cyclospora*. As an alternative, methods for the detection of *C. cayetanensis* in fresh produce (BAM Ch. 19b) and agricultural water (BAM Ch. 19c) using species-specific qPCR have been developed.

6.4.2.1. Objective

- A. Become familiar with *Cyclospora cayetanensis* and understand its significance as a public health concern.
- B. Become familiar with FDA validated methods to detect *C. cayetanensis* in food and water.

6.4.2.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be

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accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.

B. Consult the following websites for more information:

- 1) [BAM Chapter 19b](#). Detection of *Cyclospora cayetanensis* in Fresh Produce using Real-time PCR
- 2) [BAM Chapter 19c](#). Dead-end Ultrafiltration for the Detection of *Cyclospora cayetanensis* from Agricultural Water

C. Perform analysis of fresh produce samples and/or agricultural water samples using the appropriate FDA method. Successfully interpret analytical results based on guidance provided in the appropriate BAM Chapter.

6.4.2.3. Questions

- A. Explain why methods based on microscopic examination cannot be used reliably to detect and characterize *C. cayetanensis* in food and agricultural water.
- B. Explain why person to person transmission of *C. cayetanensis* does not occur.
- C. Explain the main steps included in the process for molecular identification of *C. cayetanensis* in fresh produce by PCR as described in BAM Chapter 19b.
- D. Explain the main steps included in the process for molecular identification of *C. cayetanensis* in agricultural water by PCR as described in BAM Chapter 19c.

6.4.3. Whole Genome Sequencing (WGS) (NEW)

6.4.3.1. Objectives

- A. Understand the basic principles and applications of whole genome sequencing (WGS) of bacterial isolates.
- B. Understand the role and importance of WGS in the regulatory process, including what additional information can be gathered, such as molecular serological data, virulence markers and phylogenetic relatedness to other isolates uploaded to public databases.
- C. Familiarize the trainee with the concept of metadata linked to each isolate.

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- D. Familiarize the trainee with the techniques, procedures and equipment used for WGS analyses.
- E. Introduce the use of public GenomeTrakr databases and tools to track foodborne pathogens.
- F. Introduce the trainee to the importance of the QA/QC tools available and when each tool is required.
- G. Present possible pitfalls and commonly encountered issues during the WGS workflow, from DNA extraction to data upload.

6.4.3.2. Assignment

- A. It is the trainer's primary responsibility to transfer knowledge both practical and in theory related to sample analysis. This may be accomplished through a series of designated training samples. Once the trainer is confident that the trainee can successfully and independently perform the analysis, the trainee will be issued a series of training samples.
- B. Read most recent WGS protocols and SOPs
- C. Extract and quantify DNA from isolates.
- D. Perform DNA library preparation, pooling and diluting for sequencing run.
- E. Operate MiSeq equipment, including starting sequencing run, basic washing, and instrument maintenance.
- F. Create sample sheets and program a run utilizing MiSeq Control Software.
- G. Understand metadata registration and curation processes and how to review isolate status on GenomeTrakr reports and in public databases.
- H. Perform basic QA/QC analysis using MSeq Control Software and Sequence Analysis Viewer.
- I. How to review isolate status on GenomeTrakr Reports.
- J. How to review isolate (BioSample) information on NCBI.
- K. How to compare isolate relatedness using phylogenetic trees in NCBI Pathogen Detector.
- L. Perform basic QA/QC of sequence data and data transfer procedures.

6.4.3.3. Questions

- A. Describe general steps for WGS analysis of bacterial pathogens.

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- B. What is the main difference between DNA extraction procedures for Gram positive and Gram-negative isolates?
- C. Why should index primers be rotated between sequencing runs?
- D. What is the basic sequence coverage requirement for Salmonella, Listeria and E. coli?
- E. How often should you wash your sequencer? What should you wash your machine with?
- F. Which QA/QC tools should you always run? How do you know when you need to utilize additional tools?
- G. What should you do if you suspect an isolate is a result of a contamination event involving a laboratory control or another isolate?
- H. What quality metrics should you look for during a sequencing run?

6.4.4. Select Agents

6.4.4.1. Objective

- A. Introduce the concept that select agents are biological organisms or chemical toxins capable of causing great harm or potentially lethal disease. In contrast to traditional foodborne pathogens that typically cause self-limiting gastrointestinal illness, exposure to microbiological select agents can result in serious illness or death.
- B. Understand the difference between the different Biosafety Levels, and the special requirements for BSL3 laboratories.
- C. Become familiar with safety requirements associated with the analysis of select agents in foods that are typically analyzed in a BSL3 laboratory.

The website: <http://www.cdc.gov/od/sap/docs/salist.pdf> lists the select agents and toxins covered by federal regulation. Some FDA laboratories that have BSL-3 registered laboratory space (herein this section referred to as “Laboratory facilities”) have received specialized training on working with microbiological select agents focusing on those microorganisms thought to have the most potential to be used as deliberate food contaminants. This specialized training is based on the guidelines presented in the current edition of the Biosafety in Microbiological and Biomedical Laboratories (BMBL) written by the Centers for Disease Control and Prevention and the National Institutes of Health and covers several components critical to the safe and secure handling of select agents including:

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- 1) Physical containment - Laboratory facilities must effectively contain select agents and the aerosols that could be produced during analytical manipulations. This is accomplished through design specifications of the facility and use of specialized equipment such as Biological Safety Cabinets; sealed centrifuge rotors and buckets and transport boxes.
- 2) Personal Protective Equipment (PPE) includes closed front laboratory gowns or jump suits, double gloves, shoe and hair covers and respiratory protection (N95 respirators or PAPRs).
- 3) Biosafety practices are designed to contain aerosols and prevent release of the select agents.
- 4) Methods to Identify Select Agents in various food matrices. Recovering select agents from foods can present unique challenges depending on the food type. Care must be exercised to contain aerosols during processing of the food prior to enrichment or biochemical analyses including preparation of nucleic acid templates for use in PCR and qPCR analyses.

D. Become familiar with the federal regulations governing select agents.

The Centers for Disease Control and Prevention (CDC) regulates the possession, use, and transfer of select agents and toxins that pose a severe health threat to the public. Laboratories must obtain a Select Agent Permit (SAP) from the CDC to handle these organisms.

Laboratories planning on working with or transferring microorganisms that are considered plant or animal pathogens must obtain a permit from the USDA Animal and Plant Health Inspection Service (APHIS).

All laboratories must comply with federal regulations (42 C.F.R. Part 73, 7 C.F.R. Part 331, and 9 C.F.R. Part 121) regarding biosafety and security if they anticipate working with select agents.

6.4.4.2. Assignment

Read and discuss the following websites and C.F.R. sections for more information on select agents:

- 1) www.cdc.gov/od/sap/
- 2) <http://www.cdc.gov/biosafety/publications/bmbI5/index.htm>
- 3) 42 C.F.R. Part 73
- 4) 7 C.F.R. Part 331
- 5) 9 C.F.R. Part 121

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6.4.4.3. Questions

- A. What is a select agent?
- B. Name four areas of training necessary before handling select agents.
- C. Which agency regulates the use, possession and transfer of select agents capable of posing a serious public health threat?
- D. List the PPE required to work with select agents.
- E. What work practices are to be in place when working in a Biological Safety Level (BSL)- laboratory? For BSL-3?
- F. Does the laboratory need to register with the CDC when working with regulated select agents?

7. Document History

Revision #	Status* (D, I, R)	Date	Author Name and Title	Approving Official Name and Title
1.1	I	10/01/2003	LMEB	LMEB
1.3	R	01/23/2008	LMEB	LMEB
1.4	R	02/06/2012	LMEB	LMEB
1.5	R	03/14/2013	LMEB	LMEB
1.6	R	05/02/2014	LMEB	LMEB
1.7	R	08/25/2014	LMEB	LMEB
02	R	04/27/2021	LMEB	LMEB
03	R	REFER TO QMIS	LMEB	LMEB

* - D: Draft, I: Initial, R: Revision

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8. Change History

Revision #	Change
1.2	<ul style="list-style-type: none"> • Table of Contents – deleted 2.13.3; added 2.15 • Added Section 2.1 D and 2.3 D.; revised 2.4 B.; revised 2.13, 2.13.1 C., and 2.13.2 C.
1.3	<ul style="list-style-type: none"> • Section 2.6 Viruses added • Section 2.7 Select Agents added • Section 2.17 Document/Change History added Sections 2.6 – 2.14 renumbered to 2.8 to 2.16
1.4	2.1 A. – added sentence to end of paragraph 2.1 B. – revised first paragraph 2.1 D. 2. – changed third bullet to serial dilutions 2.2 B.1. – removed “use” from end of sentence 2.2 D. – 1. updated; 2. and 3. updated weblink; added 7. 2.3 B.4. – updated reference 2.3 C. – revised 2., 8., 9., 10., 11., 14., and 16. 2.4 B.1 – updated hyperlink 2.5.1 C. – revised 2., 8., and 13 2.5.2 B. – revised third sentence in second paragraph 2.4.2 C. 6. – removed “medium” 2.5.3 B.1. – revised second sentence 2.5.3.1. C. 3. – changed media to medium 2.5.4 C. 3. and 4. – changed agar to medium 2.5.5 B.2 – removed reference 2.5.6 C.5. – revised question 2.5.7 C – 2. analyses changed to analysis; 5. revised question 2.5.12 B. – defined TSC in last paragraph; removed BAM method reference 2.5 13. B. – second paragraph deleted

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Revision #	Change
	2.6 A.2. – deleted first sentence 2.7 – revised third line 2.7 A.2. – added “USDA” 2.7 B. – second and fourth link deleted; third link updated 2.11 A.2. – changed to Acidified and Low Acid Canned Food 2.11 B. – revised and updated links 2.11 C.7. – changed to acidified and low acid canned food 2.13 B.4. – added <71> 2.13 C. 1 – revised second question 2.14 A.1. and 2. – revised 2.14 B – added <61> 2.15 – updated IOM reference; updated CFR references; added 21 CFR 111 2.15 C.3. – added “Firm” 2.16 Answer Key – revised questions 2.3 1., 2.3 8., 2.3 10., 2.3 11., 2.3 14., 2.3 16., 2.5.1 2., 2.5.1 13, 2.5.2 6., 2.5.3.1 3., 2.5.4 3., 2.5.4 4., 2.5.6 5., 2.5.7 2., 2.5.9 1., 2.8 1., 2.8 2., 2.9 4., 2.11 7., 2.13.1
1.5	Header – Division of Field Science changed to Office of Regulatory Affairs
1.6	<ul style="list-style-type: none"> • 2.4. A. – added “or electronic” • 2.13. A. 6. – revised • 2.16 Answer Key – revised questions 1.4.4 and 2.13.3
1.7	<ul style="list-style-type: none"> • 2.5.1 – Question 5 –deleted; Question 6 – “species” changed to “serovars” & added “typically”; • Question 8 – “groups” changed to “serovars” • 2.16 Appendix – Answer Key – revised questions 2.5.4, 2.5.6, & 2.5.8
02	<ul style="list-style-type: none"> • Document Reformatted – Outline, numbering and bulleting changed to better organize the document

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Revision #	Change
	<ul style="list-style-type: none"> • Established new Purpose, Scope, Responsibility, Background, References and Procedure sections for this document • Realigned the Procedures section into four subsections: Fundamentals, Basic (Tier 1), Intermediate (Tier 2), and Advanced (Tier 3); and defined each microorganism tier • Subsections 2.13 Sterility of Drugs and Medical Devices, and 2.14 Microbial Limits removed in its entirety; these subsections are covered in Volume IV Laboratory Training, Section 15 Drug and Device Microbiology • Added new subsections for Cronobacter spp., Nutritional and Dietary Supplements, Cyclospora, and Whole Genome Sequencing • Condensed Establishment Inspections subsections 2.15.1 Frozen, Chilled, Prepared Foods; Nutmeats; Shellfish, 2.15.2 Canned Foods into a more generalized Establishment Inspection section
03	<ul style="list-style-type: none"> • Fixed formatting issues • Updated broken links • Updated ORA-LAB.001 to SOP-001064 • Italicized organism names

9. Attachments

List of Attachments

Attachment A - Answer Key	49
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Attachment A - Answer Key

FUNDAMENTALS

Media, Reagent, and Supply Preparation

- A. Which media should not be steam sterilized and why?

Examples of culture media that are not steam sterilized are Selenite Cysteine Broth, Tetrathionate Broth, Bismuth Sulfite and Hektoen Enteric Agars. Autoclaving as well as boiling longer than needed destroys the selectivity of the medium.

- B. Where is agar derived from? What are the special properties of agar that make it well suited as a solidifying agent in culture media?

Once melted, what temperature should agar be kept at to prevent it from solidifying? Agar is the dried mucilaginous substance extracted from various species of algae. The plants are found primarily off the coasts of Japan, China, and southern California. Agar is insoluble in cold water but slowly soluble in hot water to give a viscous solution. A 1% solution melts at 100°C and sets at 35°C to 50°C to a firm gel. Agar should be kept at 55°C to prevent solidifying. Since agar is attacked by relatively few bacteria, it is the most satisfactory solidifying agent for the growth and isolation of bacterial and fungal species.

- C. In which media and buffer preparations is volume particularly critical?

Volume of both buffer and media is important to maintain a proper ratio of product to media or buffer. Volume is particularly critical in preparing buffer solutions intended for use in dilutions for plate counts. Incorrect volume in the buffer blank can result in erroneous plate counts.

- D. Why is pH important in media and buffer preparation?

One of the selective factors in favoring growth of one organism over another is pH. For example, media for detection of *Vibrio spp.*, such as TCBS is at pH 8.6. This high pH favors growth of the *Vibrios* while inhibiting competing organisms. Many media contain indicators which change color on the acid and basic side. The initial pH of the media is neutral with respect to the indicator. Buffer solutions are the initial diluents for many analyses and are to maintain a favorable pH environment for the desired organism.

- E. What are some safety concerns when autoclaving? What PEE should be worn?

Autoclave vessels with vented closures only. Do not use crimped seals. Use only Pyrex glass vessels and other types of autoclavable materials. Use "liquid cycles." No other cycle is safe for liquid sterilization. Follow manufacturer's instructions for proper opening of the door and end of cycle. Do not allow hot bottles to be jolted. This can

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cause hot bottle explosion. Do not move bottles if any boiling or bubbling is present. Analysts are required to wear full-length face shields, water impervious aprons and rubber gloves whenever removing materials from the autoclave.

Safety and Hazardous/Infectious Waste

- A. What are the items of personal protective equipment (PPE), minimally required, in a Biological Safety Level (BSL)-2 biological laboratory?

