USDA Food Safety and Inspection Service
U.S. DEPARTMENT OF AGRICULTURE

Understanding and Evaluating Microbiological Sampling and Testing

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Today's Presentation

- Sampling methods
- Assessing sampling plans and testing methods
- Method validations and laboratory quality assurance
- FSIS testing programs, methods and pathogen-specific issues to consider
- Industry testing activities

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Sampling Methods

- Destructive sampling – grab, cloth, N60 (excision)
  - e.g., RTE, ground comminuted products, egg products, carcass excision
- Non-Destructive sampling
  - Typically chosen when destructive sampling not an option
  - Examples:
    - Cloth
    - Carcass rinsates or sponge
    - Parts rinsate
    - Environmental sponge

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Cloth Method

- Products in combo bins / boxes / totes
  - Randomly select one combo bin from the specific production available for sampling
  - Use one cloth to sample the surface of the combo bins / boxes / totes
- Products in boxes, totes, tubs, or containers other than combo bins
  - 1 cloth for up to 5 containers from the same lot of product
  - Flip the cloth when you are half way around a combo bin or swabbed half of the number of boxes / totes
  - 45 to 60 seconds – total sampling time per lot will usually be between 1:30 to 2 minutes

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What is N60?

- N60 = number of samples (n) = 60
  - Multiple representative samples provides best option for detecting scattered contamination
  - Provides 95% confidence that no more than 5% of food pieces the size of each "n" in the entire lot are contaminated
- Keys to success
  - Must ensure that sampling is as representative as possible across the lot
  - Large composite "N60" samples typical need a larger test portion

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Common Sampling Problems

- Small sample or sampling method may not be ideal for detection
  - *e.g.*, small swab device or environmental area sampled
- Sanitizer or residual antimicrobial chemicals might interfere with the test
  - Insufficient drip time prior to carcass sample collection
  - Excessive liquid carryover for parts sample collection
- Temperature abuse for the sample prior to testing
  - Holding under refrigeration for long periods allows competing bacteria to grow
  - Freezing can kill some pathogens (*e.g.*, *Campylobacter*)

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Assessing Sampling Plans

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Sampling Methods

- All sampling plans have significant limitations
  - Relative rigor of the sampling program must be evaluated
- Best sampling plans provide the opportunity but no guarantee of detection
  - i.e., scattered contamination is difficult to detect
- Frequent sampling and sampling multiple sites/time points provides a better opportunity for detection
  Examples:
  - Multiple samples per day vs. once per month
  - N60 per lot vs. one grab sample per lot
- Does the type of sampling meet the intended need?
  - Destructive vs. non-destructive sampling

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Sampling Plans

Statistical sampling plans assume:

- Uniform manufacturing conditions
- Equal probability of contamination throughout the lot (homogeneous distribution)
- Independent, random sampling (equal probability of sampling throughout the lot)

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Why are Pathogens Hard to Detect?

- They are typically not evenly distributed
- They occur at low levels
- They are often injured when found in the product
- Detection may be inhibited by material in the food product (food matrix)
  - Example: high amounts of fat may inhibit PCR assays; spices, salt, acidulants can affect isolation and detection

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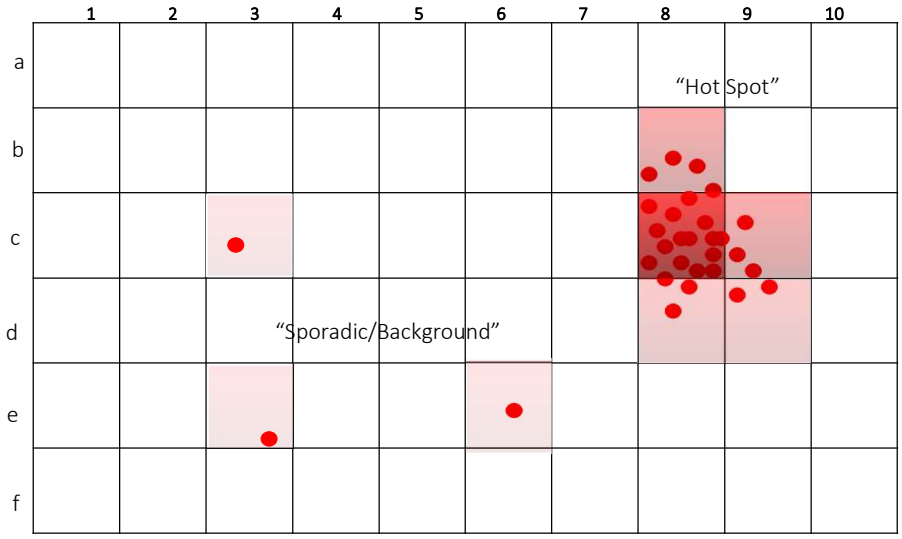
E. coli O157:H7 Contamination in a N60 Sampled Lot



