Non-Confirming Enrichments: The Dreaded False Positive (Or Is It?)

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Alex Brandt, Ph.D.
Chief Science Officer
Food Safety Net Services

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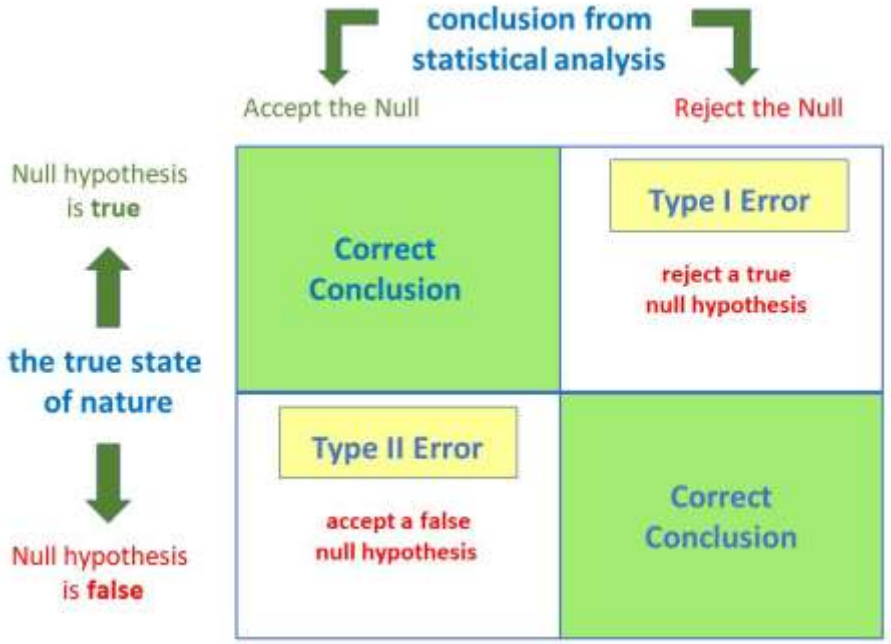
AGENDA

- Testing Errors
- Definition of False Positive
- Potential Reasons for False Positives
  - Non-Specificity of Assay
  - Non-Viable Cells
  - Viable But Non-Culturable Cells
  - Confirmation Challenges
- Case Studies
  - *E. coli* O157:H7
  - *Salmonella*
  - *L. monocytogenes*

Types of Testing Errors

- Opposite of Statistics
  - H_0 – Sample is Positive
  - H_a – Sample is Not Positive
- Type I Error: False Negative
  - Reporting a negative test result when the sample was truly positive.
- Type II Error: False Positive
  - Results indicate a positive test result when the sample was truly negative.



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Types of Testing Errors

- Which of these is the "worst" to have?
- Do these definitions only apply at the laboratory level?
- Preventing a Type I error can also help to prevent a Type II error…

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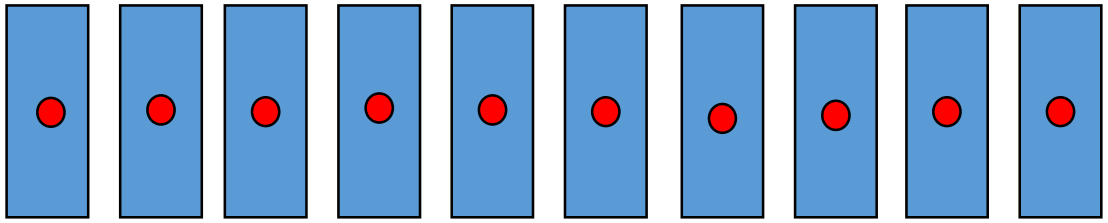
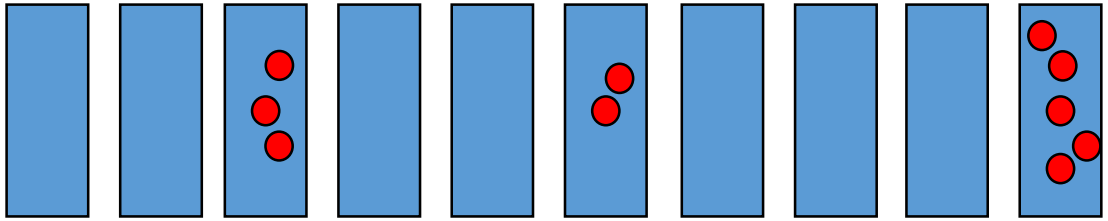
Major Factors for Testing Overall