Each person working in a biological laboratory is minimally required to be using safety glasses with shields (or goggles, face shield, other splatter guards), a protective lab coat, disposable gloves, and protective footwear (closed-toe shoes).

- B. Describe and give examples of food borne disease microorganisms that cause infections, disease, or other health hazards and identify their biosafety levels.

Laboratory access is limited or restricted. Personnel at increased risk for acquiring infection, or for whom infection would have serious consequences, are not allowed in the laboratory or animal rooms. Laboratory doors are kept closed when experiments are in process. Personnel wash their hands after handling microorganisms, when removing gloves, and before leaving the laboratory. Soap and disposable towels are readily found at sinks in the microbiology laboratories. No eating, drinking, chewing gum, smoking, handling medications, handling contact lenses, or applying cosmetics is permitted in the laboratory. All food are stored outside of the laboratory. Mouth pipetting is prohibited. There is a program for handling and disposing of sharps using special sharps' containers. Work surfaces are decontaminated on completion of work and at the end of the day. This usually involves a system of various decontamination sprays, such as bleach, iodine, quaternium ammonium materials, amphyll, etc. Any splashes or spills of viable material are immediately cleaned up with disinfectants that are effective against the organism of concern. Contaminated equipment is to be decontaminated before it is sent for repair, sent for maintenance, placed in surplus, or shipped out of the lab. When placing the equipment in surplus, the equipment is also labeled in accordance with the DHHS requirements for property management to show that the equipment is clean. All cultures, stocks, and other regulated wastes are autoclaved, or otherwise decontaminated, to destroy any viable organisms. All materials for autoclaving are placed in red (or otherwise equivalent) bags that are closed for transport out of the laboratory. These bags are set in leak-proof totes. Any materials to be decontaminated off-site are packaged in accordance with applicable local, state, and federal regulations beforehand. When working with organisms with more hazardous risk, consideration is given to chemically decontaminating the wastes within the laboratory. Insect and rodent control programs are in place. All procedures are conducted in a manner to minimize aerosols. Consideration is given to the degree of hazard when loops are used in flames, centrifugation, opening screw-capped bottles and wet petri dish covers, use of a syringe, needle, and

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septum, streaking plates, pipetting, slide agglutination, etc. Good microbiological techniques, substitution of disposable equipment, and use of primary containment devices such as biosafety cabinets reduce the risk. Laboratory management establishes policies and procedures whereby personnel in the laboratory are informed of the potential hazards and meet special entry requirements, such as immunization. A biohazard sign is posted at the entrance to any laboratory where etiologic agents are used. This label identifies what etiologic agents are used, the biosafety level, any required immunizations, the responsible person and their phone number, what PPE is worn in the laboratory, and any procedures for exiting the laboratory. Immunizations or tests are offered to personnel for the agents handled or potentially present in the lab. Baseline blood serum samples may be collected and stored, depending on what agents are handled.

Biosafety procedures are incorporated into individual SOPs, or a Biosafety Manual is adopted or prepared for the local laboratory. Personnel are advised of special hazards and are instructed to read and follow instructions on practices and procedures. Laboratory and support personnel receive training on potential hazards associated with the work performed, the precautions to avoid exposure, and exposure evaluation procedures. Training is updated annually or when the procedure or policy changes. Substitution of glassware for plasticware is recommended. A high degree of caution is taken with any procedure using needles and syringes, or any other sharps. Sharps should be restricted whenever possible. Only needle-locking syringes or disposable syringes should be used for injections, etc. Needles should not be manipulated in any manner before disposal; they should be carefully placed into a conveniently located puncture-resistant container used for sharps disposal. Non-disposable sharps are to be placed in a hard-walled container for transport to a processing area for decontamination, preferably by autoclaving. Syringes that re-sheath the needle, needleless systems, and other safety devices are used when possible. Never handle broken glassware directly by hand. Use a mechanical means, such as brush and dustpan or tongs to pick up broken glass pieces. Dispose of broken glass in special containers. Containers of broken glass, sharps, and contaminated needles are decontaminated prior to disposal, or are shipped off-site in accordance with local, state, or federal regulations. Any cultures, tissues, specimens of body fluids, or potentially infectious wastes are placed in a container with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping. All spills and accidents that result in overt exposures to infectious materials are immediately reported to the Designated Laboratory Official. Medical evaluation, surveillance, and treatment are provided as needed and written records are maintained in accordance with 29 CFR 1910.1020. Only animals involved with the work being performed are permitted in the laboratory. Correct personal protective equipment is worn in the laboratory. Disposable gloves are not reused, washed, or used when touching “clean” surfaces, such as the telephone, door handles, keyboards, etc. Properly maintained safety equipment, including biological safety

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cabinets (Class II) or other containment devices are used whenever procedures with a potential for creating infectious aerosols or splashes are conducted, or high concentrations or large volumes of infectious agents are used. High concentrations or large volumes of infectious material may be centrifuged in the open lab if sealed rotor heads or centrifuge safety cups are used and if these rotors or cups are only opened inside of the biosafety cabinet. All surfaces inside the laboratory are readily accessible for cleaning and can easily be decontaminated. No carpets or rugs are used in the laboratory. The biological safety cabinet is located where fluctuations of the room supply, and exhaust air do not cause the cabinet to operate outside its parameters for containment. The cabinet is located away from doors, from windows that can be opened, from heavily traveled laboratory areas, and from other potentially disruptive equipment to maintain the biological safety cabinets' air flow parameters for containment. Eyewashes are readily found throughout the laboratory and are flushed weekly. Outside windows are fitted with screens. Illumination is provided without glare or reflections. For BSL-3? All practices in the BSL-2 laboratory are followed in the BSL-3 laboratory. All procedures involving the manipulation of infectious materials are conducted within biological safety cabinets or other physical containment devices, or by personnel wearing correct personal protective clothing and equipment. BSL-3 laboratory has special engineering and design features, such as a double door access zone and sealed penetrations in the facility. The exhaust air from the laboratory room is discharged directly to the outdoors. The ventilation to the laboratory is balanced to provide directional airflow into the room. Access to the laboratory is restricted whenever work is in progress or the BSL-3 organism is present, and the recommended Standard Microbiological Practices, Special Practices, and Safety Equipment for BSL-3 are rigorously followed. The Designated Laboratory Official strictly controls access to the laboratory. Access is restricted to only those persons whose presence is needed for program or support purpose. The director has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory. The Designated Laboratory Official establishes policies and procedures whereby only persons who have been advised of the potential biohazard, who meet any identified entry requirements (e.g. immunization), and who comply with all entry and exit procedures, enter the laboratory or animal rooms. Laboratory and support personnel receive training on the potential hazards associated with the work involved, the precautions to prevent exposures, and the exposure evaluation procedures. Personnel receive annual updates or additional training as needed for procedural changes. The Designated Laboratory Official is responsible for ensuring that, before working with organisms at BSL-3, all personnel demonstrate proficiency in standard microbiological practices and techniques, and in the practices and operations the local laboratory facility. This might include prior experience in handling pathogens or cell cultures, or a defined training program provided by the director or other competent scientist proficient in safe microbiological practices and techniques. All open manipulations involving

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infectious materials are conducted in biological safety cabinets or other physical containment devices within the containment module. No work in open vessels is conducted on the open bench. Clean up is facilitated by using plastic-backed paper toweling on non-perforated work surfaces within biological safety cabinets. Spills of infectious materials are decontaminated, contained and cleaned up by knowledgeable professional staff, or others properly trained and equipped to work with concentrated infectious material. Spill procedures are developed and posted. Potentially contaminated equipment is decontaminated before removal from the facility for repair or maintenance or packaging for transport, in accordance with applicable regulations. Cultures, tissues, specimens of body fluids, or wastes are placed in a container that prevents leakage during collection, handling, processing, storage, transport, or shipping. All potentially contaminated waste materials (e.g. gloves, lab coats, usual waste materials) from laboratories are decontaminated before disposal or reuse. Decontamination should occur in the immediate area of the BSL-3 unit. Protective laboratory clothing such as solid-front or wrap-around gowns, scrub suits, or coveralls are worn by workers when in the laboratory. Protective clothing is not worn outside the laboratory. Reusable clothing is decontaminated before being laundered. Clothing is changed when overtly contaminated. Gloves are worn when handling infectious materials, infected animals, and when handling contaminated equipment. Frequent changing of gloves accompanied by hand washing is recommended. Disposable gloves are not reused. All manipulations of infectious materials, necropsy of infected animals, harvesting of tissues or fluids from infected animals or embryonate eggs, etc., are conducted in a Class II or Class III biological safety cabinet. When a procedure or process cannot be conducted within a biological safety cabinet, then correct combinations of personnel protective equipment (e.g., respirators, face shields) and physical containment devices (e.g. centrifuge safety cups or sealed rotors) are used. Respiratory and face protections are used when in rooms containing infected animals. The laboratory is separated from areas that are open to unrestricted traffic flow within the building, and access to the laboratory is restricted. Passage through a series of two self-closing doors is the basic requirement for entry into the laboratory from access corridors. Doors are lockable. Clothes change room may be included in the passageway. Each laboratory room contains a sink for hand washing. The sink is hands-free or automatically operated and is located near the room exit door. The interior surfaces of walls, floors, and ceilings of areas where BSL-3 agents are handled are constructed for easy cleaning and decontamination. Seams, if present, are sealed. Walls, ceilings, and floors should be smooth, impermeable to liquids and resistant to the chemicals and disinfectants normally used in the laboratory. Floors should be monolithic and slip-resistant. Consideration should be given to the use of covered floor coverings. Penetrations in floors, walls, and ceiling surfaces are sealed or capable of being sealed to facilitate decontamination. Openings such as around ducts and the spaces between doors and frames are capable of being sealed to facilitate decontamination.

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A method for decontaminating all laboratory wastes is found in the facility and utilized, preferably within the laboratory (i.e., autoclave, chemical disinfection, incineration, or other approved decontamination method). Consideration should be given to means of decontaminating equipment. If waste is transported out of the laboratory, it should be properly sealed and not transported in public corridors. Consideration is given to safe transport of samples potentially contaminated with BSL-3 organisms into the BSL-3 suite. All such samples should be contained in proper packaging to meet regulatory criteria and should not be opened except inside a Class II or III biosafety cabinet.

A complete list of all regulated organisms appears in 42 CFR 72.3.

<https://www.cdc.gov/selectagent/SelectAgentsandToxinsList.html>

Bacillus anthracis - Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities using clinical materials and diagnostic quantities of infectious cultures. Animal Biosafety Level 2 practices, containment equipment, and facilities are recommended for studies utilizing experimentally infected laboratory rodents. Biosafety Level 3 practices, containment equipment, and facilities are recommended for work involving production quantities or concentrations of cultures, and for activities with a high potential for aerosol production.

1. *Campylobacter* (*C. jejuni*/*C. coli*, *C. fetus subsp. fetus*) - Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities with cultures or potentially infectious clinical materials.
2. *Clostridium botulinum* - Biosafety Level 2 practices, containment equipment, and facilities are recommended for all activities with materials known to contain or potentially to contain the toxin. Additional primary containment and personnel precautions, such as those recommended for Biosafety Level 3, are indicated for activities with a high potential for aerosol or droplet production, and those involving production quantities of toxin. Animal Biosafety Level 2 practices, containment equipment, and facilities are recommended for diagnostic studies and titration of toxin.
3. *Escherichia coli* (Cytotoxin-producing (VTEC/SLT) organisms) - Biosafety Level 2 practices, containment equipment, and facilities are recommended for all activities utilizing known or potentially infectious clinical materials or cultures. Animal Biosafety Level 2 facilities and practices are recommended for activities with experimentally or naturally infected animals.
4. *Listeria monocytogenes* - Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities with clinical specimens and cultures known or suspected to contain the agent. Gloves and eye protection should be worn while handling infected cultures. Animal Biosafety Level 2 practices, containment equipment and facilities are recommended for activities with experimentally or naturally infected animals.

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5. *Salmonella* - all serotypes except *typhi* - Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities with clinical materials and cultures known to contain or potentially containing the agents. Animal Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities with experimentally or naturally infected animals. *typhi* - Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities with clinical materials and cultures known to contain or potentially containing the agents. Animal Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities with experimentally or naturally infected animals.
6. *Salmonella typhi* - Biosafety Level 2 practices, containment equipment, and facilities are recommended for all activities utilizing known or potentially infectious clinical materials and cultures. Biosafety Level 3 practices and procedures are recommended for activities likely to generate aerosols or for activities involving production quantities of organisms.
7. *Shigella spp.* - Biosafety Level 2 practices, containment equipment, and facilities are recommended for all activities utilizing known or potentially infectious clinical materials or cultures. Animal Biosafety Level 2 facilities and practices are recommended for activities with experimentally or naturally infected animals.
8. *Vibrio enteritis (Vibrio cholerae, V. parahaemolyticus)* - Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities with cultures or potentially infectious clinical materials. Animal Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities with naturally or experimentally infected animals.
9. *Yersinia pestis* - Biosafety Level 2 practices, containment equipment, and facilities are recommended for all activities involving the handling of potentially infectious clinical materials and cultures. Special care should be taken to avoid the generation of aerosols from infectious materials, and during the necropsy of naturally or experimentally infected rodents. Gloves should be worn when handling field collected or infected laboratory rodents, and when there is the likelihood of direct skin contact with infectious materials. Necropsy of rodents is ideally conducted in a biological safety cabinet. Additional primary containment and personnel precautions, such as those described for Biosafety Level 3, are recommended for activities with high potential for droplet or aerosol production, for work with antibiotic-resistant strains, and for activities involving production quantities or concentrations of infectious materials.
10. Bovine spongiform encephalopathy (BSE) cattle BSE prion BoPrPSc - Human prions and those propagated in apes and monkeys are manipulated at Biosafety Level 2 or 3, depending on the studies being conducted. BSE prions are likewise manipulated at Biosafety Level 2 or 3, due to the possibility that BSE prions have been transmitted to humans in Great Britain and France. All other animal prions are considered Biosafety Level 2 pathogens. Thus, based on the present understanding of prion biology

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described above, once human prions are passaged in mice and mouse PrPSc is produced, these prions should be considered Biosafety Level 2 prions, even though the human prions are Biosafety Level 3 under most experimental conditions. An exception to this statement is in the case of mice expressing human or chimeric human/mouse transgenes. These transgenic mice produce human prions when infected with human prions and should be treated as Biosafety Level 2 or 3 in accord with the guidelines described above. The mechanism of prion spread among sheep and goats developing natural scrapie is unknown. CWD, TME, BSE, FSE, and EUE are all thought to occur after the consumption of prion-infected foods.