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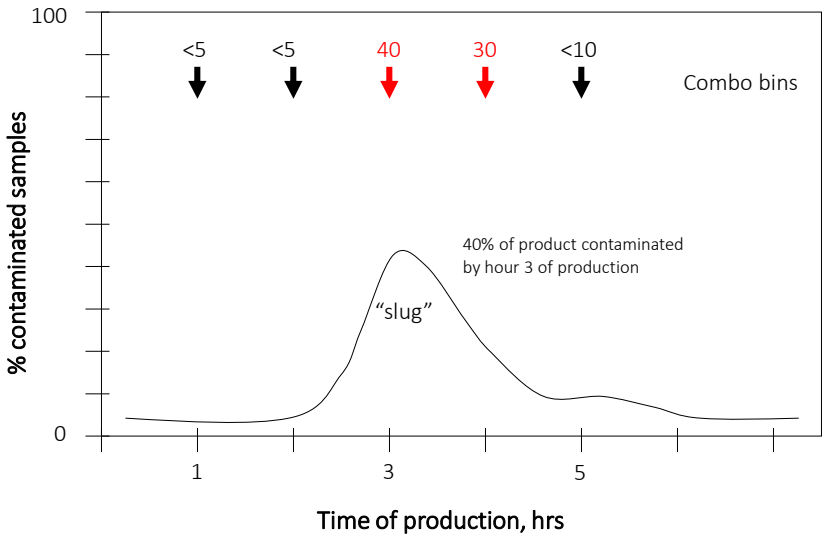
E. coli O157:H7 Contamination in Ground Beef



Assessing Testing Methods

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Key Players for Ensuring Robust Testing Methods

- The establishment that needs the testing
- The laboratory they hire
- The manufacturer of the screening test they use
- The organization validating the screening test

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Steps in Detection Methods

- Sample collection
- Sample preparation
- Enrichment for the pathogen
- Screening of the pathogen
- Confirmation of the pathogen

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Considerations for Testing Methods

- Is the method fit for the intended purpose of the analysis?
- Has the method been optimized and experimentally validated for sensitive detection of pathogens?
- Is the laboratory complying to the validated method protocol?

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Assessing Fitness for Purpose

- Is the test portion appropriate to meet the need?
- Is the method enrichment-based with the intent to detect the lowest possible numbers of stressed pathogen cells?
- Has the food matrix been validated for the method used?
- Are confirmation procedures appropriate for determining true negative samples?

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The "Test Portion"

- Laboratory sample preparation => "test portion"
  - "analytical unit" or "analytical portion"
  - Definition: the part of the "sample" that is actually tested by the laboratory
- The test portion determines the theoretical (*i.e.*, best possible) sensitivity of the test
  - *e.g.*, 1 cell/test portion
  - 25-gram test portion: detecting 0.04 cells/gram is possible
  - 325-gram test portion: detecting 0.003 cells/gram is possible

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Enrichment

- Test portion is incubated 8-48 hours in a culture broth
  - Why?
    - Contamination levels are too low for detection without enrichment
    - Must grow to high levels so very small volumes have enough pathogen present for later detection steps
- Different pathogens require different enrichment media (broth)
  - One vs. two-stage enrichment
- Primary enrichment vs. secondary enrichment
  - Resuscitation vs. selective growth

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Considerations for Proper Enrichment

- Resuscitation (lag phase) can require 2-3 hours before log-phase growth begins
  - Some samples support slower growth
- Has enrichment broth been tempered to warm temperature prior to incubation?
  - Particularly critical for large test portions or shorter incubation periods