- Definition of a “lot.”
- Sampling of the lot - is it representative?

IDEALLY…



REALITY…



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Major Factors for Testing Overall

- Lab Testing Processes
  - Aseptic technique throughout the process.
  - Lab sampling of test unit.
  - Correct media used (we are trying to grow bacteria).
  - Correct incubation used (optimal for growth).
  - Correct testing assay used.
  - Having trained personnel processing samples.
  - Reporting accurate results.

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General Considerations

- In general, with a sample that screens positive and culturally confirms negative, we have to look outside the bounds of the traditional “false positive” concept.
- Is the cultural confirmation method really a “gold” standard? Or more bronze?
- If a molecular method is more sensitive (e.g., higher copy numbers per cell, particularly with RNA methods where a cell can carry 100s to 1000s of copies of the target in each cell depending on the matrix and enrichment conditions) then is it really a “false positive” if it does not confirm culturally?
- “Non-Conforming Microbes”

OR?

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Non-Specific Binding of Primers and Probes

- Amplification of the target genetic element in a closely related non-target organism.
- Selecting a target in a coding region is ideal due to less likelihood of mutations occurring.
- It is important to select a diverse inclusivity panel for the target pathogen and a rigorous exclusivity panel (i.e., the most closely related non-target organisms) during assay development.
- In some cases two sets of primers/probes or locked nucleotides may be needed for differentiation, although locked nucleotides may be cost prohibitive.

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How Can Bacteria Share Genes?

- Gene transfer between microorganisms.
- Although it is known that horizontal gene transfer contributes to species differences, strains of the same species can differ by as much as 30% in genome composition.
- This finding has led to a perspective in which microbial genomes are composed of a core set of vertically inherited genes that are common throughout the species and a set of variable genes that are acquired horizontally and can be unique to a given strain.

It was on a short-cut through the hospital kitchens that Albert was first approached by a member of the Antibiotic Resistance.

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Screening Assay Challenges

Selection of the “Correct” and “Unique” genetic sequence.

Continued Genetic Diversification Example:

- In *Escherichia coli* strain MG1655 (Blattner et al., 1997), it was estimated that nearly 18% of its 4290 open reading frames (ORFs) were introduced in at least 234 transfer events and that the majority of the acquired genes appeared in this lineage relatively recently (Lawrence and Ochman, 1998).
- Coupled with the fact that natural isolates of *E.coli* may differ by nearly a megabase (i.e. 1,000,000 bp) in genome size (Richmond et al., 1999; Tao et al., 1999), these findings suggest that gene content is highly variable within this species, with divergent strains having gained and deleted very different arrays of genes.

