

## 2.16 Appendix - Answer Key

### 2.2 Media, Reagent, and Supply Preparation

1. **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.
2. **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.
3. **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.
4. **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.

#### 5. What are some safety concerns when autoclaving?

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 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.

### 2.3 Safety and Hazardous/Infectious Waste

1. **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).

2. **What work practices must be in place when working in a Biological Safety Level (BSL)-laboratory?**

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 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 is 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 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.

### **For BSL-3?**

All of the 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 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. 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.

3. **Describe and give examples of foodborne disease microorganisms that cause infections, disease, or other health hazards and identify their biosafety levels?** A complete list of all regulated organisms appears in 42 CFR 72.3.

<http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi?TITLE=42&PART=72&SECTION=3&YEAR=1999&TYPE=TEXT>

*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.

*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.

*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.

*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.

*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.



*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.

*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.

*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.

Vibronic 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.

*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.

Bovine spongiform encephalopathy (BSE) cattle BSE prion BoPrP<sup>Sc</sup> - 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 described above, once human prions are passaged in mice and mouse PrP<sup>Sc</sup> 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.

- 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.

4. **Describe proper procedures for disposal of used media. Describe how to use and operate an autoclave safely.** All BSL-2 cultures, stocks, and other regulated wastes are autoclaved or otherwise decontaminated, to destroy any viable organisms. All materials for autoclaving are placed in red 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. All wastes are autoclaved. All bags autoclaved are to allow the heat and steam to effectively penetrate the wastes. Metal totes can be used to further circulate the heat for effectiveness. The autoclave is professionally serviced as scheduled by the laboratory and records are maintained of this service.

Proper PPE used with this process includes a rubber apron, heat and steam resistant gloves, and a face shield. Records are maintained of the destruction of any select toxins.

For BSL-3 organisms, a method for decontaminating all laboratory wastes is found in the local facility and utilized, preferably within the laboratory (e.g. 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.

The manufacturer's instructions for safe operation of the autoclave are readily found or

posted near the autoclave.

5. **What is a MSDS (Material Safety Data Sheet)? Where are the MSDSs located? What information can be found on a chemical reagent hazard label?** Material 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.

MSDSs 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.

6. **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.

7. **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.

8. **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.

**.9. What are administrative controls? 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.

10. **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.
11. **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.
12. **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.
13. **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 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.

14. **What kind of signage is to be in place in a microbiological BSL-2 laboratory?** A biohazard sign should be posted at the entrance to any laboratory where etiologic agents are used. The hoods and biosafety cabinets should have dated certification stickers. It is recommended that refrigerator and freezers have signs that the units are not to be used for food, flammables, or other unsafe storage. Any restricted entrance rooms should be posted. The animal area has special requirements for posting which should be followed.
15. **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.
16. **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.
17. **Has 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.
18. **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.
19. **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.
20. **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.
21. **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.

## 2.4 Quality Assurance

1. **What culture controls are used for an *Escherichia coli* enumeration analysis? For a *Staphylococcus aureus* analysis?** 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 is able to 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.

2. **What does RODAC mean?** R.O.D.A.C. stands for replicate organism detection and counting. These convex-surfaced contact plates are used for environmental monitoring of surfaces. A potential biohazard contamination exists when a sample container is opened for analysis. The contamination can occur from the environment to the sample. Laboratories that perform microbiological and sterility analyses need to demonstrate and monitor the bioburden in areas where analysis occurs.

The RODAC plate contains letheen agar which inactivates some disinfectants. It is pressed against a previously disinfected surface and incubated. Colonies are counted and gram stained, and compared to pre-determined action levels. There should typically be <10 colonies, no gram negative rods, no fungi.

3. **Why are temperatures recorded for incubators, water baths etc.?** Checks of the temperature of instruments such as incubators and waterbaths 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 waterbaths be checked daily.)

Microorganisms only grow at certain temperature ranges, and each has a particular optimum temperature. If an incubator or waterbath 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.

- 4. 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.

- 5. 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.

## **2.5 Food Pathogens and Indicator Organisms**

### **2.5.1 Salmonella**

- 1. 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 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).

- 2. Why is more than one type of 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 in order 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 in order 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.

- 3. 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 back 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).

- 4. 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.

5. **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.
6. **What is indole?** Indole is a component of the amino acid tryptophan. Some bacteria have the ability to break down tryptophan for nutritional needs using the enzyme tryptophanase. When tryptophan is broken down, the presence of indole can be detected through the use of Kovacs' reagent. Kovacs' 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.