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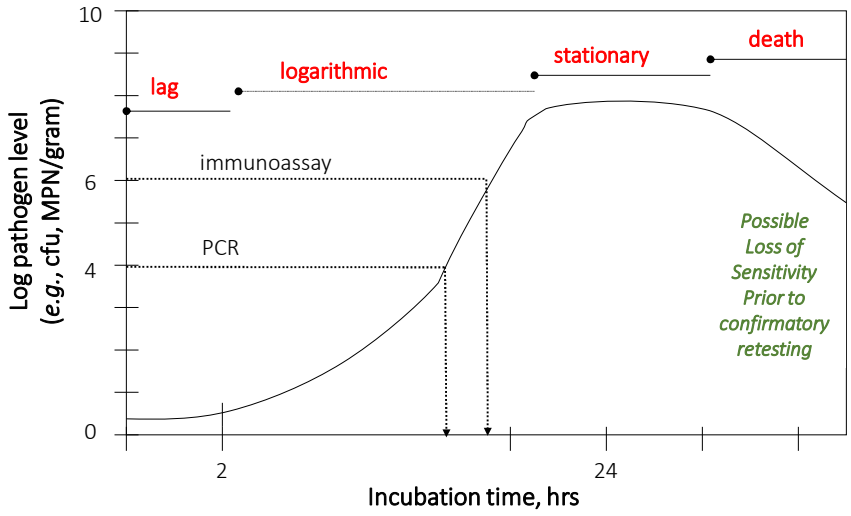
Pathogen Growth During Enrichment



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Enrichment Period

- Different screening tests require different levels of enriched pathogen
- Shorter incubation periods (<15 hours) may warrant additional scrutiny of laboratory compliance to the validated protocol
- Has enrichment/screening combination been validated for a larger test portion?
  - Particular concern for large test portions incubated for shorter periods - *e.g.*, 375-gram test portion incubated for 8 hours
- Proposed incubations <8 hours may warrant OPHS review

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Confirmatory Testing

- Non-culture confirmation (*e.g.*, PCR)
- Culture confirmation (*e.g.*, FSIS confirmation)
  - Plating the enrichment on selective and differential agar media
  - Immunomagnetic separation (IMS) necessary prior to plating for *E. coli* O157:H7 and non-O157 STECs
    - Suspect colonies = "presumptive positive"
- Purification and confirmatory identification tests including:
  - Biochemical (*e.g.*, identifies "*E. coli*")
  - Serological (*e.g.*, identifies "O157" and "H7")
  - Genetic (*e.g.*, identifies "*stx*" = Shiga toxin genes)

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Concerns for Confirmation

- Do not re-sample the lot or sample reserve!
- Non-culture confirmation
  - Same considerations as the screening test
  - Used under validated conditions
  - Transport and storage of enrichment
- Culture confirmation- carefully assess!
  - Typically expect that methods comply with a validated procedure (*e.g.*, MLG, FDA-BAM, ISO)
  - Small changes can affect ability to recover pathogen of interest

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Quantitative Testing

Two options:
- MPN
- Direct plating

NOTE: Quantitative testing typically cannot accommodate larger test portions and provide the opportunity for detection that a qualitative test can provide

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Most Probable Number (MPN) Enumeration Analysis

- Traditional enrichment-based analyses are performed on three or more dilutions, each typically in triplicate, from a single sample homogenate (*i.e.*, MPN = method format, not a specific method per se)
- Advantages:
  - Better sensitivity (lower LOD) than direct plating
- Disadvantages:
  - Very resource intensive/expensive
- Application:
  - For quantifying low levels of pathogens (*e.g.*, *Salmonella*, *E. coli* O157:H7, *L. monocytogenes*)

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Direct Plating Enumeration Methods

- Product is homogenized in diluent and small volume is directly dispensed onto agar media (*i.e.*, sometimes there is a 1-2 h "resuscitation" step, but enrichment is never used prior to plating)
- Advantages:
  - Allows easy inexpensive quantitative analysis
- Disadvantages:
  - Accommodates only a very small test portion
  - Higher LOD (*i.e.*, often 100 CFU/g) not suitable for detecting low levels of pathogens
- Application:
  - Expedient for higher level analytes (*e.g.*, indicators, *Campylobacter*, *S. aureus*, *C. perfringens*, *B. cereus*)

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Method Validations

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Value of Validation

- Determines performance characteristics of the method in comparison to a gold standard (reference) method (*e.g.*, usually FSIS or FDA method)
- Independent evaluation provides credibility
- Rigor varies (multilab vs. single lab, # tests, etc)
- Still must consider fitness for purpose and how the method is applied
  - *e.g.*, some AOAC-validated methods are not consistent with FSIS goals or Compliance Guidelines