11. Hantaviruses - Biosafety level 2 practices and procedures are recommended for laboratory handling of sera from persons potentially infected with the agents of HPS. Potentially infected tissue samples should be handled in BSL-2 facilities following BSL-3 practices and procedures. Cell-culture virus propagation should be carried out in a BSL-3 facility following BSL-3 practices and procedures. Large-scale growth of the virus, including preparing and handling viral concentrates should be performed in BSL-4 containment facilities. Experimentally infected rodent species known not to excrete the virus can be housed in ABSL-2 facilities using ABSL-2 practices and procedures. BSCs and other primary physical containment devices should be used whenever procedures with high potential for generating aerosols are conducted. Serum or tissue samples from potentially infected rodents should be handled at BSL-2 using BSL-3 practices and procedures. All work involving inoculation of virus containing samples into *P. maniculatus* or other permissive species should be conducted at ABSL-4.

- C. What is a SDS (Safety Data Sheet)? Where are the SDSs located? What information can be found on a chemical reagent hazard label?

Safety Data Sheets are required documents in OSHA's Hazard Communication standard prepared by manufacturers or distributors of chemicals received by a facility to relay information about the hazards of those chemicals, etc. SDSs are located where identified for the laboratory and on-line at the web sites of the various manufactures or distributors. Chemical labels must relate the manufacturer, his address, and phone number in addition to the major hazards of the chemical. The health hazards disclosed on the label include, whether the material is a carcinogen, a reproductive toxin, a toxic material, a corrosive, damaging to organs, a central nervous system depressant, a corrosive, an irritant or sensitizer. Any conditions that adversely affect the chemical are also relayed.

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central nervous system depressant, a corrosive, an irritant or sensitizer. Any conditions that adversely affect the chemical are also relayed.

- D. Does the local state regulate medical waste? What laboratory wastes are permitted to enter the sewer? What laboratory wastes are to be incinerated?

EPA no longer regulates medical waste and its destruction, but many states have adapted previous EPA requirements and these requirements are still valid.

- E. What are the guidelines for handling food microorganisms (mostly bacteria or their toxins) in the BMBL?

Food organisms are mostly BSL-2 and are handled as any other BSL-2 organism described above.

- F. What are engineering controls?

Controls include use of biological safety cabinets, isolated work areas, use of equipment and procedures that reduce manipulation, and employment of ventilation and air filtering systems. Describe the proper use of engineering controls in a food microbiology laboratory. Properly maintained safety equipment, including biological safety cabinets (Class II) or other containment devices are used whenever procedures with a potential for creating infectious aerosols or splashes are conducted, or high concentrations or large volumes of infectious agents are used. Materials that are potentially not contained are managed in the biosafety cabinet. For example, infectious material may be centrifuged in the open lab if sealed rotor heads or centrifuge safety cups are used and if these rotors or cups are only opened inside of the biosafety cabinet. Any operation that potentially generates an aerosol is handled in the biosafety cabinet. Plastic backed absorbent paper may be used in the cabinet to contain some of the aerosol and reduce an extensive cleanup. The cabinet is always decontaminated before and after use with an effective disinfectant. The sash is preset to 8-10 inches to minimize any air turbulence in the room that might adversely affect the cabinet's performance. Arm movements in and out of the cabinet are slow and minimal to not disrupt the performance. All supplies are placed so they are readily found during use of the cabinet. No air grills are blocked during use. All contaminated materials are laid in a flat container with disinfectant for later decontamination. No flames are used in the cabinet; disposable loops or electronic heaters are used for loops. Personnel are dissuaded from walking in front of the cabinet during use. A contractor certifies the cabinets annually.

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- G. What are administrative controls?

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Describe administrative controls designed to minimize the risk of hazardous agent exposure to those personnel that are not directly involved with their manipulation. List those administrative controls that assist in the maintenance of quality control. Cleaning crews should not be allowed in the lab except when scheduled. Only authorized personnel should be allowed in the labs and even tighter controls used for animal suites. Everything infectious that is transported should be contained. Use of plasticware is recommended to reduce the potential for breakage. All wastes are autoclaved to ensure nothing infectious enters the sewer system in the building. All materials shipped offsite are specially packaged to meet federal requirements. All equipment is disinfected before service or surplus.

H. What safety equipment is found within a microbiological laboratory?

Eyewashes, sinks, telephones, paper towels, and liquid soap should be readily found in every laboratory. Cabinets used for spill supplies, first aid boxes, safety showers, fire pull stations, and fire extinguishers should be found in various locations throughout the microbiology area.

I. What types of laboratory procedures have the potential to generate aerosols? How can these procedures be contained? How can the generation of aerosols be minimized?

Blending, opening containers of live organisms, using needles and syringes, splattering or spilling infectious liquids, injecting needle through septum, opening food containers under pressure, splattering droplets of infectious materials in a flame, etc. Analysts should use good technique and when needed use containment equipment such as biosafety cabinets.

J. What decontamination procedures are in place and when are they performed?

Work surfaces are decontaminated on completion of work and at the end of the day. This usually involves a system of various decontamination sprays, such as bleach, iodine, quaternium ammonium materials, amphyll, etc. Contaminated equipment is decontaminated before it is sent for repair, sent for maintenance, placed in surplus, or shipped out of the lab. When placing the equipment in surplus, the equipment must be also labeled in accordance with the DHHS requirements for property management to show that the equipment is clean.

K. Describe how spills are handled? Are the cleanups following a spill documented and the cleanup verified?

Spills are decontaminated with an effective disinfectant and then cleaned up with absorbent pads. Any splashes or spills of viable material are immediately cleaned up with disinfectants that are effective against the organism of concern. The material is collected as chemical waste if chemicals are also present. If radioactive materials are present, the spill is then handled as radioactive waste. The area is monitored to verify no hazardous agents are present afterwards by plating swipes of the area. The

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decontaminated microbial spill is handled as a non-hazardous spill if no organisms are present. All areas are routinely decontaminated at the end of a project and at the end of the day in the microbiological lab. Routine settling plates and air monitoring is done to evaluate if there are any infectious issues in the lab. All spills and accidents that result in overt exposure to infectious materials should be immediately reported to laboratory management. Medical evaluation, surveillance, and treatment are provided as needed and written records are maintained in accordance with 29 CFR 1910.1020.

L. What are the potential routes of exposure when working with infectious organisms?

The most common potential routes of exposure are inhalation, ingestion, punctures, and through the skin absorption and exposure to the mucous membrane of the eye. Any cuts in the skin are problematic when working with organisms.

M. Why is it necessary for a minimum of two people to be working in the laboratory at any given time?

This is a standard policy for laboratories.

N. Has enough training to perform the assigned tasks been completed and has this training been documented?

All laboratory personnel receive regularly scheduled training on a revolving annual basis on safety issues and waste management. This is also part of the training given to all new hires. FDA has started the new ORA U on-line training program with web-based training; completion of these modules is documented.

O. Is the facility designed to prevent infectious organisms from being accidentally released to other areas in the building?

Laboratories have different features. Answers should be reflective of the local lab.

P. Is there a sharps safety program in place to reduce hazards when handling syringes or pipettes or other sharps?

The answer for all labs should be yes and defined for that lab.

Q. Are animals used to test infectious agents? Identify what program and laws that address the use of animals in research studies?

Yes. The Animal Welfare Act, IACUC, USDA requirements, and other guidance as needed.

Quality Assurance

A. What culture controls are used for an *Escherichia coli* enumeration analysis? For a *Staphylococcus aureus* analysis?

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In general, both analyses employ positive and negative cultural controls. The positive cultural control assures that conditions at the time of analysis were satisfactory for the recovery of the target organism and that the media and biochemical tests give correct results. The negative cultural control demonstrates the atypical results for biochemical tests and growth characteristics and assures that differential media can give both typical and atypical reactions.

E. coli analysis:

Biochemically typical *E. coli* ATCC 25922 is used as a positive cultural control to demonstrate the typical growth characteristics and reactions for *E. coli*: turbidity and gas from LST; turbidity, gas, and growth at 45.5° C; gas in BGLB; dark flat colonies on EMB agar; indole+, VP-; methyl red+, citrate -.

Enterobacter aerogenes ATCC 13048 is used as a negative cultural control to demonstrate the opposite reactions, so that the analyst is assured that growth conditions are satisfactory for *E. coli*, and our differential media and biochemical reagents are working properly for the atypical reaction as well as the typical reaction. *E. aerogenes* does not grow at 45.5° C, produces pink moist colonies on EMB, is indole-, VP +, methyl red- , citrate +.

Staphylococcus aureus analysis:

Staphylococcus aureus ATCC 6538 is used as a positive cultural control to demonstrate the typical growth characteristics and reactions for *S. aureus*: growth in 10% salt; black colonies surrounded by an opaque zone on BP agar; coagulase +.

Staphylococcus epidermidis ATCC14990 is used as a negative cultural control to demonstrate atypical reactions on BP, i.e. poor growth with no opaque zone, and negative coagulase reaction.

- B. What is the significance of environmental monitoring within an accredited microbiology laboratory? How does your laboratory perform environmental monitoring?

Check Local Guidance for local responses.

- C. Why are temperatures recorded for incubators, water baths etc.?

Checks of the temperature of instruments such as incubators and water baths are needed to maintain confidence in the calibration status of the instrument. For instruments that maintain certain temperatures to encourage microbial growth, these checks are to be performed frequent. (The ALACC recommends incubators be checked twice daily (AM and PM) and water baths be checked daily.)

Microorganisms only grow at certain temperature ranges, and each has a particular optimum temperature. If an incubator or water bath fails to provide that temperature, the target pathogen may not be able to recover. It is critical to never exceed the temperature range of the organism or it may die off.

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D. Why are worksheets reviewed and signed by an analyst who has not worked on the sample?

Worksheets are our “work product” and are used in regulatory actions by the agency. They are written by the lead analyst or electronically generated by the web application and subsequently reviewed by a check analyst, supervisor, director, compliance officer, and in some cases by our legal staff, judges, jury, etc. Not all the readers are scientists; therefore, the worksheet should be presented clearly enough for all to understand. A poorly presented worksheet will not hold up in court, despite the elegant analytical science that may have gone into it.

It is critical that the worksheet be flawless before it leaves the laboratory. The lead analyst or additional analysts may not have detected all the mistakes on the worksheet. Therefore, the worksheet is thoroughly reviewed by a peer analyst who did not work on the sample, is more objective, and thus is less likely to overlook an error. If a peer analyst cannot decipher a worksheet, then a non-scientist has no chance. Worksheets are checked for method suitability, accuracy of calculations, accuracy of transcribed data, and completeness.

E. Why use validated methods?

21CFR2.19 states that it is the policy of FDA to use official methods published in the AOAC, whenever possible. These methods have been validated to provide documented evidence which provides a high degree of assurance that a procedure does what it purports to do (the requirements for an intended use are fulfilled) and is reproducible by other laboratories. Use of validated methods ensures consistency within the various laboratories in the agency. Use of validated methods ensures that our analytical work will hold up in court in defense of our regulatory actions.

The techniques of validation include using reference standards, comparison of results with other methods, interlaboratory comparisons, assessment of factors that influence the results, and assessment of uncertainty of the results. Methods are to be characterized for detection limit, selectivity, linearity, limit of repeatability and/or reproducibility, robustness, and interference from the matrix. Microbiological methods are to achieve a low number of false positives, a low number of false negatives, high sensitivity, high specificity, and a sensitive limit of detection.

F. What is the purpose of QMIS? What are some of the actions/uses of QMIS?

QMIS is the Quality Management Information System. Uses for QMIS include but are not limited to SOP/WI/Guidance document edits/creation/archival, Issue documentation and tracking, audit finding documentation and tracking, training documentation and tracking, storage for documentation, etc.

G. What is your laboratory’s current external auditing body?

This response can change every few years so check with local documentation.

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Polymerase Chain Reaction (PCR)

A. What are some of the benefits to using PCR to detect microbial pathogens in food?

Benefits: It's fast. Can have results in 1-2 days after sample set-up. It's sensitive.

Can detect and amplify very small quantities of target DNA. Problems: Inhibition. Inhibition can come from food matrix and will prevent the PCR reaction from working correctly. PCR amplifies any target DNA present. DNA can be from living or dead cells. An analyst may detect target DNA in a food product, but there may not be viable organisms to recover.

B. What are the three major steps (processes) involved in the PCR cycle?

Denaturation, annealing (complementary binding of primers), and extension.

C. Why would using an enrichment procedure before PCR analysis be useful?

If there are low numbers of injured or stressed bacteria, an enrichment procedure would allow the bacteria to recover and grow and improve the chances of detecting the organism's target DNA with PCR. Using a pre-enrichment may also allow enough bacteria to grow to facilitate the recovery of viable organisms from the enrichment.

D. What components are needed in the PCR reaction mixture and what function does each element serve?

Template: provides source of target DNA

Primers: bind to specific locations on the target DNA

Nucleotides: provide the building blocks of DNA structure

Polymerase: extends the DNA strands

E. Why is selection of the primers so important to the success of the reaction?

The primers will bind on either side of the target DNA. Where they bind will determine what portions of the DNA strand are amplified and how large the PCR product will be.

F. Why are there forward and reverse primers?

Primers are short DNA sequences complementary to the single-stranded DNA templates from which the polymerase begins replicating. PCR uses forward and reverse primers that bind to opposite strands of DNA, flanking the region that is to be amplified. DNA synthesis at one primer is directed toward the other, resulting in the replication of the desired sequence.

G. Why does a microbiologist need an excess quantity of primers in the reaction mixture?

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To ensure the denatured DNA strands anneal to the primers, not to each other. The quantity of primers in the reaction also partly determines how many times the DNA can be amplified.

H. What is real-time PCR? How does it differ from conventional PCR?

Real time PCR (ex. TaqMan) allows for DNA amplification and measurement to occur within the same reaction tube. The machine will measure the increasing fluorescence intensity that occurs as increasing amounts of target DNA are amplified. This eliminates the step of running the PCR reaction product on a gel to determine if target DNA has been amplified.

More detailed description of what happens during real-time PCR...

A probe is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome (usually 6-carboxyfluorescein, 6-FAM) and a quencher fluorochrome (6-carboxy-tetramethylrhodamine, TAMRA) added at any T position or at the 3' end. If both fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as TAQ polymerase extends the primer, the intrinsic nuclease activity of TAQ degrades the probe, releasing the reporter fluorochrome. Thus, the amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. An optical fiber inserted through a lens is positioned over each well and laser light is directed through the fiber to excite the fluorochrome in the PCR solution. Emissions are sent via the fiber to the CCD camera where they are analyzed by the software's algorithms. Collected data is subsequently sent to the computer. The software calculates the threshold cycle (CT) for each reaction with which there is a linear relationship to the amount of starting DNA.