E. coli and _Salmonella_ approximately 5 million base pairs

Listeria approximately 3 million base pairs

Campylobacter approximately 1.6 million base pairs

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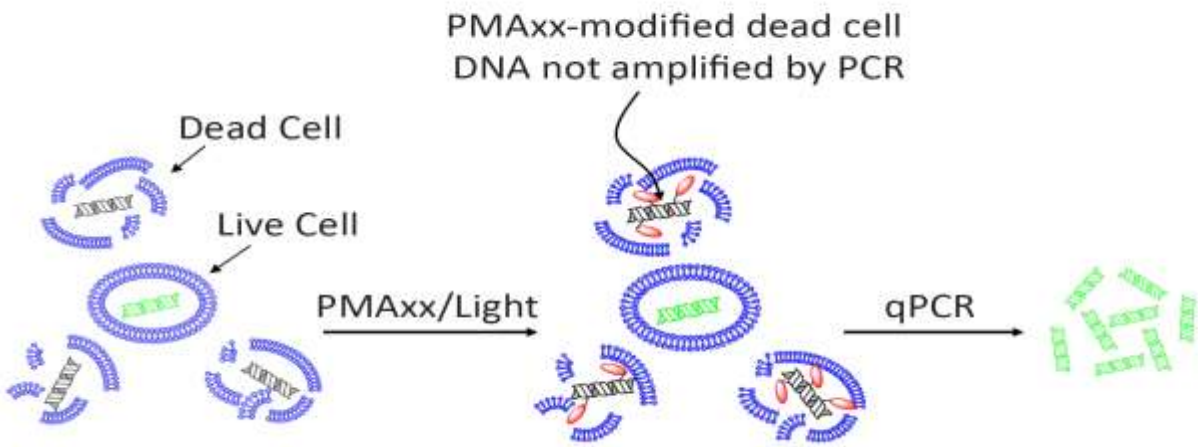
Amplification Of DNA From Non-Viable Cells In The Matrix

- Likely only an issue with samples that initially contained high levels of the pathogen before going through a lethality step (e.g., spices, rendered product, pet food, etc.).
- Can be managed with modifications to protocols such as the addition of PMA (Propidium monoazide) which binds to DNA that is not inside of live cells and prevents the PCR reaction from occurring on that DNA.



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Viable But Non-Culturable (VBNC)

- Refers to bacteria that are in a very low state of metabolic activity and do not divide, but are alive and have the ability to multiply once resuscitated under the right conditions. Then can cause disease.
- Typically, do not grow on standard growth media.
- Can become this way due to stress: **(all your plant interventions!)**
  - Limited nutrients
  - Temperature extremes
  - Limited oxygen
  - Chemical damage
- Morphologically Smaller
- Reduced Nutrient Transport Into the Cell
- Reduced Rate of Respiration
- Reduced Rate of Macronutrient Metabolism

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Species Known To Enter VBNC State

- *Campylobacter* (*coli*, *jejuni*, *lari*)
- *Enterococcus* (*faecalis*, *hirae*, *faecium*)
- *Escherichia coli*
- *Klebsiella* (*aerogenes*, *pneumoniae*, *planticola*)
- *Listeria monocytogenes*
- *Pseudomonas* (*aeruginosa*, *syringae*)
- *Salmonella enterica*
- *Shigella*
- *Vibrio*
- *Yersinia pestis*

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What To Do With VBNCs?

- Allow additional time to grow in the culture media.
- Additional regrowth with other media to resuscitate.
- Screen sample with alternative more sensitive methodology to provide additional information.
- With congruent results from other screening methods, can we accept the results as they are?
- Develop a decision tree with the concept that this is a potentially recurring event; it could happen again…

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Lab Interference

- DNA amplicon contamination with free DNA from the entire genome or from specific amplified sequences.
- Cross-contamination from controls or client sample positives (normally due to pipetting challenges, poor handling of positive controls).
- Contamination of transfers if applicable: poor techniques, gross contamination of enrichments bags, poor lab sanitation.

Confirmation Challenges

- **Selective Media Challenges**
  - Media may be too selective for injured cells.
  - Media may not provide correct nutrients for all strains within a certain group of diverse organisms.
  - Chromogenic and color variances.
- **Colony Selection**
  - Shape and overall morphology.
  - Over crowded plates (mixed culture when selecting).
  - Isolation challenges.
- **Growth Rates**
  - Not every bacterial strain grows at same rate.
  - Competitive inhibition (what else is in the enrichment?).

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Confirmation Challenges, Cont.