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Method Validation

- Recognized independent method validation organizations:
  - Government: FSIS (MLG) and FDA (BAM)
  - AOAC International (U.S.A.)
    - AOAC Official Methods of Analysis (OMA) validations
    - AOAC-RI "Performance Tested Method" validations
  - AFNOR (France)
    - *e.g.*, bioMerieux Vitek biochemical confirmation tests
  - Others (ISO, MicroVal, NordVal, AENOR, etc.)
- However, past validations conducted by these organizations may not be relevant to larger test portions or other testing scenarios

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Foodborne Pathogen Test Kits Validated by Independent Organizations

- FSIS maintains a list, updated quarterly, of methods that have been validated by independent organizations
  - https://www.fsis.usda.gov/sites/default/files/media_file/2021-05/Validated-Test-Kit.pdf
- None of the test kits listed are implicitly approved by USDA FSIS
  - A validated test kit must also be fit for purpose and appropriate for the specific application in a food safety program

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Process for Validating Qualitative Pathogen Methods

- Series of laboratory experiments using inoculated samples under controlled conditions
- Inoculate portions with pathogen strain at very low level where only 20-80% of samples are positive (*i.e.*, fractional recovery)
- Statistically compare percent of positive samples in alternative method to reference method (FSIS MLG)

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Considerations for Validation Data

- Was method compared to an appropriate reference method (*e.g.*, FSIS MLG; FDA)?
- If not performed by AOAC, AFNOR, etc., is supplemental validation data available?
  - May require additional scrutiny

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Testing Method Specifications

- Sensitivity: probability that truly positive samples are detected as positive by analytical test
  - 100 – false negative rate
- Specificity: probability that truly negative samples detected as negative by analytical test
  - 100 – false positive rate
- Level of detection (LOD): lowest level of contamination reliably detected by analytical test
  - LOD expressed as ratio of organisms to quantity tested material (*e.g.*, CFU per gram, MPN per mL, CFU per square-ft) but definitions vary (e.g., LOD95, POD)

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Factors Impacting Detection and Method Specifications

- Detection as measured by sensitivity, specificity, and LOD can vary based on:
  - Specific strains of pathogen
  - Intrinsic factors for the sample matrix
    - Levels of competing bacteria
    - Fat, salt, pH and additives
- Experimental design for the validation study (*e.g.*, cell stress, etc.)

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Complying with the Validated Protocol

- Do AOAC/AFNOR/ISO citations match the protocol in use?
  - Modifications are common, and some contribute to greater potential for false negative result
- Compare the lab procedure to the validated protocol (*i.e.*, package insert)
- If culture confirmation is used, verify that it follows validated method as well

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Methods not Validated by Recognized Organizations

- "Supplemental" or "extension" validations
- *E. coli* O157:H7 and non-O157 STEC testing for 325-375g test portions
  - Modifications required for AOAC validated procedures based on 25g
  - Instructions for sample preparation may not be clear for the lab

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Laboratory Accreditation and Quality Assurance

- ISO 17025 = protocol for establishing and documenting a microbiology laboratory quality program (*i.e.*, "HACCP" for labs)
- Accrediting bodies = A2LA and others
- Accreditation implies robust quality program but does not necessarily indicate methods meet FSIS expectations
  - Laboratories are able to perform the methods they use as expected, but methods are not "accredited" to be fit for purpose
- Laboratories are not required to be ISO accredited, but should have quality assurance programs that ensure results are reliable and accurate

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FSIS Testing Programs

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FSIS Microbiological Sampling Program Objectives

- Assess effectiveness of industry process controls
- Provide critical feedback to industry
- Monitor compliance with performance standards, zero-tolerance policies
- Allow FSIS to monitor industry-wide trends
- Serve as a strong incentive to reduce the occurrence of pathogens in products
- Capture pathogen characterization information (*i.e.*, serotype, speciation, antimicrobial resistance, whole genome sequencing)

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FSIS Sampling Programs

- Sampling plans measure compliance with performance standards:
  - *Salmonella* and *Campylobacter* verification programs (raw poultry)
- Zero-tolerance policies for food pathogens
  - *E. coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli* (non-O157 STEC) (raw non-intact beef or components of raw ground beef)
  - *Listeria monocytogenes* in RTE and pasteurized egg products and on food contact surfaces
  - *Salmonella* in RTE and pasteurized egg products