I. Why always run a reagent control?

“DNA free” reagent control should always be run to show that there was no DNA present in the reagent to contaminate the PCR reaction.

J. List three ways to prevent contamination when performing PCR.

- Prepare PCR solutions in an area different from the amplification area. Use pipettes and supplies that are dedicated for PCR use only.
- Use gloves.
- Aliquot stock solutions and prepared solutions into small quantities and small containers to avoid contamination of a large volume of solution.
- Avoid spilling and splashing of reagents. Use filtered pipette tips.

K. In what ways can PCR be inhibited?

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PCR inhibitors can interfere with the cell lysis needed for extraction of DNA, interfere by nucleic acid degradation or capture, and can inhibit polymerase activity of amplification of target DNA. Potential PCR inhibitors include components of body fluids (e.g. hemoglobin, urea, and heparin), food constituents (organic and phenolic compounds, glycogen, and fats), environmental compounds (phenolic compounds, humic acids, and heavy metals), constituents of bacterial cells, non-target DNA, pollen, glove powder, laboratory plastic ware, and cellulose.

Reference: Wilson, I. G. 1997. Minireview Inhibition and Facilitation of Nucleic Acid Amplification, Applied and Environmental Microbiology Vol. 60, No. 10 p. 3741-3751.

Establishment Inspections

NO QUESTIONS

BASIC (TIER 1) ORGANISMS

Salmonella spp.

- A. Why are different pre-enrichment media used for different foods? Give examples. Why is a pre-enrichment step needed for the recovery of *Salmonella*?

Different food matrices inherently involve different intrinsic growth factors. Pre-enrichment in non-selective mediums takes into account the different intrinsic parameters of foods that affect microbial growth thereby, allowing for cell repair, diluting toxic or inhibitory substances and providing nutritional requirements for growth. The pre-enrichment procedure was devised for use with specified food products to promote the growth of injured cells of *Salmonella*. This step favors the recovery of microorganisms from the injured state in which some are believed to exist in many foods. A pre-enrichment step is needed because *Salmonella* organisms may be present in small numbers and/or in competition with microflora. *Salmonella* organisms may also be injured in food-processing procedures, which include exposure to low temperatures, sub marginal heat, drying, radiation, preservatives, and sanitizers. When attempting to initiate growth of inactive bacteria, conditions for their growth such as favorable temperature and pH and the nutritional requirements should be maintained during analysis.

Example. Lactose broth – egg containing products, crustaceans, cheese, fresh frozen dried fruits and vegetables; T-soy broth – spices; Nonfat Dry Milk (NFDM) – candy; BG water – dry milk

By using a pre-enrichment such as Lactose Broth, other organisms will utilize the lactose resulting in a lower pH. This lower pH serves to hold in check the other competing organisms which may be present in greater numbers. The lower pH does

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not appear to be sufficient to affect the growth or to be lethal for *Salmonella* recovery. Other pre-enrichment broths have been shown to increase the recovery of *Salmonella* in certain products (e.g. TSB for spices, NFDM for chocolate).

B. Why is more than one type of selective enrichment (Rappaport-Vassilades, selenite cystine and tetrathionate broths) used instead of just one?

More than one type of enrichment (RV, TT, and SC) is used instead of just one to increase the chances of recovery. These different enrichment broths showed different recoveries depending upon the food matrix and the different serotypes involved. Some serotypes may be more sensitive to one medium than another, so more than one enrichment broth is used to recover as many serotypes as possible.

Rappaport-Vassiliadis: This medium selectively enriches for *Salmonellae* because bacteria, including intestinal bacteria, are high resistant to or inhibited by malachite green, high osmotic pressure and/low pH. It is used widely for analyzing milk and milk products, raw flesh foods, highly contaminated foods, and animal feeds.

Selenite Cystine: As a selective enrichment medium, Selenite Cystine broth allows the proliferation of *Salmonella* while inhibiting the growth of competing non-*Salmonella* bacteria. Selenite broth favors the growth of *Salmonella* while reducing growth of fecal coliforms and enterococci. Sodium Acid Selenite inhibits gram-positive bacteria and most enteric gram-negative bacteria except *Salmonella*.

Tetrathionate: This selective enrichment is used for the cultivation of *Salmonella* species that may be present in small numbers. This medium inhibits or kills the coliform organisms and permits the typhoid and paratyphoid bacilli to grow almost unrestrictedly.

C. Describe sugar reactions in triple sugar iron agar (TSI) tubes.

Lactose and sucrose are present at 1%, while glucose is present at only 0.1%.

Salmonella ferment glucose but not lactose or sucrose. Phenol red, the pH indicator in TSI, is red under alkaline conditions and yellow under acidic conditions. At first, the slant and butt turns yellow due to glucose fermentation (acidic). Since the lactose and sucrose cannot be utilized, when the glucose is used up, the *Salmonella* depends on the aerobic de-amination of the amino acids in the medium (alkaline). The slant reverts to red because of the alkaline aerobic reaction, while the butt remains yellow due to the acidic glucose (anaerobic) fermentation.

An alkaline slant-acid butt (red/yellow) indicate fermentation of dextrose only.

An acid slant-acid butt (yellow/yellow) indicates fermentation of dextrose, lactose, or sucrose. (Sucrose is added to TSI to eliminate some sucrose-fermenting non-lactose fermenters such as *Proteus* and *Citrobacter* spp.)

An alkaline slant-alkaline butt (red/red) indicates that neither dextrose nor lactose was fermented (non-fermenter).

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D. Why use three primary plating media instead of one?

The use of 2 or more selective plating media is recommended to facilitate the recovery of strains of *Salmonella* that may be inhibited by a particular plating medium. Some *Salmonella* serotypes grow poorly or fail to grow on certain selective agars (e.g. *S. typhi* is inhibited by BG agar). Selective plating is the procedure requiring the use of solid selective media (BS, HE, XLD) that restrict growth of bacteria other than *Salmonella* and provide visual recognition of pure, discrete colonies suspected to be *Salmonella*.

Bismuth Sulfite (BS) – A highly selective medium used for the isolation of *S. typhi* and other *Salmonella*. Gram positive bacteria and members of the coliform group are inhibited. The inhibitory reaction to gram positive and coliform organisms permits the use of much greater inoculum. The use of larger inoculum increases the possibility of recovering pathogens especially if present in low numbers.

Hektoen Enteric (H E) – A selective, differential medium for the isolation and differentiation of *Salmonella* from gram negative pathogens (The lactose positive colonies are differentiated from the lactose negative colonies) while at the same time ensuring inhibition of gram-positive microorganisms.

Xylose Lysine Deoxychocolate (XLD) – A selective, differential plating medium. Inhibits the growth of gram-positive organisms. *Salmonella* are differentiated from the other xylose fermenting enteric organisms with the addition of lysine. *Salmonella* organisms decarboxylate lysine causing a reversion to alkaline conditions. Alkaline reversion by other lysine positive organisms is prevented by excess acid production from fermentation of lactose and sucrose.

E. Which *Salmonella* serovars do not typically produce hydrogen sulfide?

S. ser. Paratyphi and *S. ser. choleraesuis*. Other serotypes may also have mutations which produce hydrogen sulfide negative strains.

F. What is indole?

Indole is a component of the amino acid tryptophan. Some bacteria can break down tryptophan for nutritional needs using the enzyme tryptophanase. When tryptophan is broken down, the presence of indole can be detected using Kovacs' reagent. Kovac's reagent, which is yellow, reacts with indole and produces a red color on the surface of the test tube. *Salmonella* cultures give a negative test (lack of deep red color at surface of broth).

Indole test: 0.2 - 0.3ml of Kovacs reagent is added to a 5 ml aliquot of a 24-hour tryptophan broth culture.

G. Can all serovars of *Salmonella* be identified with antisera? Give reason.

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No. *Salmonella* is not identified with antisera alone. Identification calls for answers from biochemical reactions as well as serological reactions. When all pieces are put together, one has an identification. An example is the two non-motile species (*S. bioseer Gallinarum*, and *S. bioseer Pullorum*). Both contain the same O antigens, but no H antigens. They are to be identified biochemically.

H. Describe symptoms and onset time of *Salmonella* food borne disease.

Acute symptoms - Nausea, vomiting, abdominal cramps, diarrhea, fever, and headache. Chronic consequences -- arthritic symptoms may follow 3-4 weeks after onset of acute symptoms.

Onset time – 6 to 72 hours.

Infective dose - As few as 15-20 cells; depends upon age and health of host, and strain differences among the members of the genus.

Duration of symptoms - Acute symptoms may last for 1 to 2 days or may be prolonged, again depending on host factors, ingested dose, and strain characteristics. Cause of disease - Penetration and passage of *Salmonella* organisms from gut lumen into epithelium of small intestine where inflammation occurs; there is evidence that an enterotoxin may be produced, perhaps within the enterocyte.

I. Describe and give examples of foods in Food Category I, II and III. (See BAM chapter 1 Food Sampling.)

Food Category I. - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption and are intended for consumption by the aged, the infirm, and infants.

Food Category II. - (Numbers in chart represent industry codes) - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption.

<u>2</u>	Milled grain products not cooked before consumption (bran and wheat germ)
3	Bread, rolls, buns, sugared breads, crackers, custard- and cream-filled sweet goods, and icings
5	Breakfast cereals and other ready-to-eat breakfast foods
<u>7</u>	Pretzels, chips, and other snack foods
9	Butter and butter products, pasteurized milk and raw fluid milk and fluid milk products for direct consumption, pasteurized and unpasteurized concentrated liquid milk products for direct consumption, dried milk and dried milk products for direct consumption, casein, sodium caseinate, and whey
<u>12</u>	Cheese and cheese products
13	Ice cream from pasteurized milk and related products that have been pasteurized, raw ice cream mix and related unpasteurized products for direct consumption

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14	Pasteurized and unpasteurized imitation dairy products for direct consumption
15	Pasteurized eggs and egg products from pasteurized eggs, unpasteurized eggs and egg products from unpasteurized eggs for consumption without further cooking
16	Canned and cured fish, vertebrates, and other fish products; fresh and frozen raw shellfish and crustacean products for direct consumption; smoked fish, shellfish, and crustaceans for direct consumption
17	Meat and meat products, poultry and poultry products, and gelatin (flavored and unflavored bulk)
20- 22	Fresh, frozen, and canned fruits and juices, concentrates, and nectars; dried fruits for direct consumption; jams, jellies, preserves, and butters
23	Nuts, nut products, edible seeds, and edible seed products for direct consumption
24	Vegetable juices, vegetable sprouts, and vegetables normally eaten raw
26	Oils consumed directly without further processing; oleomargarine
27	Dressings and condiments (including mayonnaise), salad dressing, and vinegar
28	Spices, flavors, and extracts
29	Soft drinks and water
30	Beverage bases
31	Coffee and tea
33	Candy (with and without chocolate; with and without nuts) and chewing gum
34	Chocolate and cocoa products
35	Pudding mixes not cooked before consumption, and gelatin products
36	Syrups, sugars, and honey
37	Ready-to-eat sandwiches, stews, gravies, and sauces

Food Category III. - Foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption.

2	Whole grain, milled grain products that are cooked before consumption (corn meal and all types of flour), and starch products for human use
3	Prepared dry mixes for cakes, cookies, breads, and rolls
4	Macaroni and noodle products
16	Fresh and frozen fish; vertebrates (except those eaten raw); fresh and frozen shellfish and crustaceans (except raw shellfish and crustaceans for direct consumption); other aquatic animals (including frog legs, marine snails, and squid)
18	Vegetable protein products (simulated meats) normally cooked before consumption
24	Fresh vegetables, frozen vegetables, dried vegetables, cured and processed vegetable products normally cooked before consumption

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26	Vegetable oils, oil stock, and vegetable shortening
35	Dry dessert mixes, pudding mixes, and rennet products that are cooked before consumption

J. Why is the pH of the enrichment broth adjusted after addition of sample?

The pH for optimum growth is around neutrality, with values above 9.0 and below 4.0 being bactericidal. For best growth, the *Salmonella* require a pH between 6.6 and 8.2.

K. What does the VIDAS assay detect?

VIDAS *Salmonella* is an enzyme immunoassay for the detection of *Salmonella* antigens using the ELFA (Enzyme Linked Fluorescent Assay) method. Vidas assay detects both motile and non-motile *Salmonella*.

The SPR (Solid Phase Receptacle) is coated with anti-*Salmonella* antibodies. *Salmonella* antigens present in the sample will be bound. Unbound sample components are washed away. Antibodies conjugated with alkaline phosphatase are cycled in and out of the SPR and will bind to the *Salmonella* antigen bound by the SPR. A final wash step removes unbound conjugate. A fluorescent substrate, 4-methyl-umbelliferylphosphate, is introduced in the SPR. This enzyme will catalyze the conversion of the substrate to the fluorescent product, 4methyl-umbelliferone. The intensity of the fluorescence is measured by the optical scanner in the VIDAS.

L. Why does the sample need to be boiled before performing the VIDAS assay?

The culture needs to be boiled before performing the VIDAS assay to kill the organism. Otherwise, the VIDAS would become contaminated. The boiling procedure disrupts the cell wall thereby, making accessible the antigen for the antigen-antibody reaction.

M. Which organism is most likely to cause a VIDAS false positive result?

The organism most likely to cause a false positive result is Citrobacter. Citrobacter may contain common O, H, and Vi antigens. The pH for optimum growth is around neutrality, with values above 9.0 and below 4.0 being bactericidal. For best growth, the *Salmonellae* require a pH between 6.6 and 8.2.

Listeria monocytogenes

A. What is unusual about the appearance of the motility of *Listeria*?

A characteristic motility of *Listeria* is described as tumbling.

B. At what high temperatures and low temperatures will *Listeria* grow?

Listeria can grow between 2.5 and 44°C

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C. Describe the CAMP reactions of different *Listeria* species. What do the letters “CAMP” stand for in the CAMP test?

“CAMP” stands for Christie-Atkins-Munch-Peterson.

Hemolytic Interaction		
Species	<i>Staphylococcus aureus</i>	<i>Rhodococcus equi</i>
<i>L. monocytogenes</i>	+	- (a)
<i>L. ivanovii</i>	-	+
<i>L. innocua</i>	-	-
<i>L. welshimeri</i>	-	-
<i>L. seeligeri</i>	+	-

^a Some strains are weakly R+, especially after 48hr incubation, but the R+ reaction is much less pronounced than that of *L. ivanovii*

D. Why is *Rhodococcus equi*, and not other *Rhodococcus* species used in the CAMP test?

There is a synergistic hemolytic effect based on the diffusion of "R. equi factors," phospholipase C and cholesterol oxidase which help enhance hemolysis of some species of *Listeria* when performing the camp test.