- **Extended Storage Prior to Confirmation**
  - Possible loss of genetic targets located on mobile elements (e.g., *stx* genes on prophages or targets carried on plasmids being cured by selective agents in enrichment media).
  - Die off of organisms.
- **Immunomagnetic Separation Overwhelmed**
  - May not bind the target if background organism levels are high.
- **Number of Colonies Selected**
  - Sometimes if you just pick more colonies or deviate from the standard cultural confirmation you can eventually tease out a cultural confirmation.
- **Cells Numbers Too Low In Enrichment (45-50 Amplification Cycles for PCR Assays Can Detect Low Levels).**
- **Homogenization Issues of the Enrichment**

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Selective Media Challenges

- Example: Not all *Salmonella* are the same and do not always metabolize nutrients in the same manner.
- Variance in growth rates among serotypes.
- Media that is too selective can inhibit growth of the target microbe.
- Less selectivity allows for too many competitor organisms to grow and possibly crowd plates or just plain outgrow the target organism that is in the enrichment.

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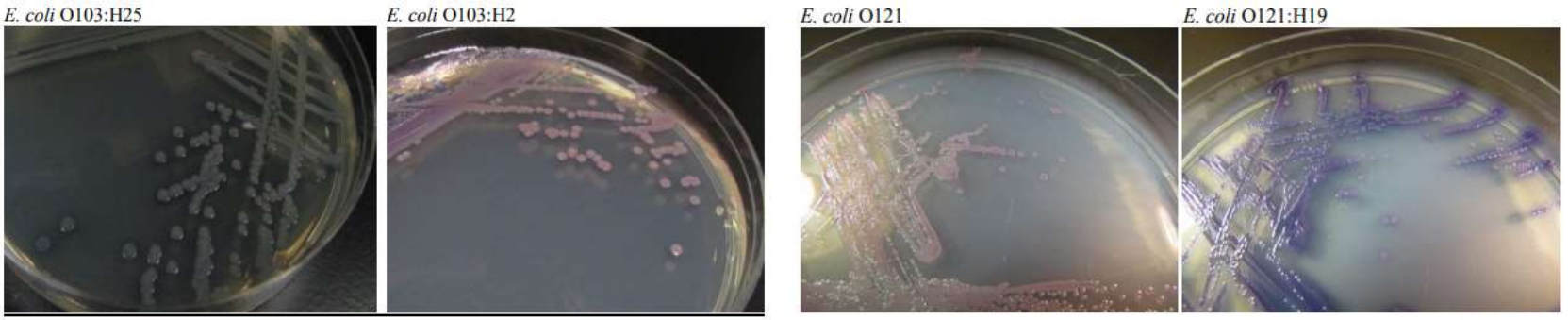
Picking The Right Colony…

- **Color of Colony**
  - With chromogenic media there can be shades of colors
  - Color variance due to pH change in and around the colony
- **Shape and Overall Morphology**
- **Overcrowded Plates**
- **Isolation**
- **Variability in Degree of Hemolysis**



VARIOUS STECs ON RAINBOW AGAR

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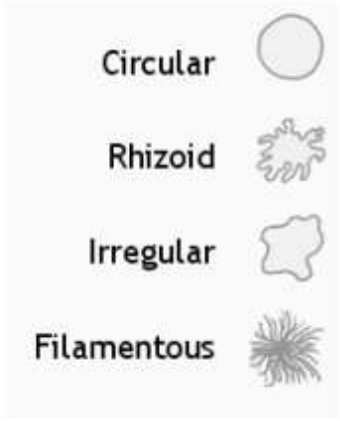
Colony Morphology Factors