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FSIS Methods and Pathogen-specific Issues to Consider

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Shiga Toxin-Producing E. coli (STEC) Testing

Includes:

E. coli O157:H7 and the six non-O157 Shiga toxin-producing *E. coli* (STEC) (non-O157 STEC) - O26, O45, O103, O111, O121, and O145

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O157 STEC Program

- Strain must have:
  - O157(+)
  - *stx*(+) OR *stx*(-) and H7(+)
  - biochemical(+) or Bruker MALDI Biotyper
- Currently FSIS only analyzes beef manufacturing trimmings (MT60) for non-O157 STECs
- FSIS plans to expand non-O157 STEC verification testing (85 FR 34397; June 2020):
  - Ground beef (MT43), bench trim (MT65), raw ground beef components other than trim (MT64)
  - Responding to comments; final rule; grace period, etc.

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Non-O157 STEC Program

- Six non-O157 STEC = O26, O45, O103, O111, O121, O145
  - Strain must have:
    - *stx*(+) and *eae*(+) genes
    - one of the six O-groups
    - biochemical(+) or Bruker MALDI Biotyper
  - Currently FSIS only analyzes beef manufacturing trimmings (MT60) for non-O157 STECs
    - Phased rollout – MT65 – MT64 – MT43

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E. coli Top Seven STEC Analysis (MLG 5C.03)

Day 1	Sample Prep and Primary Enrichment 42°C±1 for 15-24 hours	Enrichment
Day 2	Perform PCR All samples that do not test PCR negative are carried forward for further analysis Immunomagnetic Bead Capture & Rainbow Agar Plating	Screening negative or potential (+) – stx(+) eae(+) O group(+)
Day 3	Latex Agglutination & Sheep Blood Agar Plating	Negative – no growth, agglutination (-) or agglutination(-)/rapid screen(-) presumptive (+) – agglutination(+) and rapid screen (+) or inconclusive
Day 4 / 5	Perform -stx/eae gene analysis -latex agglutination & genetic serological test - Bruker MALDI Biotyper	

Confirmation – next page

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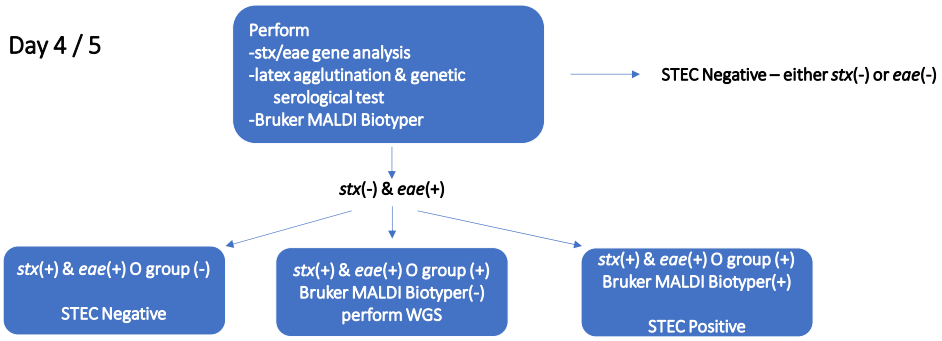
E. coli Top Seven STEC Analysis (MLG 5C.03) - continued



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Larger *E. coli* O157:H7 and Non-O157 Test Portions

- Larger test portions (325-375 grams) are important for composite (grab) samples when multiple samples are collected throughout the production day
- Methods must be adapted, optimized and validated for effective use with 325-375 gram test portions

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E. coli O157:H7 and Non-O157 STEC Testing Concerns

- Supplemental validation and special instructions for testing larger test portions
  - For enrichment periods <15 hours
  - 325-375g test portions typically often require longer minimum enrichment period than 25g
- Culture-based detection and confirmation requires immunomagnetic separation (IMS)

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Listeria testing

Includes:

L. monocytogenes testing (FSIS)
Listeria-like or *Listeria* spp. testing (industry)

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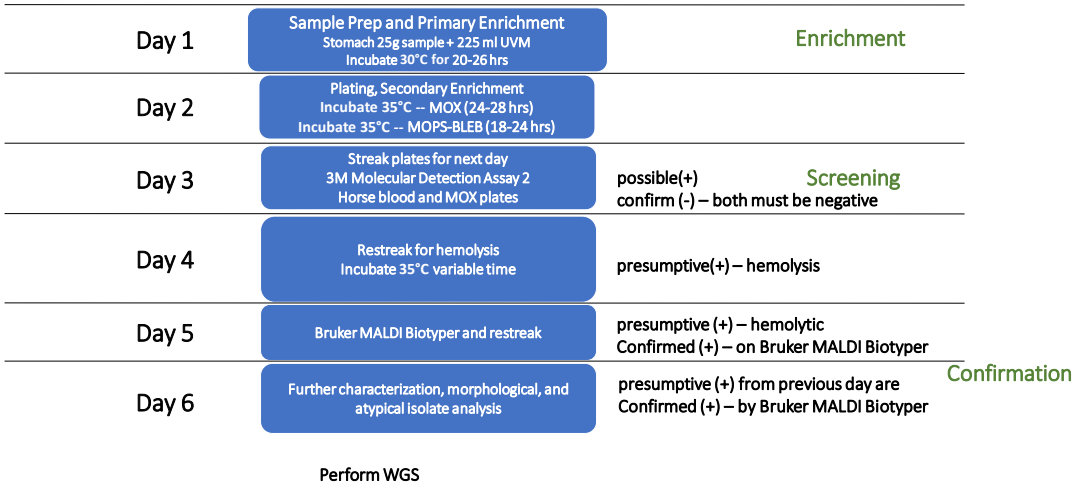
Listeria monocytogenes (MLG 8.13)



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Expectations for *Listeria* Environmental Testing Equivalence

- Compliance Guidelines – "Controlling *Listeria monocytogenes* in Post-Lethality Exposed Ready-To-Eat Meat and Poultry Products" (FSIS-GD-2014-0001; Jan 2014)
- For optimal sensitivity of detection, method for food contact surface testing must:
  - Validated by a recognized body (*e.g.*, AOAC, AFNOR)
  - Be enrichment-based
  - Enrich the entire sponge/swab sample
    - *e.g.*, an aliquot from sponge/swab does not provide opportunity to detect bacteria trapped in the sponge

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Analytes for Industry Food Contact or Environmental Surface Testing

Establishment laboratories test for one of the following:

- *Listeria monocytogenes*
  - Use internationally recognized **enrichment-based method** that biochemically confirms culture as *L. monocytogenes*
- *Listeria* spp.
  - Use internationally recognized **enrichment-based method** that uses ELISA, PCR or other screening technology to provide more rapid but less specific *Listeria* spp. result
- "*Listeria*-like" indicator bacteria
  - Use the first part of an internationally recognized enrichment-based method to find suspect *Listeria* colonies (*e.g.*, darkened colonies on MOX using the FSIS method)

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Salmonella Testing

- Raw products
  - Meat and turkey carcass sponge samples
  - Chicken carcass/parts rinsates
  - Raw meat and comminuted poultry
- Processed products
  - RTE (325 g portion)
  - Pasteurized egg

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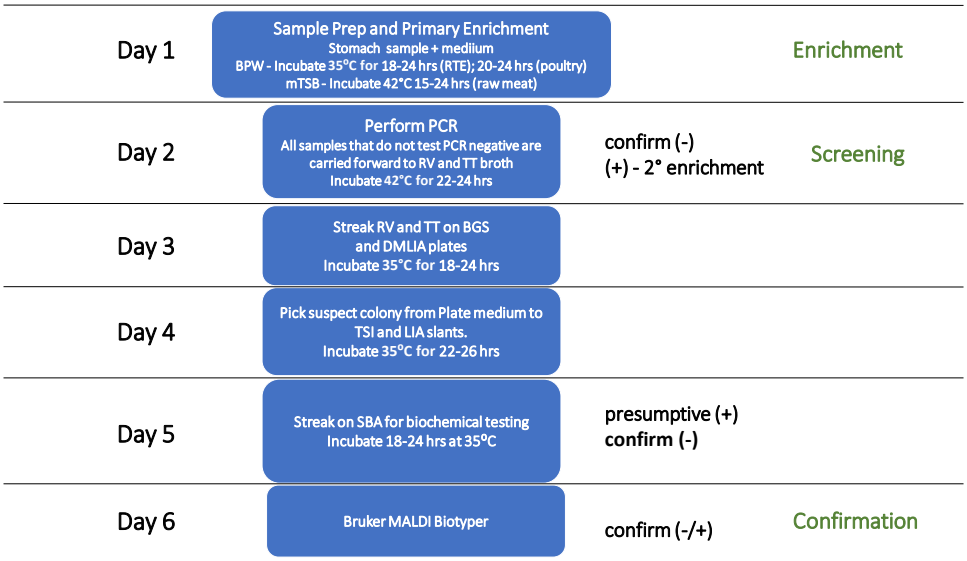
Salmonella (MLG Ch. 4.13)