7. **Can all serovars of *Salmonella* be identified with antisera? Give reason.** No. *Salmonella* is not identified with antisera alone. Identification calls for answers from biochemical reactions as well as serological reactions. When all of the pieces are put together, one has an identification. An examples is the two non-motile species (*S. bioaser Gallinarum*, and *S. bioaser Pullorum*). Both contain the same O antigens, but no H antigens. They are to be identified biochemically.
8. **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.

9. **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.

2	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
7	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
12	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
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

38	Soups
39	Prepared salads
54	Nutrient supplements, such as vitamins, minerals, proteins, and dried inactive yeast

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

10. **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 *Salmonellae* require a pH between 6.6 and 8.2.

11. **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* antigen 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, 4-methyl-umbelliferone. The intensity of the fluorescence is measured by the optical scanner in the VIDAS.

12. **Why does the sample need to be boiled before performing the VIDAS assay?** The culture needs to be boiled before performing the VIDAS assay in order 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.

13. **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.

## 2.5.2 *Listeria monocytogenes*

1. **What is unusual about the appearance of the motility of *Listeria*?** A characteristic motility of *Listeria* is described as tumbling.
2. **At what high temperatures and low temperatures will *Listeria* grow?** *Listeria* can grow between 2.5 and 44°C
3. **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>	+	- <sup>(a)</sup>
<i>L. ivanovii</i>	-	+
<i>L. innocua</i>	-	-
<i>L. welshimeri</i>	-	-
<i>L. seeligeri</i>	+	-

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

4. **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.
5. **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).
6. **Describe they 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.
7. **Describe how acid production from various carbohydrates (mannitol, rhamnose, xylose) typically differs between *Listeria monocytogenes* and *Listeria innocua*.**

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

<sup>b</sup> V, variable

8. **What role does the addition of selective agents in pre-enrichment and enrichment media play?** To increase the number of *Listeria* by minimizing background microflora.
9. **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*

### 2.5.3 *Escherichia coli*

1. **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-1 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.

2. **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.
3. **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.
4. **In the Tree nut Method, why is there a "rest period" between shaking of the original dilution?** The "rest period" is needed to allow the product to settle to the bottom of the dilution bottle, so that it will not be taken up into the pipette and alter the volume inoculated into media tubes.
5. **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.

6. **Describe the quadrant streaking technique to obtain isolated colonies.**

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:

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.

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.

Flame loop, rotate plate, and repeat procedure for quadrants three and four.

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

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

8. **What does a typical *E. coli* isolate look like on L-EMB?** Dark centered and flat, with or without metallic sheen

9. **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 4-methylumbelliferone (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 on the basis of gas production from lactose. *E. coli* are presumptively identified by fluorescence in the medium under longwave UV light

10. **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.

### 2.5.3.1 Enterohemorrhagic *E. coli* (EHEC)

1. **Do all *E. coli* strains ferment sorbitol?** No. Unlike typical *E. coli*, isolates of O157:H7 do



not ferment sorbitol.

**CAUTION:** Although most *E. coli* are sorbitol fermenters, about 6% of the isolates will not ferment sorbitol. These atypical strains may be found in foods and will appear identical to O157:H7 colonies on the TC SMAC agar.

- 2. What does TC SMAC stand for?** TC SMAC stands for Tellurite-Cefixime-Sorbitol MacConkey agar. Cefixime and Potassium tellurite are supplements added to Sorbitol-MacConkey agar to increase the selectivity for *E. coli* O157:H7 and suppresses the remaining accompanying flora.
- 3. Why is TC SMAC a better medium than HC agar for detecting *E. coli* O157:H7?** Comparative analysis of the TC SMAC procedure with the HC agar method using a variety of naturally contaminated and seeded foods showed that the TC SMAC procedure was superior to the HC agar method in the recovery of O157:H7 bacteria.  
(Weagant, S.D., J.L. Bryant, and K.G. Jinneman. 1995. An improved rapid technique for isolation of *Escherichia coli* O157:H7 from foods. *J. Food Prot.* 58:7-12)
- 4. What is the advantage of streaking at 6 and 24hr?** Advantages to adding a 6-hr streak in addition to a 24-hr streak are enhanced ability to identify Enterohemorrhagic *E. coli* in food matrices with a high background microflora that may overgrow and mask the O157:H7 colonies. Also, the 6-hr streak gives the advantage of a quicker confirmation of O157:H7 if colonies are isolated.
- 5. Describe the symptoms of hemorrhagic colitis and the onset time.** Hemorrhagic colitis is the name of the acute disease caused by *E. coli* O157:H7. The illness is characterized by severe cramping (abdominal pain) and diarrhea which is initially watery but becomes grossly bloody. Occasionally vomiting occurs. Fever is either low-grade or absent. The illness is usually self-limited and lasts for an average of 8 days. In some cases, individuals may exhibit watery diarrhea only. Onset time of symptoms after exposure ranges from 1 to 8 days but usually is 3 to 4 days

#### 2.5.4 *Staphylococcus aureus*

- 1. 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.
- 2. 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.