E. Describe the symptoms and onset time of *Listeria* foodborne disease.

Flu-like illness with fever, myalgia or headache and diarrhea. Onset can be days to weeks except for gastroenteritis which has a rapid onset (hours).

F. Describe the appearance of typical *Listeria monocytogenes* colonies on selective plating media including but not limited to Oxford, Palcam, and BCM.

Listeria monocytogenes has a similar appearance on Oxford and Palcam with black pin-point colonies with a black halo. *L. monocytogenes* has a blue colony on BCM.

G. Describe how acid production from various carbohydrates (mannitol, rhamnose, xylose) typically differs between *Listeria monocytogenes* and *Listeria innocua*.

Acid Production			
Species	Mannitol	Rhamnose	Xylose
<i>L. monocytogenes</i>	-	+	-
<i>L. innocua</i>	-	V ^(b)	-

^b V, variable

H. What role does the addition of selective agents in pre-enrichment and enrichment media play?

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To increase the number of *Listeria* by minimizing background microflora.

I. Name two beta-hemolytic and two non beta-hemolytic *Listeria* species.

Beta-Hemolytic: *L. monocytogenes* and *L. ivanovii*

Non Beta-Hemolytic: *L. innocua* and *L. welshimeri*

Escherichia coli

A. Describe different types of *E. coli* that cause food borne disease. List methods used for their determination.

- Enterotoxigenic *E. coli* (ETEC)-gastroenteritis, traveler's diarrhea
- ETEC strains produce two types of toxins: heat-labile toxin (LT) and heat-stable toxin (ST). LT can be detected by the Y-I tissue culture test, and ST can be detected by the infant mouse test. These toxins also can be detected by ELISA, and genes coding for them can be detected by gene probes.

- Enteropathogenic *E. coli* (EPEC)-infant diarrhea

Although there are no definitive tests for EPEC strains, some methods, which are still experimental, are found in the literature or from communications with researchers. Confirmation of a putative EPEC strain involves serogrouping and serotyping and consideration of case symptomology. Further complexity concerns the fact that some EPEC strains behave like EHEC strains.

- Enterohemorrhagic *E. coli* (EHEC)-hemorrhagic colitis

The toxins produced by EHEC are detected by tissue culture assays. However, DNA probe and polymerase chain reaction assays have also been developed to detect the presence of SLT gene in HEC isolates.

- Enteroinvasive *E. coli*-(EIEC)-bacillary dysentery

A tissue culture (HeLa cell) test is found in the BAM to screen isolates for invasive potential before confirming invasiveness by the Sereny test. An in vitro staining technique using acridine orange to stain for intracellular (invasive) bacteria in HeLa cell monolayers is also an effective assay to determine invasiveness of pathogenic *E. coli*.

- Enteroadherent *E. coli* (EAEC)-newly added category

Is not fully characterized in the BAM and so no methodology is not found there.

B. Is there an acceptable way to minimize foaming when shellfish are homogenized?

Yes, limit the blending to 60-90 seconds. Excessive grinding can cause overheating which leads to the foaming.

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C. What percent foam is aspirated when pipetting sample from homogenate? Would this affect the results?

Less than 5% is aspirated. The BAM specifies that the solution should be allowed to settle. The top is not to be pipetted. The effect of the foam is that it destroys any protein produced by the bacteria. This is important in characterization that involves the protein.

D. Describe the bottled water definition how would one initiate analysis.

In addition to "bottled water" or "drinking water", in 21 CFR Part 103 FDA also defines various types of bottled water that meet certain criteria. These identities include "artesian or artesian well water", "ground water", "mineral water", "purified or demineralized water", "sparkling bottled water", "spring water" and "well water". Under the current bottled water quality standard, FDA has established a microbiological quality requirement that is based on coliform detection levels. These levels may be obtained by membrane filtration (MF) or by 10-tube MPN analysis of ten 10-mL analytical units.

E. Describe how a Gram stain is performed and the meaning of the results.

Gram Stain Procedure

- Add crystal violet stain over the fixed culture. Let stand for 10 to 60 seconds; for thinly prepared slides, it is usually acceptable to pour the stain on and off immediately. Pour off the stain and gently rinse the excess stain with a stream of water. Note that the objective of this step is to wash off the stain, not the fixed culture.
- Add the iodine solution on the smear, enough to cover the fixed culture. Let stand for 10 to 60 seconds. Pour off the iodine solution and rinse the slide with running water.
- Shake off the excess water from the surface.
- Add a few drops of decolorizer so the solution trickles down the slide. Rinse it off with water after 5 seconds. The exact time to stop is when the solvent is no longer colored as it flows over the slide. Further delay will cause excess decolorization in the gram-positive cells, and the purpose of staining will be defeated.
- Counterstain with basic fuchsin solution for 40 to 60 seconds. Wash off the solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may be shaken to remove most of the water and air-dried.
- Meaning of the results - If the stained culture retains the primary dye (the purplish crystal violet dye), then the culture is gram positive. However, if the culture retains the counterstain (the red fuchsin solution) the culture is gram negative.

F. Describe the quadrant streaking technique to obtain isolated colonies.

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Purpose:

The streak plate technique is the most widely used method of obtaining isolated colonies from a mix of cultures.

Principle:

The streak plate technique is essentially a method to dilute the number of organisms, decreasing the density. This allows for individual colonies to be isolated from other colonies. Each colony is considered "pure," since theoretically, the colony began with an individual cell.

Procedure:

- a. Begin with inoculating the first, or primary, quadrant of the agar plate. Use a light touch. Don't penetrate or scrape the agar surface. Cover plate with lid. Flame the loop, cool by touching an uninoculated portion of the surface.
- b. Now rotate the plate. Open lid and streak again. Remember, the analyst is picking up growth from quadrant one, and using this as an inoculum for quadrant two.
- c. Flame loop, rotate plate, and repeat procedure for quadrants three and four.

G. What is the IMViC pattern for *E. coli*?

IMViC patterns +++- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*.

H. What does a typical *E. coli* isolate look like on L-EMB?

Dark centered and flat, with or without metallic sheen

I. When does one use LST-MUG? How does LST-MUG work?

Use LST-MUG when detecting *E. coli* in chilled or frozen foods exclusive of bivalve molluscan shellfish.

The LST-MUG assay can presumptively identify *E. coli* within 24 h. The assay is based on the presence of GUD in *E. coli*, which cleaves the MUG substrate to release 4methylumbelliferone (MU). When exposed to longwave (365 nm) UV light, MU exhibits a bluish fluorescence which is easily visualized. When MUG is incorporated into LST medium, coliforms can be enumerated based on gas production from lactose. *E. coli* are presumptively identified by fluorescence in the medium under longwave UV light

J. What time period is allowable between sample shaking and inoculation of tubes?

Not >15 min should elapse from the time test sample is blended until all dilutions are made in prescribed culture media.

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Enterohemorrhagic *E. coli* (EHEC)

- A. Are all STEC also Enterohemorrhagic *E. coli* (EHEC)?

No, only a subset of Shiga-toxin producing *E. coli* (STEC) are involved in causing illness in humans and are referred to as enterohemorrhagic *E. coli* (EHEC). The more severe cases are characterized by bloody diarrhea and can progress to hemolytic uremic syndrome (HUS) or death.

- B. Do all *E. coli* strains ferment sorbitol?

More than 85% of *E. coli* strains do ferment sorbitol. Significantly, however, almost all *E. coli* O157:H7 do not ferment sorbitol.

- C. Why is tellurite cefixime sorbitol MacConkey agar (TCSMAC) particularly useful for the isolation of *E. coli* O157:H7?

The incorporation of sorbitol as the primary sugar source in MacConkey agar aids in the differentiation of sorbitol negative *E. coli* O157:H7 (tan to light gray colony appearance) from most other *E. coli* (magenta colony appearance) that do ferment sorbitol.

- D. Are all STEC hemolytic?

Approximately 89 % of STEC and only 15% of non-STEC produce an active enterohemolysin and are hemolytic. Therefore the use of agar such as SHIBAM can be useful to detect those STEC that are hemolytic.

- E. Why is it important to use multiple differential and selective agars to recover *E. coli* O157:H7 and other non-O157 STEC?

As a group STEC are very phenotypically diverse. The use of several agars that can distinguish between these different phenotypic characteristics are useful to improve the isolation and recovery of STEC.

- F. Explain why the PCR to confirm the presence of shiga toxin genes (*stx*) must be done as part of the isolate confirmation process even if the PCR was used during the initial screening of the food enrichment.

When the food enrichment is screened for *stx* genes this is only an indication that these gene targets may be present in one or more different genomes and that the analysis should continue to isolate and recover one or more STEC strains. After recovery it is essential to reconfirm the *stx* genotype for each STEC isolate recovered individually.

- G. Why is it important to have confirmed STEC isolates further characterized for complete virulence profile and serotype by methods such as microarray or whole genome sequencing (WGS)?

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The assessment of STEC health risk is complicated by the existence of many non-O157 STEC serotypes that do not have the right combination of virulence determinants and therefore, do not have the potential to cause severe illness. As a result, it is necessary to evaluate the health risk of a particular STEC isolate to human health, regardless of serotype, by compiling complete laboratory generated data including genetic data that can be evaluated for virulence profiles, serotype and similarity to strains involved in human disease.

Staphylococcus aureus

- A. Describe the symptoms and onset time of staphylococcal food borne disease.

Primary symptoms may include nausea, vomiting, abdominal cramps, diarrhea, headache, and muscular cramps. Secondary symptoms may include retching, dehydration, sweating, weakness, and prostration. Symptoms typically develop within 6 h after ingestion of contaminated food. Symptoms usually last for 1 to 2 days.

- B. Describe the difference between intoxication and infection. Which one is associated with *S. aureus*?

Intoxication is associated with ingestion of pre-formed toxin and does not need growth of the organism in the host to produce illness. Infection requires the establishment of a pathogen within or on a host after invasion, where the organism may begin to grow and cause illness. Staphylococcal foodborne illness is considered an intoxication since it does not need growth of the pathogen in the host to cause illness.

- C. Which ingredient/s of Baird Parker medium help injured organisms grow?

This medium contains lithium chloride and tellurite to inhibit the growth of accompanying microbial flora, whereas pyruvate and glycine selectively stimulate the growth of *Staphylococci*.

- D. What are typical observations of coagulase positive *Staphylococcus aureus* when plated on Baird Parker medium? What is indicated by the presence or absence of a halo around an isolated colony on Baird Parker medium?

Coagulase positive *S. aureus* colonies are typically circular, smooth, convex, 2-3 mm gray to jet-black colonies with an outer clear halo zone surrounding the colony. Colonies have a buttery to gummy consistency when touched with a needle. *Staphylococcus* colonies show two characteristic features when grown in Baird Parker medium. The first would be characteristic halos which are formed because of lipolysis and proteolysis. Second, reduction of tellurite to tellurium producing a gray to jet-black coloration. The egg-yolk reaction and tellurite reduction are usually

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found to occur together with a positive coagulase reaction and can thus serve as an index for the latter.

- E. Name the staphylococcal enterotoxin type and which is the most common cause of food borne disease.

There are many different designations for SE types on classification schemes based on antigenicity. SEA remains the most common toxin implicated in foodborne intoxication.

Coliforms

- A. Why is sodium thiosulfate added to jars used to collect water samples?

(Investigations Operations Manual (IOM), current edition) To prevent chlorine from acting on the bacteria and assures when sample is analyzed, the bacterial load is the same as when collected.

- B. Explain the difference between coliforms and fecal coliforms.

E. coli and the coliforms are both Gram-negative, rod-shaped facultatively anaerobic bacteria, however, coliforms produce gas from glucose (and other sugars) and ferment lactose to acid and gas within 48 h at 35°C, but fecal coliforms (and *E. coli* as a coliform) produce gas from glucose (and other sugars) and ferment lactose to acid and gas within 48 h at 45.5°C. Also, coliforms are found throughout the environment and fecal coliforms are found in the intestinal tract of warm-blooded vertebrates.

- C. What organisms are considered coliforms?

Escherichia, Enterobacter, Klebsiella, Serratia, Citrobacter, Proteus.

Aerobic Plate Count

- A. Name foods that may have natural high counts. Name foods that should have low counts.

One example of a high natural APC count would be sprouts. An example of a food with a low natural APC count would be tree fruits such as oranges and lemons.

- B. Why are APC counts of interest to FDA?

The aerobic plate count (APC) is intended to indicate the level of live microorganisms in a product at the time of analysis. By modifying the temperature of incubation, the analyst can selectively screen for microorganisms such as thermophiles/mesophiles/psychrotrophiles, to name a few.

- C. Why do "spreaders" sometimes form when doing an APC? At the air/agar interface? At the agar/glass interface?

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Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth so that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreaders.

D. How long and at what temperature can one "thaw" a frozen food? Discuss.

Normally, a sample can be thawed at 2-5°C within 18 h. If rapid thawing is desired, thaw the sample at less than 45°C for not more than 15 min. When thawing a sample at elevated temperatures, agitate the sample continuously in thermostatically controlled water bath.

E. Will there be a change to the APC results with repeated freezing and thawing of product?

One theory is that the viability and subsequent growth on agar plates of the organisms may be compromised due to disruption and destruction of bacterial DNA through repeated freezing and thawing.

F. What is the proper procedure for manually mixing dilution blanks?

Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 seconds.

G. At what temperature should pour plates be dispensed?

Desired temperature is 45 ± 1°C.

H. How should the analyst interpret plates with more than 250 colony-forming units (CFU's)?

When number of CFU per plate exceeds 250, for all dilutions, record the counts as too numerous to count (TNTC) for all but the plate closest to 250, and count CFU in those portions of plate that are representative of colony distribution. See BAM Chapter 3 for detailed guidelines. Mark calculated APC with EAPC to denote that it was estimated from counts outside 25-250 per plate range. When plates from both dilutions yield more than 250 CFU each (but fewer than 100/cm²), estimate the aerobic counts from the plates (EAPC) nearest 250 and multiply by the dilution.

Example:

Colonies		
1:100	1:1000	EAPC/ml (g)
TNTC	640	640,000
TNTC = too numerous to count. EAPC = estimated aerobic plate count.		

I. How should the analyst interpret plates with less than 25 CFU's?

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When plates from both dilutions yield fewer than 25 CFU each, record actual plate count but record the count as less than 25 x 1/d when d is the dilution factor for the dilution from which the first counts were obtained.

Example:

Colonies		
1:100	1:1000	EAPC/ml (g)
18	2	<2,500
0	0	<2,500

Yeast and Mold Count

A. Why are yeast and mold count of interest to FDA?

Both yeasts and molds can cause various degrees of deterioration and decomposition of foods. Also, several foodborne molds and some yeasts can produce mycotoxins which are hazardous to human and animal health.