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Campylobacter Testing

Qualitative

- Enrichment-based (as opposed to direct plating) since Aug 27, 2018 - exception: "other raw chicken parts" (EXP_CPT_OT01 and LO_CPT_OT01)

Targets

- *C. jejuni*, *C. lari* or *C. coli*

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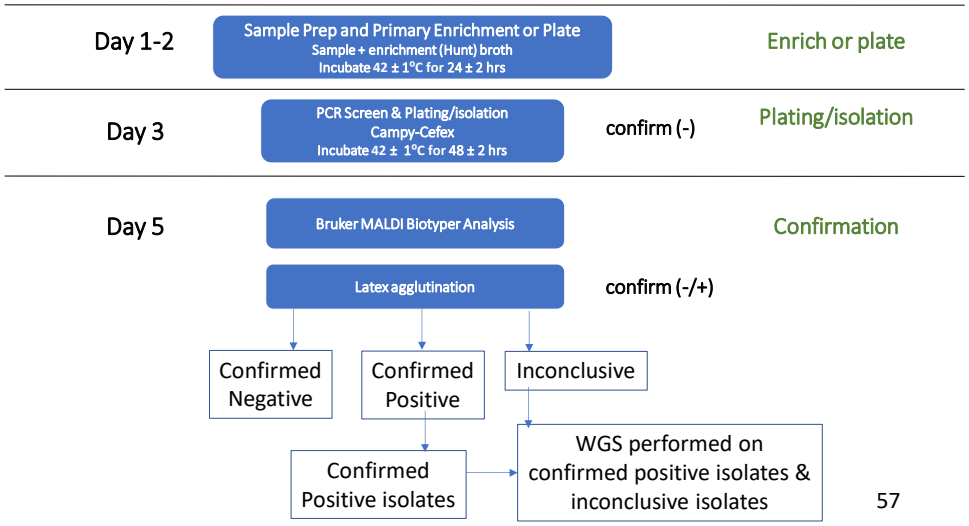
Campylobacter (MLG 41.07) - Qualitative



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Issues for *Campylobacter* Testing

- *Campylobacter* is highly vulnerable to freezing
  - Do not freeze samples
- Can be a challenging test (inconsistent results across labs)

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Industry Testing Programs

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Establishment Documentation for Testing Methods

- Does the establishment have the necessary documentation?
  - Can the establishment provide the method used for microbial detection?
  - Can the establishment provide evidence that the method used was properly validated by an independent body?
  - Can the establishment explain why the method fits the need?

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Issues for Industry Labs

- On-site vs. off-site labs
  - Shipment of samples/handling during shipment
- Overarching concerns for on-site labs
  - Is testing effective?
  - Is testing safe in that facility?
    - Enrichment of pathogens in an establishments
- Evaluate the following:
  - Are personnel qualified?
  - Does the lab have proper equipment and materials for testing and disposal of contaminated media?
  - Do they follow the validated testing protocol?

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Establishment Responsibilities for Laboratory Testing

- The establishment is ultimately responsible for the testing they request from private laboratories
- Has the establishment properly conveyed testing needs?
  - *e.g.*, test portion equivalent to FSIS as opposed to the default 25 g in protocols
- Is the laboratory aware of FSIS expectations?
  - Directives, Notices and guidance
- Establishment should provide documented detailed methodology and validation information for FSIS review

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FSIS Verification of Establishment Sampling and Testing Programs

Effectiveness verified by FSIS

- Reviews/observations of EIAOs during FSA
- Establishment provides supporting documentation
- Technical and policy support provided through askFSIS
- Establishment, not laboratory, is responsible for implementing effective program

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FSIS Verification of Establishment Sampling and Testing Programs

Focus of FSIS' evaluation

- Is the method fit for the intended purpose?
- Does the method support the hazard analysis decisions?
- Is the method comparable to the appropriate FSIS method (or is there justification for an alternative)?
- Is a comparable or appropriate test portion used?
- Is the method validated and used under validated conditions?
- Does the laboratory assure the quality of the results?

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