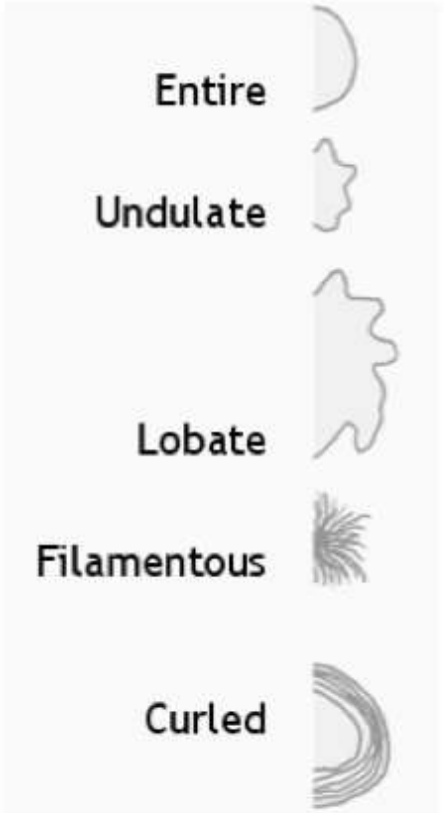
Colony Size

Punctiform: <1 mm
Small: 1-2 mm
Medium: 3-4 mm
Large: >5 mm

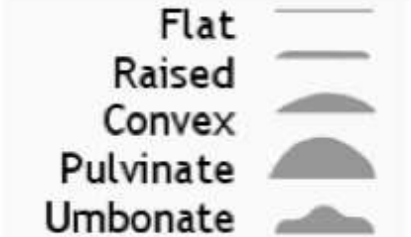
Colony Forms



Colony Margins



Colony Elevations



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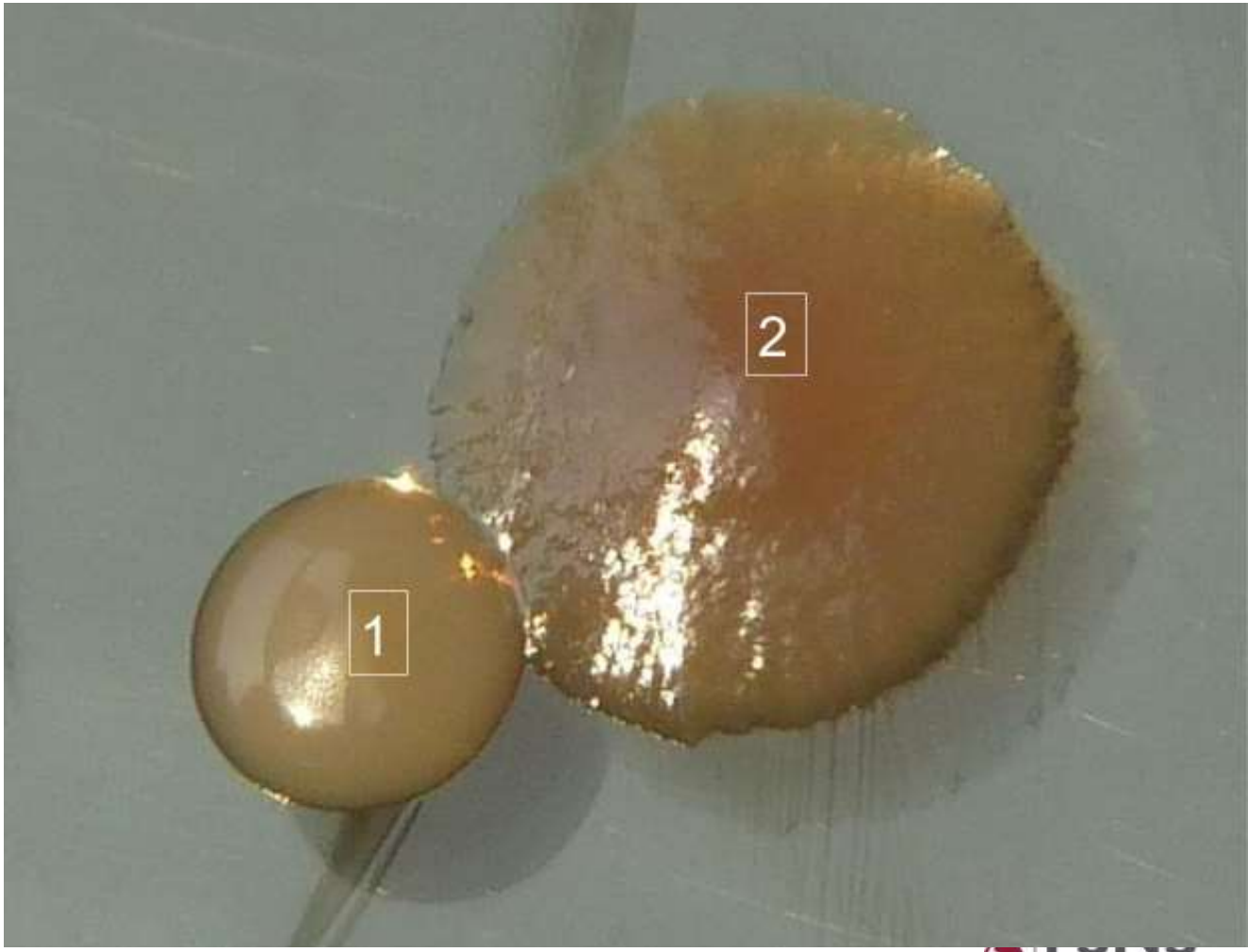
Morphology and Proximity