B. What is the medium of choice for yeast and mold analysis?

Potato Dextrose Agar.

C. What medium is especially useful for analyzing samples containing "spreader" molds?

Potato Dextrose-Salt Agar.

D. What agent/agents are added to the agar to inhibit bacterial growth?

Antibiotics (Chlortetracycline-HCl, Chloramphenicol, or Streptomycin) or Sterile 10% Tartaric Acid.

E. Why should the plates be left undisturbed until the incubation period is complete?

Handling plates could result in secondary growth from dislodged spores which would make counts invalid.

INTERMEDIATE (TIER 2) ORGANISMS

Vibrio spp.

A. Describe the foodborne illness and onset times of the *Vibrio spp.* that were studied.

Vibrio cholerae:

Cholera is an acute, diarrheal illness caused by infection of the intestine with the bacterium *Vibrio cholerae*. The infection is often mild or without symptoms, but

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sometimes it can be severe. Symptoms of Asiatic cholera may vary from a mild, watery diarrhea to an acute diarrhea, with characteristic rice water stools. Additional symptoms may include vomiting, leg cramps, nausea, dehydration, and shock. After severe fluid and electrolyte loss, death may occur. Onset of the illness is generally sudden, with incubation periods varying from 6 hours to 5 days. Symptoms usually last 2 to 3 days although in some patients they can continue up to 5 days. Most people are infectious from the onset of illness until a few days after recovery.

Vibrio parahaemolyticus:

When ingested, *V. parahaemolyticus* causes watery diarrhea often with abdominal cramping, nausea, vomiting fever and chills. Usually these symptoms occur within 24 hours of ingestion. Illness is usually self-limited and lasts 3 days. Severe disease is rare and occurs more commonly in persons with weakened immune systems. *V. parahaemolyticus* can also cause an infection of the skin when an open wound is exposed to warm seawater.

Vibrio vulnificus:

V. vulnificus can cause disease in those who eat contaminated seafood or have an open wound that is exposed to seawater. Wound infections may lead to skin breakdown and ulceration. Among healthy people, ingestion of *V. vulnificus* can cause vomiting, diarrhea, and abdominal pain. (In healthy individuals, gastroenteritis usually occurs within 16 hours of ingesting the organism). In immunocompromised persons, particularly those with chronic liver disease, *V. vulnificus* can infect the bloodstream, causing a severe and life-threatening illness characterized by fever and chills, decreased blood pressure (septic shock), and blistering skin lesions. *V. vulnificus* bloodstream infections are fatal about 50% of the time.

B. What are the advantages and disadvantages of using a PCR method?

Advantages: PCR analysis is rapid and can be performed within 48 hours including sample enrichment. Only enterotoxigenic *Vibrio spp.* will yield a positive result. It will detect enterotoxigenic *V. mimicus* that may be missed in the standard BAM bacteriological analysis. (Colonies of *V. mimicus* appear green on TCBS selective media).

Disadvantages: A positive PCR result does not indicate the viability of the organisms detected. (Therefore, viable cells containing the ctxAB target sequence may not be able to produce enterotoxin in culture media. Certain food matrices may inhibit PCR reactions and yield false negative results. A positive food spike control should be analyzed with each new food tested.)

C. Why is NaCl added to media used for *Vibrio spp.*?

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Vibrio spp. other than *V. cholerae* and *V. mimicus* are halophilic and do not grow in media that lack NaCl.

- D. Why do some methods enumerate the organism and other methods only check for the presence of the organism?

There are guidance tolerance levels for some *Vibrio spp.* organisms and zero tolerance for others. For example, enterotoxigenic *V. cholerae* has a zero-tolerance level whereas violative levels of *V. parahaemolyticus* are $\geq 1 \times 10^4$ organisms/gram food.

- E. What are the characteristics of *V. mimicus*? What are the similarities and differences of this organism compared to *V. cholerae*?

V. mimicus colonies are sucrose negative on TCBS agar whereas *V. cholerae* are sucrose positive. *V. mimicus* are also urease negative. *V. cholerae* are urease positive.

- F. What are the major factors in the pathogenesis of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus*?

V. cholerae- cholera toxin (CT) or cholera-like toxin (non01/0139).

V. parahaemolyticus - produces a thermostable direct hemolysin also referred to as the Kanagawa phenomenon-associated hemolysin. Epidemiological evidence suggests that it is a virulence factor. All strains produce thermolabile hemolysin (species specific). Some produce thermostable direct hemolysin (TDH) some produce thermostable related hemolysin (TRH). Some produce both. *V. vulnificus* - produces cytotoxin/hemolysin.

V. mimicus - produces a cholera-like toxin.

Discussion: Cholera symptoms result from the action of cholera toxin (CT), which is a chromosomally mediated, heat-labile enterotoxin. CT has a high level of similarity to the plasmid mediated, heat-labile enterotoxin (LT) of *Escherichia coli*. CT is an oligomeric protein composed of an A subunit and five B subunits. The B subunits bind to ganglioside GM1 receptors on the membranes of intestinal epithelial cells, while the A subunit activates adenylate cyclase, producing increased levels of cyclic AMP and resulting in the hypersecretion of water and electrolytes. The outpouring of water and electrolytes into the lumen of the intestine causes copious purging, resulting in the severe dehydration associated with the classical clinical presentation of cholera. With rare exceptions, Non-01 and 0139 *V. cholerae* strains do not produce CT and rarely cause epidemic disease. Several other toxins have been identified from non-01/0139 strains. These include a hemolysin stable enterotoxin very similar to that produced by *E. coli* referred to as NAG-ST; and a hemolysin termed NAG-rTDH which is related to the thermostable direct hemolysin (TDH) of *V. parahaemolyticus*.

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Bacillus cereus

- A. Describe the symptoms and onset times for the two types of *B. cereus* food borne diseases. What foods have been implicated in each type?
- Type 1: Diarrheal type – diarrhea, abdominal pain, nausea, some vomiting, usually fever with an onset time usually 8-16 hours. Implicated foods include but are not limited to meats, vegetables, milk, cream pastries, and soups. Type 2: Emetic type – primary symptom is vomiting with an onset time usually between 1 – 5 hours. Implicated foods include but are not limited to fried or cooked rice and another starchy food such as potatoes.
- B. Can *B. thuringiensis* produce diarrheal antigens? Diarrheal disease?
- Yes.
- C. The symptomatic profile of emetic type of food poisoning produced by some strains of *B. cereus* most closely mimics that of *Clostridium perfringens* or *Staphylococcus aureus*?
- Staphylococcus aureus* intoxication.
- D. The toxins of *B. cereus* most commonly associated with food poisoning are:
- Diarrheal and emetic components.
- E. Explain the purpose for each ingredient used in MYP agar and how *B. cereus* can be interpreted on MYP agar.
- Ingredients included in MYP agar include beef extract, mannitol, peptone, NaCl, phenol red, and agar. Check plates for lecithinase production as indicated by zone of precipitation surrounding growth. Mannitol is not fermented by isolate if growth and surrounding medium are eosin pink. (Yellow color indicates that acid is produced from mannitol.) *B. cereus* colonies are usually lecithinase-positive and mannitol-negative on MYP agar.
- F. When is the Plate Count Method recommended? When is Most Probable Number (MPN) recommended?
- The MPN technique is recommended for enumerating *B. cereus* in foods that are expected to contain fewer than 10 *B. cereus* organisms/g. It may also be preferred for examining certain dehydrated starchy foods for which the plate count technique is not recommended.
- G. When interpreting test results (motility, hemolytic activity, plating characteristics, and crystal production) what are typical results for each pertaining to *B. cereus*?
- Most strains of *B. cereus* are motile; however, some non-motile strains exist. *B. cereus* exhibit hemolysis (sheep RBC). *B. cereus* exhibit development of rhizoid growth, which is characterized by production of colonies with long hair or root-like

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structures that may extend several centimeters from site of inoculation. Rough galaxy-shaped colonies are often produced by *B. cereus* strains and should not be confused with typical rhizoid growth, which is the definitive characteristic of *B. mycoides*. *B. cereus* and other members of the *B. cereus* group do not produce protein toxin crystals. *B. thuringiensis* usually produces protein toxin crystals that can be detected by the staining technique either as free crystals or parasporal inclusion bodies within the exosporium.

Campylobacter

- A. Describe the symptoms and onset time of *Campylobacter* food borne disease.
- Onset of disease is 2-10 days. Symptoms include fever of greater than 40°C, abdominal pain, nausea, vomiting, and mild to severe diarrhea. Diarrhea is self-limiting, 2-7 days.
- B. Describe methods used to obtain microaerobic conditions.
- Microaerobic conditions can be achieved by bubbling with or shaking in a modified gas mixture of 5% O₂, 10% CO₂, 85% N₂. Anaerobe jars evacuated with the modified gas mixture or containing a Campy Pak or large jars with a single Anaerobe Pak will also provide a microaerobic environment.
- C. What are the oxygen-quenching compounds added to *Campylobacter* media?
- FBP (ferrous sulfate, sodium metabisulfite, sodium pyruvate), hemin, blood, and charcoal are oxygen-quenching compounds.
- D. Which biochemical test is used to differentiate between *C. jejuni* and *C. coli*?
- Hippurate hydrolysis.
- E. What are the advantages and disadvantages of using rapid methods such as the Dryspot Campy Test or Alert for *Campylobacter*?
- The Dryspot Test gives a quick confirmation that the analyst has isolated one of the most commonly found *Campylobacters* (*C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. fetus*); however, it will not serotype the isolate nor speciate it. Additionally, it will not react with all *C. fetus* strains. (FYI-The reference to Alert will be removed from future BAM/LPM editions.)

Yersinia enterocolitica

- A. Describe the symptoms and onset time of *Yersinia enterocolitica* illness.

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Symptoms of yersiniosis include gastroenteritis with diarrhea and/or vomiting. The usual onset time is 4 to 7 days after exposure with the illness lasting 1 to 3 weeks or longer. Right-sided abdominal pain and fever are the hallmark symptoms.

B. Does *Yersinia* grow and survive during refrigerated storage?

Yersinia may survive and grow during refrigerated storage.

C. What enrichment broths and selective media are used to culture *Yersinia enterocolitica*?

Yersinia enterocolitica is enriched in peptone sorbitol bile broth (PSBB) or on spread plate (MacConkey and CIN - Cefsulodin Irgasan Novobiocin agars) if high levels are suspected.

D. What enrichment broths and selective media are used to culture *Yersinia pseudotuberculosis*?

Y. pseudotuberculosis is enriched in PMP broth and streaked onto MacConkey and YSA/CIN agar plates.

E. Describe the biochemical characteristics of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*.

Y. enterocolitica: Characteristic biochemical reactions include anaerogenic fermentation of glucose, positive urease, and positive motility below 30°C but negative at 35°C. *Y. enterocolitica* are variable in their indole reaction; they do not ferment lactose, and do not utilize citrate. They are also phenylalanine deaminase negative and lysine decarboxylase negative.

Y. pseudotuberculosis: Characteristic biochemical reactions include fermentation of glucose, positive urease, and positive motility below 30°C but negative at 35°C. *Y. pseudotuberculosis* is indole negative; they do not ferment ornithine, sorbitol and sucrose, and do not utilize citrate. They are also phenylalanine deaminase negative and lysine decarboxylase negative. They can ferment mannitol and rhamnose.

F. What is the relationship of plasmids and *Yersinia*?

Yersinia spp. that cause human yersiniosis carry a plasmid (41- 50Mdal).

G. What types of tests are used to determine pathogenicity?

There are several traits related to virulence:

- autoagglutination in certain media at 35°C-37°C
- inhibition of growth in calcium deficient media
- binding of crystal violet dye at 35°C-37°C
- increased resistance to normal human sera

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- production of a series of outer membrane proteins at 35°C-37°C ability to produce conjunctivitis in guinea pig or mouse (Serenytest)
- lethality in adult and suckling mouse by intraperitoneal injection of live organisms

Clostridium perfringens

A. Describe the symptoms and onset time for *C. perfringens* foodborne disease.

Affected individuals usually only develop diarrhea and severe abdominal pain. Less common side effects are vomiting, fever, shivering and headache. Onset usually will develop between 8 to 14 hours after ingestion of contaminated food and usually resolve spontaneously within 12 to 14 hours.

B. How does one test the organism for toxin production?

Utilize a specially designed enterotoxin toxin testing kit such as Reverse Passive Latex Agglutination (RPLA) kit.

C. Tell when each enumeration method would be use.

Laboratory capability will determine enumeration method. Analysts are to always utilize official validated methodology

D. How do anaerobe jar commercial systems produce anaerobic conditions? How can one tell if it worked?

Inoculated media is placed in the anaerobe jar with or without palladium catalyst and with a generator envelope. The system is then sealed with an airtight lid. The envelopes are H₂ + CO₂ generator envelopes with or without a palladium catalyst. Note* catalyst is to be in envelope or jar for successful conversion to anaerobic conditions. Some systems either have the catalyst in the lid of the jar, or the catalyst is contained in the generator envelope. To visually see if anaerobic conditions are produced through the anaerobic container system, there are commercially purchasable indicator strips which will turn colors (usually blue when exposed to oxygen and will turn white when O₂ is depleted from the system.

E. Describe stormy fermentation and how to test *C. perfringens* for stormy fermentation?

After inoculation into media such as Iron Milk Medium (M68), coagulation of milk to produce an acid curd with subsequent disruption or fracturing of the spongy curd by a large volume of gas produced by the organism exhibits stormy fermentation.

F. Describe key characteristics of *C. perfringens* including motility, gram-reaction, nitrate reduction, and lecithinase activity.

Non-motile.

Gram-positive spore-forming *Bacillus*

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Reduce nitrates to nitrites

C. perfringens colonies on TSC/EY - 2-4 mm opaque, white zone surrounding
Exhibits lecithinase activity

Clostridium botulinum

A. What are the symptoms and onset time of botulism?

The classic symptoms of botulism include double vision, blurred vision, drooping eyelids, slurred speech, difficulty swallowing, dry mouth, and muscle weakness. Infants with botulism appear lethargic, feed poorly, are constipated, and have a weak cry and poor muscle tone. These are all symptoms of the muscle paralysis caused by the bacterial toxin. If untreated, these symptoms may progress to cause paralysis of the arms, legs, trunk and respiratory muscles. In foodborne botulism, symptoms generally begin 18 to 36 hours after eating a contaminated food, but they can occur as early as 6 hours or as late as 10 days.

B. What is wound botulism and infant botulism?

Wound botulism is caused by toxin produced from a wound infected with *Clostridium botulinum*. Infant botulism is caused by consuming the spores of the botulinum bacteria, which then grow in the intestines and release toxin.