3. **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.
4. **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 as a result 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 found to occur together with a positive coagulase reaction and can thus serve as an index for the latter.
5. **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.

### 2.5.5 Coliforms

1. **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.
2. **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.
3. **What organisms are considered coliforms?** *Escherichia*, *Enterobacter*, *Klebsiella*, *Serratia*, *Citrobacter*, *Proteus*

## 2.5.6 Aerobic Plate Count

1. **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.
2. **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.
3. **Why do "spreaders" sometimes form when doing an APC? At the air/agar interface? At the agar/glass interface?** 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.
4. **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.
5. **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.
6. **What is the proper procedure for manually mixing dilution blanks?** Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 seconds.
7. **At what temperature should pour plates be dispensed?** Desired temperature is  $45 \pm 1^\circ\text{C}$ .
8. **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  $100/\text{cm}^2$ ), 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.

9. **How should the analyst interpret plates with less than 25 CFU's ?** When plates from both dilutions yield fewer than 25 CFU each, record actual plate count but record the count as less than  $25 \times 1/d$  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

## 2.5.7 Yeast and Mold Count

1. **Why are yeast and mold counts 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 are able to produce mycotoxins which are hazardous to human and animal health.
2. **What is the medium of choice for yeast and mold analysis?** Potato Dextrose Agar.
3. **What medium is especially useful for analyzing samples containing "spreader" molds?** Potato Dextrose-Salt Agar.
4. **What agent/agents are added to the agar to inhibit bacterial growth?** Antibiotics (Chlortetracycline-HCl, Chloramphenicol, or Streptomycin) or Sterile 10% Tartaric Acid.
5. **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.

## 2.5.8 *Vibrio*

1. **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 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.

2. **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.)

3. **Why is NaCl added to media used for *Vibrio* spp? *Vibrio* spp. other than *V. cholerae* and *V. mimicus* are halophilic and do not grow in media that lack NaCl.**

4. **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.
5. **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.
6. **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 (non-01/0139).  
*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 GM<sub>1</sub> 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*.

### 2.5.9 *Bacillus cereus*

1. **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 onset time usually between 1 – 5 hours. Implicated foods include but are not limited to fried or cooked rice and another starchy foods such as potatoes.

2. **Can *B. thuringiensis* produce diarrheal antigens? Diarrheal disease? Yes.**
3. **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*? *Staphylococcus aureus* intoxication.**
4. **The toxins of *B. cereus* most commonly associated with food poisoning are?** Diarrheal and emetic components.
5. **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.
6. **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.
7. **When interpreting test results in particular (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 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.

### 2.5.10 *Campylobacter*

1. **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.

2. **Describe methods used to obtain microaerobic conditions.** Microaerobic conditions can be achieved by bubbling with or shaking in a modified gas mixture of 5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>. 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
3. **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.
4. **Which biochemical test is used to differentiate between *C. jejuni* and *C. coli*?** Hippurate hydrolysis.
5. **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.)

### 2.5.11 *Yersinia*

1. **Describe the symptoms and onset time of *Yersinia enterocolitica* illness.** 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.
2. **Does *Yersinia* grow and survive during refrigerated storage?** *Yersinia* may survive and grow during refrigerated storage.
3. **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.
4. **Describe the biochemical characteristics of *Yersinia 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.
5. **What is the relationship of plasmids and *Yersinia*?** *Yersinia* spp. that cause human yersiniosis carry a plasmid (41- 50Mdal).
6. **What types of tests are used to determine pathogenicity?**  
There are a number of 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  
production of a series of outer membrane proteins at 35°C-37°C  
ability to produce conjunctivitis in guinea pig or mouse (Sereny test)  
lethality in adult and suckling mouse by intraperitoneal injection of live organisms

### 2.5.12 *Clostridium perfringens*

1. **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.
2. **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.
3. **Tell when each enumeration method would be use.** Laboratory capability will determine enumeration method. Analyst are to always utilize official validated methodology
4. **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 H<sub>2</sub> + CO<sub>2</sub> 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 O<sub>2</sub> is depleted from the system.
5. **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.
6. **Describe key characteristics of *C. perfringens* including motility, gram-reaction, nitrate reduction, and lecithinase activity.**

Non-motile

- Gram-positive spore-forming bacillus
- Reduce nitrates to nitrites
- C. perfringens* colonies on TSC/EY - 2-4 mm opaque, white zone surrounding
- Exhibits lecithinase activity

### 2.5.13 *Clostridium botulinum*

1. **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.
2. **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.
3. **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.
4. **Describe how the MLD is calculated.** Dilutions that kill and dilutions that do not kill are needed in order 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.
5. **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.