Which One Do You Pick?



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Case Studies: The Real World

The Case of the *Listeria monocytogenes* positive late crosser which did not confirm culturally (repeatedly) and tested negative for *Listeria spp.* by PCR

All assays have false positive and false negative rates

Often a trade off between false negatives and false positives

Situation recurred for 2 years: **$50,000 per each false positive, based upon down time and investigational samples while waiting for cultural confirmation**

Kit manufacturer recommended this approach in this case after extensive investigation and discussion

Retest all questionable PCR melt curve results with 4 lysates from the same sample enrichments in place of cultural confirmation

Precedent: USDA MLG (USDA's Microbiology Laboratory Guidebook, e.g. section 4.6.a, regarding *Salmonella in cases where data is suspect)*

Math
2% false and false negative rates for a molecular assay
Assay detection limit is based upon 10,000 CFU/ml of enrichment (detects 7.5 cells per ml)
Probability of false negative in this case (poison statistics): 0.06% with the current assay per manufacturer

IF sample sensitivity is set lower than designed (e.g. 1000 cfu/ml -<1 cells per ml after dilutions) probability of false negative is 0.47%

4 assays in a row after SUSPICIOUS amplification curve, then probability of missing Listeria when have 4 subsequent negatives is 0.474 or <5% a level 10 X below the designed assay detection level.

KORNACKI MICROBIOLOGY SOLUTIONS, INC.

Salmonella Case Study

The Situation: 40 lots of beef trim screened positive for *Salmonella* using a PCR platform. All were subjected to cultural confirmation per customer specifications. All samples confirmed NEGATIVE.

The Problem: Would expect at least 75-85% cultural confirmation rate on the first attempt using standard USDA MLG confirmation procedures.

What Happened: Conducted an intense investigation:

1. **Subjected all samples to two other DNA tests, one RNA test, and one Immunoassay. All samples were POSITIVE. This indicated that specificity and sensitivity of the original assay was good.**
2. **Subjected all samples to confirmation in three other laboratories where a variety of other plating, temperatures, and times were used to culturally confirm. An isolate was obtained on about 50% of the samples.**
3. **Remaining samples were plated to non-selective media and different colony morphologies were selected and tested with the *Salmonella* DNA test. All positive colonies were then confirmed.**
4. **Eventually 100% of samples were confirmed.**
5. **WGS of isolates showed that isolates from all samples were identical *Salmonella* Dublin.**

Conclusions: Molecular screen was accurate. Cultural confirmation was not accurate. *Salmonella* in some cases would not grow on certain media. They needed more time and lower temperatures. Determined to be *Salmonella* Dublin, which is known to have challenges.

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E. coli O157:H7 Case Study

The Situation: Beef slaughter client testing for *E. coli* O157:H7 with a PCR platform requested that MLG cultural confirmation be performed on every sample that screened positive on the PCR platform.