C. Name the different types of toxins and which have been implicated in human botulism.

There are seven types of botulism toxin designated by the letters A through G; only types A, B, E and F cause illness in humans.

D. Describe how the MLD is calculated.

Dilutions that kill and dilutions that do not kill are needed to establish an endpoint or the minimum lethal dose (MLD) as an estimate of the amount of toxin present. The MLD is contained in the highest dilution killing both mice (or all mice inoculated). From these data, the number of MLD/ml can be calculated.

E. What is the difference between preformed and formed toxin?

Preformed toxin is when botulin toxin is detected in a suspect food, whereas formed toxin is when botulin toxin is produced during growth in a culture medium.

Alkaline Phosphatase

A. Why is the presence of alkaline phosphatase determined in dairy products?

To detect improper pasteurization, a negative phosphatase test indicates most of the milk used has been properly heated during pasteurization.

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B. What is the reason for heating the control blank?

The control blank is heated to identify the amount of color development which may be due to reagent contamination and/or interfering color material in the sample so that it can be subtracting from sample colorimetric readings ensuring the remaining color development can be attributed to the presence of alkaline phosphatase.

C. Why is a buffer used in the analysis?

To maintain optimal pH of between 9.85 and 10.2 for the reaction to occur.

D. In the AOAC confirmation method, why are different buffer and precipitant concentrations used for different cheeses?

Different strength buffers are used for different types and ages of cheese.

E. What parameters need to be controlled during analysis for the phenol to be liberated from the disodium phenyl phosphate substrate?

A pH range of 9.85 to 10.2 and incubation time.

F. To what compound is the alkaline phosphatase enzyme activity proportional?
Phenol.

G. Describe the reaction that takes place for phenol to be measured colorimetrically?

To measure phenol either 2,6-dichloroquinonechlorimide (CQC) in the screening method or 2,6 dibromoquinonechlorimide (BQC) in the confirmation method reacts in the presence of catalyst (CuSO₄) to form indophenol blue. The indophenol blue can then be measured colorimetrically.

Canned Food and Can Seam Examination

A. What is a Flipper, Springer, Soft Swell and Hard Swell?

Flipper: A can that normally appears flat. When brought down sharply on its end on a flat surface, one end flips out. When pressure is applied to this end, it flips in again and the can appears flat. Springer: A can with one end permanently bulged. When sufficient pressure is applied to this end, it will flip in, but the other end will flip out. Soft Swell: A can bulged at both ends, but not so tightly that the ends cannot be pushed in somewhat with thumb pressure. Hard Swell: A can bulged at both ends, and so tightly that no indentation can be made with thumb pressure. The next stage is the bursting of the can.

B. What is flat-sour spoilage? What bacteria can cause this?

A type of microbial spoilage characterized by a markedly lowered pH, sour and abnormal odor, and sometimes cloudy liquor. Gas is not usually produced therefore the can remains flat, hence the name "flat-sour". Flatsour organisms include *Bacillus*

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thermacidurans (Bacillus coagulans) and *Bacillus stearothermophilis*, both being thermophilic bacteria, growing and causing flat-sour spoilage at elevated temperatures (50-55°C).

- C. Does microbial spoilage of a food always result in a swollen can? Explain microbial spoilage of a food does not always result in a swollen can.

The following are examples.

Flat-sour spoilage as explained in question #2.

Sulfide spoilage: H₂S gas is produced which is absorbed (soluble) by the product. Usually blackened, "rotten egg" odor. The can remains flat. Thermophilic, spore forming anaerobes are primarily responsible for this spoilage. *Clostridium nigrificans* is considered the type species of this group.

Proteolytic bacteria such as *Clostridium botulinum* Type A may not produce enough gas to swell the can.

- D. Why are certain canned food media incubated at 55°C?

Media is incubated at 55°C to detect thermophilic bacteria which cause flat-sour and sulfide spoilage.

- E. Explain the microbiology and physical attributes of each of the following:

Underprocessing, leaker spoilage, hydrogen swell and overfill. Underprocessing caused by undercooking. Either the temperature and/or time of the cook were short. This is the most serious processing problem. Characterized by pure cultures of Gram + rods and intact seams. If cans missed the retort altogether, there could be a high rate of swell and a mixed flora of rods and cocci. Leaker Spoilage: Caused by post-processing contamination, usually characterized by defective seams, microleaks and a mixed flora of rods and cocci. Hydrogen Swell: Caused by the acid of the product reaction with the metal of the can, resulting in hydrogen gas. Most prevalent in acid foods such as canned tomatoes which have exceeded the normal shelf life of the product. Overfill: Caused by too much product filled into the can, resulting in not enough headspace and lack of proper vacuum.

- F. Explain the construction of a metal can. Name the components of the double seam.

The metal can is made of either steel or aluminum. It consists of the can body and two lids (3 piece can), or the can body and one lid (2 piece drawn can). The can body is joined together by a weld. The interior of a steel can is usually coated with a protective enamel, which inhibits rusting and interaction of the product with the steel. The lid is attached by a double seaming operation. The double seam consists of 5 thicknesses of plate plus a thin layer of sealing compound. The seam is formed by two rolling operations.

- G. Explain water activity and its significance in an acidified and low acid canned food.

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Water activity is a number which provides information about the vapor pressure of water in a system. It's measured by dividing the equilibrium vapor pressure of the product by that of pure water, which is taken to be 1.0. The higher the water activity (A_w), the more free water there is for microbial growth. Water activity is part of the definition of a Low Acid Canned Food and Acidified Low Acid Canned Food. In both, according to 21 CFR, parts 113 and 114. the A_w must be >0 .

Cronobacter spp.

- A. What species belong to genus Cronobacter and which species have been involved in human illnesses?

Cronobacter sakazakii, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, and *Cronobacter* genomospecies I (later renamed as *Cronobacter universalis*). All species, except *Cronobacter* genomospecies I, have been involved in clinical cases.

- B. What is the morphology of typical colonies on chromogenic agars?

Presumptive *Cronobacter* colonies on DFI agar appear either dark green, weak green, or brownish green. Some colonies only have a green center with a white/yellow border. Presumptive *Cronobacter* colonies on R&F agar appear blue to black, or blue to grey with the red background. The red background can appear purplish red with different strain or under different light conditions. *Cronobacter* does not change the color of R&F agar, but in the presence of background microflora which change the color of R&F agar from red to yellow, *Cronobacter* colonies appear blue to green.

- C. Can the method be performed without running the qPCR assay?

The cultural portion of this method is a complete detection/isolation method, so it can be used as a stand-alone method if PCR technology is unavailable. The PCR portion of the method is a screening method, whose positive results should always be confirmed with the cultural method. The PCR method may be used to confirm pure cultures as *Cronobacter* spp.

Cosmetic Analysis

- A. Describe the sample preparation techniques for liquids and semi powders; solids and powders; preparations with petroleum base; aerosols of powders and liquids; and aerosols of soaps and other foamy liquids.

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Liquids: Decimally dilute 1 ml liquid directly into 9 ml modified letheen broth (MLB) in 20 x 150 mm screw-cap test tube for the 10-1 dilution. Solids and powders: Aseptically remove and weigh 1 g sample into 20 x 150 mm screw-cap test tube containing 1 ml sterile Tween 80. Disperse product in Tween 80 with sterile spatula. Add 8 ml sterile MLB and mix thoroughly. This will be the 10-1 dilution. Cream and oil-based products: Aseptically remove and weigh 1 g sample into 20 x 150 mm screw-cap tube containing 1 ml sterile Tween 80 plus five to seven 5-mm glass beads (or ten to fifteen 3-mm glass beads). Mix total contents with Vortex mixer. Adjust total volume to 10 ml with sterile MLB (8 ml) for the 10-1 dilution. Aerosols of powders, soaps, liquids, and other materials: Decontaminate nozzle of spray can as much as possible by swabbing with gauze pad moistened with 70% (v/v) aqueous ethanol. Expel some product to flush out nozzle; then spray reweigh. This will be a 10-1 dilution if exactly 1 g of sample was obtained.

B. What is the purpose of dilutions and other added ingredients?

Dilutions and other added ingredients partially inactivate preservative systems commonly found in cosmetic products.

C. How do *Pseudomonas* and *Klebsiella* differ biochemically? What characteristics clearly separate them?

Pseudomonas is a non-fermenting microorganism and *Klebsiella* is a fermenter. *Pseudomonas* can be separated from *Klebsiella* in that it is oxidase positive and does not ferment glucose while *Klebsiella* is oxidase negative and ferments glucose.

D. If, biochemically, a culture was indicated to be *Pseudomonas* but it did not produce a fluorescent yellow or blue pigment, would a microbiologist still consider the culture as *Pseudomonas*? Explain.

The culture could still be a *Pseudomonas* based on other identifying characteristics. Production appears to be somehow involved in iron acquisition. Approximately 90% of *Pseudomonas spp.* are fluorescent. The pigments are useful differentiating markers. The classification of *Pseudomonas aeruginosa* is based on the production of a number of pigments including a pyocyanin (blue) and fluorescein (yellowish green). Some strains also produce pyorubin (red), pyomelanin (brown), two other red pigments (aeruginosin A and B) and various other pigments.

E. Which organisms are considered pathogenic in the eye area? On the skin?

Pathogens or opportunistic pathogens whose incidence would be of particular concern, especially in eye-area cosmetic products, include *S. aureus*, *Streptococcus pyogenes*, *P. aeruginosa* and other species, and *Klebsiella pneumoniae*. Some microbes normally regarded as nonpathogenic may be opportunistically pathogenic (e.g. in wounds).

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Nutritional and Dietary Supplements

A. What are the controls used in this experiment?

Controls used: for *Total Aerobic Microbial Count*, inoculate duplicate plates with 25–250 cfu of *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 8739), and *Bacillus subtilis* (ATCC No. 6633) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For the *Sabouraud Dextrose Agar Medium* used for *Total Combined Molds and Yeasts Count*, inoculate duplicate plates with 25–250 cfu of *Candida albicans* (ATCC No. 10231) and *Aspergillus brasiliensis* (ATCC No. 16404) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For *Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)*, appropriate dilutions of *Escherichia coli* (ATCC No. 8739) and *Salmonella typhimurium* (ATCC No. 13311) are used.

B. What are some ways to neutralize inhibitory substances?

The following are examples of ingredients and their concentrations that may be added to the culture medium to neutralize inhibitory substances present in the sample: soy lecithin, 0.5%; and polysorbate 20, 4.0%. Alternatively, repeat the test as described in the preceding paragraph, using *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where inhibitory substances are contained in the product and the latter is soluble, a suitable, validated adaptation of a procedure set forth under *Procedure* using the *Membrane Filtration Method* may be used.

If, despite the incorporation of suitable inactivating agents and a substantial increase in the volume of diluent, it is still not possible to recover the viable cultures described above, and where the article is not suitable for the employment of membrane filtration, it can be assumed that the failure to isolate the inoculated organism is attributable to the bactericidal or bacteriostatic activity of such magnitude that treatments are not able to remove the activity. This information serves to indicate that the article is not likely to allow proliferation or contamination with the given species of microorganism. Monitoring should be continued in order to determine the inhibitory range and bactericidal activity of the article.

C. What is Purified Water?

Purified Water is water obtained by distillation, ion-exchange treatment, reverse osmosis, or other purifying process. It is prepared from water complying with the regulations of the federal Environmental Protection Agency with respect to drinking water. It contains no added substance.

D. How does one determine what microbial test to run on products?

The microbial test run is that specified by the USP monograph for that product.

E. What is the preparatory test?

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The preparatory test is done to demonstrate that the test specimens do not of themselves, inhibit the multiplication, under the test conditions of microorganisms that may be present.

ADVANCED (TIER 3) ORGANISMS

Viruses

A. List two ways that viruses differ from bacteria?

1. Viruses require a host or cell line for replication where bacteria can replicate outside the host.
2. Viruses contain a capsid or protein coat.

B. What is the genetic material found in viruses?

Viruses contain either RNA or DNA, their genetic material can either be single stranded or double stranded.

C. What process is required before RNA viruses can be detected by qPCR?

RNA Viruses must be converted to cDNA (complimentary DNA) during a process called reverse transcriptase before the gene can be amplified via PCR.

D. How are viruses removed and concentrated from fruit, finfish, and molluscan shellfish?

Fruit and finfish are surface contaminated and are rinsed in a filter bag at a high pH to remove the small virus particles from the product. The eluted virus can fit through the filter and can be removed physically removed from large food particles. Moderate centrifugation separates the even smaller food particles. Molluscan shellfish are filter feeders and viruses concentrated in their digestive glands. The digestive glands are dissected from the rest of the animal and composited from many animals. This composite is then homogenized, and treated through series of absorption, elution, and ultra-speed centrifugation to concentrate the virus. A chloroform extraction step is used on the concentrated samples to remove organic matter prior to a second series of absorption, double elution, and concentration. Internal contamination allows for more inhibitors to be mixed with the sample concentrate and this method uses multiple cycles of Ultracentrifugation and double elution of the chloroform cleanup to reduce these inhibitors.

E. What is the importance of pH in removing viruses from foods and what characteristic of the target virus is important in this approach?

The isoelectric point of a virus is the pH at which it absorbs to food particles. This pH is used to determine the pH needed for various steps of the extraction and

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concentration steps to release the virus from the food (higher pH) or absorb (lower pH) the virus to foods.

- F. How do you determine if the amount of inhibition is too high to be able to rely on negative PCR results for a given virus target?

If the average Ct value (cycle # at which target detection fluorescence crosses the pre-defined threshold set by the method) of the IAC (internal control) in the sample wells is more than 4 cycles delayed from the Ct of the internal control in the No Template Control (NTC) then there is too much inhibition to rely on a negative result for the target organism and the inhibition must be resolved as per the method.

Cyclospora

- A. Explain why methods based on microscopic examination cannot be used reliably to detect and characterize *C. cayetanensis* in food and agricultural water.

<https://www.fda.gov/food/laboratory-methods-food/bam-chapter-29-cronobacter>

Microscopy lacks the sensitivity and specificity to detect low concentrations of oocysts or differentiate between morphologically identical species of *Cyclospora*.

- B. Explain why person to person transmission of *C. cayetanensis* does not occur.

Individuals infected with *C. cayetanensis* shed unsporulated oocysts in their stools.

These unsporulated oocysts are not infective when freshly passed and require a week or longer in the environment at optimal temperatures to sporulate and become infective.

- C. Explain the main steps included in the process for molecular identification of *C. cayetanensis* in fresh produce by PCR as described in BAM Chapter 19b.

The process for molecular identification of *C. cayetanensis* in fresh produce by PCR can be broken down into the following steps: 1. Produce wash procedure, 2. Isolation of DNA from produce wash, and 3. Identification by PCR amplification.