## 2.6 Viruses

1. **List two ways that viruses differ from bacteria.** Bacteria can reproduce autonomously while viruses are required to invade a host cell to reproduce. Bacteria can be readily enriched in culture medium while viruses require mammalian cells in tissue culture to propagate.
2. **What is the genetic material found in viruses?** Viruses can either RNA or DNA as their genetic material

3. **What step is required to be performed before performing PCR on RNA viruses?** RNA virus detection requires the use of reverse transcriptase prior to amplification in PCR reactions

## 2.7 Select Agents

1. **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.
2. **Name four areas of training necessary before handling select agents.** Physical containment, PPE, Biosafety Practices, Microbiological Characterization of Select Agents.
3. **Which agency regulates the use, possession and transfer of select agents capable of posing a serious public health threat?** The CDC.
4. **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.

## 2.8 Alkaline Phosphatase

1. **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.
2. **What is the reason for heating the control blank?** Account for any color development which may be due to reagent contamination and/or interfering color material in the sample.
3. **Why is a buffer used in the analysis? In the AOAC confirmation method, why are different buffer and precipitant concentrations used for different cheeses?** To maintain optimal pH of between 9.85 and 10.2 for the reaction to occur different strength buffers are used to match different types and ages of cheese.
4. **What parameters need to be controlled for the phenol to be liberated from the disodium phenyl phosphate substrate?** A pH range of 9.85 to 10.2 and incubation time.
5. **To what compound is the alkaline phosphatase enzyme activity proportional?** Phenol.
6. **Describe the reaction that takes place in order for phenol to be measured colorimetrically?** To phenol either 2,6-dichloroquinonechlorimide (CQC) for the screening method or 2,6 dibromoquinonechlorimide (BQC) for the confirmation method reacts in the presence of catalyst ( $\text{CuSO}_4$ ) to form indophenol blue.

## 2.9 Polymerase Chain Reaction (PCR)

1. **What are some of the benefits to using PCR to detect microbial pathogens in food? What are some of the potential problems to using PCR to detect microbial pathogens in foods?** 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.
2. **What are the three major steps (processes) involved in the PCR cycle?** Denaturation, annealing (complementary binding of primers), and extension.
3. **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.
4. **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

5. **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.
6. **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.  
Reference: [http://biology.uoregon.edu/Biology\\_WWW/Biospheres/Spring95/keller.html](http://biology.uoregon.edu/Biology_WWW/Biospheres/Spring95/keller.html)
7. **Why does one need an excess quantity of primers in the reaction mixture?** 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.

8. **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-tetramethyl-rhodamine, TAMRA) added at any T position or at the 3' end. As long as 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.

Reference: <http://www.lsc.psu.edu/stf/naf/Quantitative.html>

9. **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.

10. **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.

11. **In what ways can PCR be inhibited?** 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.

## 2.10 Pulsed Field Gel Electrophoresis (PFGE)

1. **Describe briefly the preparation of agarose plugs.** Prepare cell suspensions of each isolate, utilizing Cell Suspension Buffer and reaching the correct turbidity. Make sure that the plug agarose is completely melted, and tempered to the correct temperature before using. Mix correct amounts of cell suspension, lysozyme, and plug agarose quickly. Dispense into labeled plug wells without the introduction of air bubbles.
2. **What are the conditions for plug lysis?** Shaking water bath, 54°C, vigorous shaking, at least two hours shaking.
3. **Describe the steps needed for plug washing.**

Preheat water and TE buffer to 50°C. Warm shaking water bath to 50°C.

Two water washes, 15 minutes, vigorous shaking, 50°C.

Four TE washes, 15 minutes, vigorous shaking. 50°C.