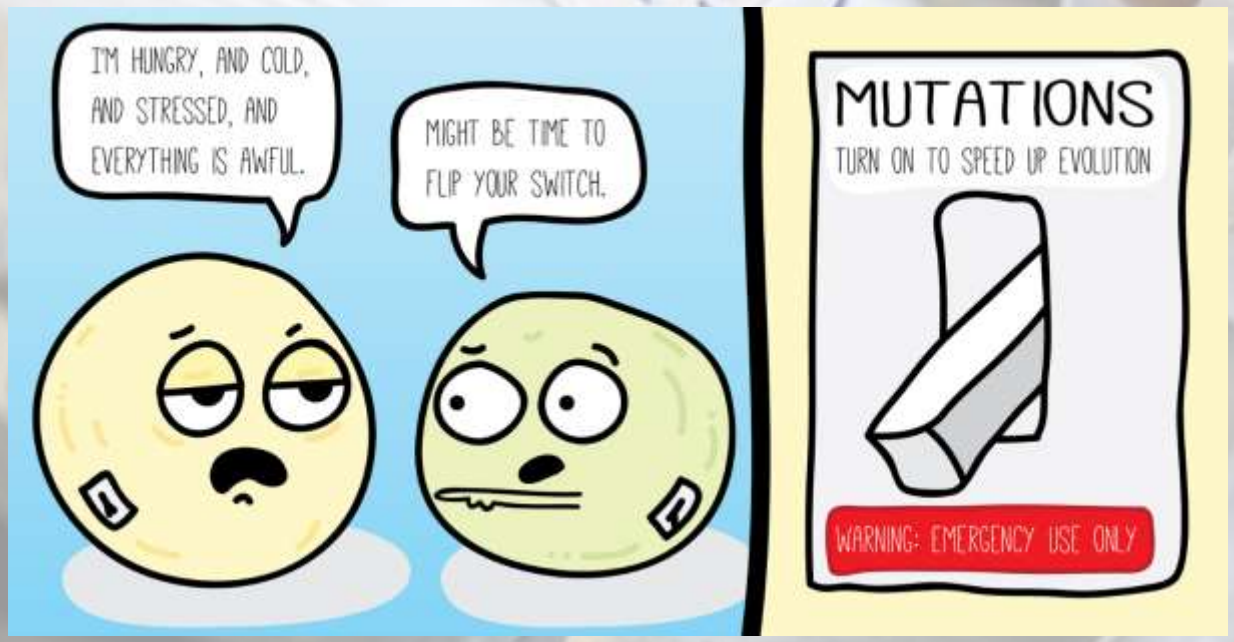
The Problem: Normal screen positive rates (0.27%), but cultural confirmation rates were low (61%). Would expect ~80%. Client began to question PCR platform performance and laboratory practices.

What Happened: Used additional molecular confirmation techniques to determine what was going on.

1. Subjected 225 enrichments to Neogen NeoSeek STEC Identification. Also performed MLG cultural confirmation on these samples. Cultural confirmation found 138 positives (61%).
2. Neogen NeoSeek STEC Identification added 23 additional samples that did not originally culturally confirm positive. Improved confirmation rate to 72% (increase of 11%).
3. Demonstrated that cultural confirmation was missing a significant percentage of pathogenic *E. coli* O157:H7. Maybe due to shortcomings of the media, picking colonies, etc.
4. An additional 73 samples had a non-*stx E. coli* O157:H7 signal. Previously believed that non-stx *E. coli* O157:H7 were rare (~1%). This shows they are common (~32%).
5. In all, an *E. coli* O157:H7 signal of some kind was found in 210 out of 225 samples (93%).

Conclusions: Cultural confirmation had shortcomings (i.e. the gold standard may not be that golden…). PCR assay was accurately finding *E. coli* O157:H7, but there was more non-stx *E. coli* O157:H7 in the population than ever previously expected. Thus, are now using a molecular confirmation to screen for *stx* after initial *E. coli* O157:H7 positive result is obtained to make disposition.

Remember… Microbiology Is Always Evolving…



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