- D. Explain the main steps included in the process for molecular identification of *C. cayetanensis* in agricultural water by PCR as described in BAM Chapter 19c.

1. Collect 10L sub-samples using the dead-end ultrafiltration procedure described in part I of BAM 19c (Dead-end Ultrafiltration).
2. *C. cayetanensis* oocysts are recovered from ultrafilters by backflushing and concentration into a pellet following the protocol of Part II of 19c.
3. DNA from recovered oocysts within the pellet is extracted using the FastPrep-24 bead beater and the FastDNA SPIN Kit for Soil.
4. Detection of *C. cayetanensis* DNA is performed using a species-specific qPCR assay targeting the 18S rRNA gene.

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Whole Genome Sequencing (WGS)

A. Describe general steps for WGS analysis of bacterial pathogens.

Extract and quantitate DNA from a single colony from a pure culture bacterial isolate. Dilute input DNA and tagment DNA fragments. Generally, this process uses a transposase enzyme to simultaneously fragment and inserts adapter sequences into dsDNA. Attach specified index primers to fragments by PCR amplification. PCR cleanup to remove unincorporated index primers and purify amplified product. Library normalization. GenomeTrakr protocol uses a bead-based approach and PulseNet protocols use a quantitation and dilution approach. This step is intended to refine the library size and prepare appropriate concentrations from each isolate to be included in the sequencing pool. Pool libraries from all isolates to be included in sequencing run and load onto sequencing cartridge. Load cartridge and flow cell on MiSeq instrument and initiate sequencing run. After sequencing run has completed check basic run quality metrics. This includes cluster density, Q30 and approximate genome coverage for each isolate included in the run. Transfer data for upload to NCBI. Wash and maintain MiSeq instrument between sequencing runs.

B. What is the main difference between DNA extraction procedures for Gram positive and Gram-negative isolates?

The extraction of Gram-positive organisms includes an additional enzymatic lysis step using lysozyme.

C. Why should index primers be rotated between sequencing runs?

Index primer combinations should be rotated to avoid sample carryover and cross-contamination from a previous run.

D. What is the basic sequence coverage requirement for *Salmonella*, *Listeria* and *E. coli*?

Salmonella - 30X, *Listeria* – 20X, and *E. coli* – 40X

E. How often should you wash your sequencer? What should you wash your machine with?

Each lab has their own local variations to this process so please check local guidance or with your local SME. Illumina provides minimal requirements as follows: the Miseq will prompt you to perform a post-run wash after each sequencing run. Additionally, they recommended maintenance washes to be performed at least monthly, a standby wash to be performed if the instrument will remain idle for a least 7 days and every 30 days after while it remains idle. Many labs have elected to run a maintenance wash between runs or as needed. The machines are washed using Tween 20 (Sigma-Aldrich, catalog # P7949) mixed with Laboratory-grade water. The instructions provided by illumina to create your tween 20 wash solution is as follows: Add 5 ml of 100% Tween 20 to 45ml laboratory grade water. These volumes result in 10% Tween 20. Add 10% Tween 20 to 475 ml laboratory grade water. This results in a 0.5%

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Tween 20 wash solution. Mix solution well and add 6ml wash solution to each section of the wash tray, as well as 350 mls to the 500ml wash bottle. Repeat this each time for the three-step wash cycle. Some Illumina representatives will recommend replacing some of these washes with only laboratory grade water so remember to consult on this process locally.

- F. Which QA/QC tools should you always run? How do you know when you need to utilize additional tools?

You should always run the GalaxyTrakr tool MicroRunQC. If the ST value of your isolate matches any of your internal controls or any of your labs, in the last six months, you need to run the SNP Pipeline.

- G. What should you do if you suspect an isolate is a result of a contamination event involving a laboratory control or another isolate?

You should report to your supervisor and WGS Program Coordinators and initiate an issue in QMIS so that you can investigate and root causes analysis.

- H. What quality metrics should you look for during a sequencing run?

Sequence Analysis Viewer and MicroRunQC results.

Select Agents

- A. What is a select agent?

A select is a microorganism or chemical toxin capable of causing serious harm or death due to exposure through inhalation.

- B. Name four areas of training necessary before handling select agents.

Physical containment, PPE, Biosafety Practices, Microbiological Characterization of Select Agents.

- C. Which agency regulates the use, possession and transfer of select agents capable of posing a serious public health threat?

The CDC

- D. List the PPE required to work with select agents.

Closed front gown or jump suit, double gloves, respirator or face mask, hair and shoe coverings.

- E. What work practices are to be in place when working in a Biological Safety Level (BSL) laboratory? For BSL-3?

Laboratory access is limited or restricted. Personnel at increased risk for acquiring infection, or for whom infection would have serious consequences, are not allowed in the laboratory or animal rooms. Laboratory doors are kept closed when experiments are in process. Personnel wash their hands after handling microorganisms, when removing gloves, and before leaving the laboratory. Soap and disposable towels are readily found at sinks in the microbiology laboratories. No eating, drinking, chewing

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gum, smoking, handling medications, handling contact lenses, or applying cosmetics is permitted in the laboratory. All foodstuffs are stored outside of the laboratory. Mouth pipetting is prohibited. There is a program for handling and disposing of sharps using special sharps' containers. Work surfaces are decontaminated on completion of work and at the end of the day. This usually involves a system of various decontamination sprays, such as bleach, iodine, quaternium ammonium materials, amphyll, etc. Any splashes or spills of viable material are immediately cleaned up with disinfectants that are effective against the organism of concern. Contaminated equipment is to be decontaminated before it is sent for repair, sent for maintenance, placed in surplus, or shipped out of the lab. When placing the equipment in surplus, the equipment is also labeled in accordance with the DHHS requirements for property management to show that the equipment is clean.

All cultures, stocks, and other regulated wastes are autoclaved, or otherwise decontaminated, to destroy any viable organisms. All materials for autoclaving are placed in red (or otherwise equivalent) bags that are closed for transport out of the laboratory. These bags are set in leak-proof totes. Any materials to be decontaminated off-site are packaged in accordance with applicable local, state, and federal regulations beforehand. When working with organisms with more hazardous risk, consideration is given to chemically decontaminating the wastes within the laboratory. An insect and rodent control program are in place. All procedures are conducted in a manner to minimize aerosols. Consideration is given to the degree of hazard when loops are used in flames, centrifugation, opening screw-capped bottles and wet petri dish covers, use of a syringe, needle, and septum, streaking plates, pipetting, slide agglutination, etc. Good microbiological techniques, substitution of disposable equipment, and use of primary containment devices such as biosafety cabinets reduce the risk. Laboratory management establishes policies and procedures whereby personnel in the laboratory are informed of the potential hazards and meet special entry requirements, such as immunization. A biohazard sign is posted at the entrance to any laboratory where etiologic agents are used. This label identifies what etiologic agents are used, the biosafety level, any required immunizations, the responsible person, and their phone number, what PPE is worn in the laboratory, and any procedures for exiting the laboratory. Immunizations or tests are offered to personnel for the agents handled or potentially present in the lab. Baseline blood serum samples may be collected and stored, depending on what agents are handled. Biosafety procedures are incorporated into individual SOPs, or a Biosafety Manual is adopted or prepared for the local laboratory. Personnel are advised of special hazards and are instructed to read and follow instructions on practices and procedures. Laboratory and support personnel receive training on potential hazards associated with the work performed, the precautions to avoid exposure, and exposure evaluation procedures. Training is updated annually or when the procedure or policy changes. Substitution of glassware for plasticware is recommended. A high degree of caution is taken with any procedure using needles and syringes, or any other sharps. Sharps should be restricted

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whenever possible. Only needle-locking syringes or disposable syringes should be used for injections, etc. Needles should not be manipulated in any manner before disposal; they should be carefully placed into a conveniently located puncture-resistant container used for sharps disposal. Non-disposable sharps are to be placed in a hard-walled container for transport to a processing area for decontamination, preferably by autoclaving. Syringes that re-sheath the needle, needleless systems, and other safety devices are used when possible. Never handle broken glassware directly by hand. Use a mechanical means, such as brush and dustpan or tongs to pick up broken glass pieces. Dispose of broken glass in special containers. Containers of broken glass, sharps, and contaminated needles are decontaminated prior to disposal, or are shipped off-site in accordance with local, state, or federal regulations.

Any cultures, tissues, specimens of body fluids, or potentially infectious wastes are placed in a container with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping. All spills and accidents that result in overt exposures to infectious materials are immediately reported to the Designated Laboratory Official. Medical evaluation, surveillance, and treatment are provided as needed and written records are maintained in accordance with 29 CFR 1910.1020. Only animals involved with the work being performed are permitted in the laboratory. Correct personal protective equipment is worn in the laboratory. Disposable gloves are not reused, washed, or used when touching “clean” surfaces, such as the telephone, door handles, keyboards, etc. Properly maintained safety equipment, including biological safety cabinets (Class II) or other containment devices are used whenever procedures with a potential for creating infectious aerosols or splashes are conducted, or high concentrations or large volumes of infectious agents are used. High concentrations or large volumes of infectious material may be centrifuged in the open lab if sealed rotor heads or centrifuge safety cups are used and if these rotors or cups are only opened inside of the biosafety cabinet. All surfaces inside the laboratory are readily accessible for cleaning and can easily be decontaminated. No carpets or rugs are used in the laboratory. The biological safety cabinet is located where fluctuations of the room supply, and exhaust air do not cause the cabinet to operate outside its parameters for containment. The cabinet is located away from doors, from windows that can be opened, from heavily traveled laboratory areas, and from other potentially disruptive equipment so as to maintain the biological safety cabinets' air flow parameters for containment. Eyewashes are readily found throughout the laboratory and are flushed weekly. Outside windows are fitted with screens. Illumination is provided without glare or reflections.

BSL 3?

All practices in the BSL-2 laboratory are followed in the BSL-3 laboratory. All procedures involving the manipulation of infectious materials are conducted within biological safety cabinets or other physical containment devices, or by personnel wearing correct personal protective clothing and equipment. BSL-3 laboratory has special engineering and design features, such as a double door access zone and

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sealed penetrations in the facility. The exhaust air from the laboratory room is discharged directly to the outdoors. The ventilation to the laboratory is balanced to provide directional airflow into the room. Access to the laboratory is restricted whenever work is in progress or the BSL-3 organism is present, and the recommended Standard Microbiological Practices, Special Practices, and Safety Equipment for BSL-3 are rigorously followed. The Designated Laboratory Official strictly controls access to the laboratory. Access is restricted to only those persons whose presence is needed for program or support purpose. The director has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory. The Designated Laboratory Official establishes policies and procedures whereby only persons who have been advised of the potential biohazard, who meet any identified entry requirements (e.g. immunization), and who comply with all entry and exit procedures, enter the laboratory or animal rooms. Laboratory and support personnel receive training on the potential hazards associated with the work involved, the precautions to prevent exposures, and the exposure evaluation procedures. Personnel receive annual updates or additional training as needed for procedural changes. The Designated Laboratory Official is responsible for ensuring that, before working with organisms at BSL-3, all personnel demonstrate proficiency in standard microbiological practices and techniques, and in the practices and operations the local laboratory facility. This might include prior experience in handling pathogens or cell cultures, or a defined training program provided by the director or other competent scientist proficient in safe microbiological practices and techniques. All open manipulations involving infectious materials are conducted in biological safety cabinets or other physical containment devices within the containment module. No work in open vessels is conducted on the open bench. Clean up is facilitated by using plastic-backed paper toweling on non-perforated work surfaces within biological safety cabinets. Spills of infectious materials are decontaminated, contained, and cleaned up by knowledgeable professional staff, or others properly trained and equipped to work with concentrated infectious material. Spill procedures are developed and posted. Potentially contaminated equipment is decontaminated before removal from the facility for repair or maintenance or packaging for transport, in accordance with applicable regulations. Cultures, tissues, specimens of body fluids, or wastes are placed in a container that prevents leakage during collection, handling, processing, storage, transport, or shipping. All potentially contaminated waste materials (e.g. gloves, lab coats, usual waste materials) from laboratories are decontaminated before disposal or reuse. Decontamination should occur in the immediate area of the BSL-3 unit. Protective laboratory clothing such as solid-front or wrap-around gowns, scrub suits, or coveralls are worn by workers when in the laboratory. Protective clothing is not worn outside the laboratory. Reusable clothing is decontaminated before being laundered. Clothing is changed when overtly contaminated. Gloves are worn when handling infectious materials, infected animals, and when handling contaminated equipment. Frequent changing of gloves accompanied by hand washing is recommended.

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Disposable gloves are not reused. All manipulations of infectious materials, necropsy of infected animals, harvesting of tissues or fluids from infected animals or embryonate eggs, etc., are conducted in a Class II or Class III biological safety cabinet. When a procedure or process cannot be conducted within a biological safety cabinet, then correct combinations of personnel protective equipment (e.g., respirators, face shields) and physical containment devices (e.g. centrifuge safety cups or sealed rotors) are used. Respiratory and face protections are used when in rooms containing infected animals

The laboratory is separated from areas that are open to unrestricted traffic flow within the building, and access to the laboratory is restricted. Passage through a series of two self-closing doors is the basic requirement for entry into the laboratory from access corridors. Doors are lockable. A clothes change room may be included in the passageway. Each laboratory room contains a sink for hand washing. The sink is hands-free or automatically operated and is located near the room exit door. The interior surfaces of walls, floors, and ceilings of areas where BSL-3 agents are handled are constructed for easy cleaning and decontamination. Seams, if present, are sealed. Walls, ceilings, and floors should be smooth, impermeable to liquids and resistant to the chemicals and disinfectants normally used in the laboratory. Floors should be monolithic and slip-resistant. Consideration should be given to the use of covered floor coverings. Penetrations in floors, walls, and ceiling surfaces are sealed or capable of being sealed to facilitate decontamination. Openings such as around ducts and the spaces between doors and frames are capable of being sealed to facilitate decontamination. A method for decontaminating all laboratory wastes is found in the facility and utilized, preferably within the laboratory (i.e., autoclave, chemical disinfection, incineration, or other approved decontamination method). Consideration should be given to means of decontaminating equipment. If waste is transported out of the laboratory, it should be properly sealed and not transported in public corridors. Consideration is given to safe transport of samples potentially contaminated with BSL-3 organisms into the BSL-3 suite. All such samples should be contained in proper packaging to meet regulatory criteria and should not be opened except inside a Class II or III biosafety cabinet.

F. Does the laboratory need to register with the CDC when working with regulated select agents?

If the lab receives or ships any of these agents for analysis or research or participates in a collaborative study, then the lab must be registered with CDC to ship or receive any select agents.