4. **What restriction enzyme is used for *E. coli* and *Salmonella*?** Both methods utilize *Xba I*.
5. **What are the two methods for loading plugs into the gel wells?** Plugs can be loaded onto the gel comb before pouring the gel, or they can be inserted into the cast wells of the solidified gel.
6. **Briefly describe equipment set up for running a PFGE gel.** Running buffer: 2.2 liters of 0.5X TBE, pre-cooled to 14°C and circulating in leveled gel box at a setting of 70. Gel on base plate setting in gel frame in gel box with wells toward top of gel box. Electrophoresis unit parameters set for correct organism.
7. **What is the chemical agent used to stain PFGE gels, and what safety precautions are to be used to handle this agent?** Staining is done using ethidium bromide, which is toxic and mutagenic. It should always be handled with care, wearing gloves. All waste should be disposed of as hazardous, including gels.

## 2.11 Canned Food and Can Seam Examination

1. **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.

- 2. 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 so the can remains flat, hence the name “flat-sour”. Flat-sour organisms include *Bacillus thermacidurans* (*Bacillus coagulans*) and *Bacillus stearothermophilis*, both being thermophilic bacteria, growing and causing flat-sour spoilage at elevated temperatures (50-55°C).
- 3. 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: H<sub>2</sub>S 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 sufficient amounts of gas to swell the can.

- 4. 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.
- 5. 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.
- 6. 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.
- 7. Explain water activity and its significance in an acidified and low acid canned food.**

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  $>0$

## 2.12 Cosmetic Analysis

- 1. 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.** 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.
- 2. What is the purpose of dilutions and other added ingredients?** Dilutions and other added ingredients partially inactivate preservative systems commonly found in cosmetic products.
- 3. 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.
- 4. 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.
- 5. 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).

## 2.13 Sterility of Drugs and Medical Devices

1. **What is the purpose of the bacteriostatic/fungistatic test?** What is the inoculation level in CFUs to be used to inoculate product in media? The B&F is the validation step for the USP Sterility test. One inoculates between 10-100 CFU.
2. **Describe what is done to ensure the work area is acceptable for sterility testing?** The area is wiped with 70 % filtered sterilized EtOH before working. The area is uncluttered and exposed to the HEPA filtered air. Opened media controls and exposure plates are prepared in advance and used at the time of analysis.
3. **What should one do if the air sample plates grew several different kinds of organisms?** Inform the supervisor immediately of this finding. He/she will advise the analyst to identify the microorganisms and record them on the worksheets or in the web application.
4. **When would the incubation time be extended to 30 days?** If the product was sterilized by irradiation.
5. **What determines if a product is a SVP or LVP?** According to the USP a Small Volume Parenteral (SVP) is a product volume of 100 ml or less, A Large Volume Parenteral is a product volume greater than 100 ml.
6. **What is the purpose of using two different media?** The SCD broth is used to culture aerobic bacterial and yeast and mold. The thioglycollate broth is used to cultivate anaerobic or microaerophilic bacteria.
7. **How does Fluid Thioglycollate maintain anaerobic conditions?** Sodium thioglycollate lowers the oxidation-reduction potential of the media and the low percentage of agar reduces the diffusion of oxygen into the liquid media
8. **What is the function of the red indicator in Fluid Thioglycollate?** The red indicator (resazurin) indicates the status of the oxidation or aerobiosis of the broth.
9. **What is meant by the D value, Z value, F° value?** D-value - Time at a given temperature needed to reduce the number of microorganisms by 90% (one logarithmic reduction). Z-value - The number of degrees temperature change to change the D value by a factor of ten. F° value - The equivalent time at 121°C delivered to a container for the purpose of sterilization.
10. **How is a sterility test by membrane filtration different from a sterility test by direct**

**transfer? A Millipore Steritest system would be used for which of these methods?**

Sterility test by membrane filtration allows the liquid drug product to pass through a membrane filter to collect the microorganisms from the product and subsequently cultured in an enrichment broth. In Sterility testing by direct inoculation the product is added directly to the enrichment broth. The Millipore Steritest system is a membrane filtration method.

- 11. What is the difference between Fluid A, D, and K? When would one use one over the other?** Fluid A is 1 gram of peptic digest of animal tissue in water to make 1 liter. Fluid D is the same as Fluid A except it contains 1 ml of polysorbate 80 added to the 1 liter. Fluid K is 5 grams of peptic digest of animal tissue, 3 grams of beef extract and 10 gram of polysorbate 80 added to 1 liter of water.

## 2.14 Microbial Limits Test

- 1. What are the controls used in this experiment?** Controls used are the following microorganisms: *Candida albicans*, *Aspergillus niger*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*.
- 2. What are some ways to neutralize inhibitory substances?** The following are examples of ingredients and their concentration that may be added to the culture medium to neutralize inhibitory substances that may be present in the sample: soy lecithin, 0.5% and polysorbate 20 4.0%.
- 3. 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.
- 4. 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.
- 5. What is the preparatory test?** 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.