Intro on new tests and how to pick new tests

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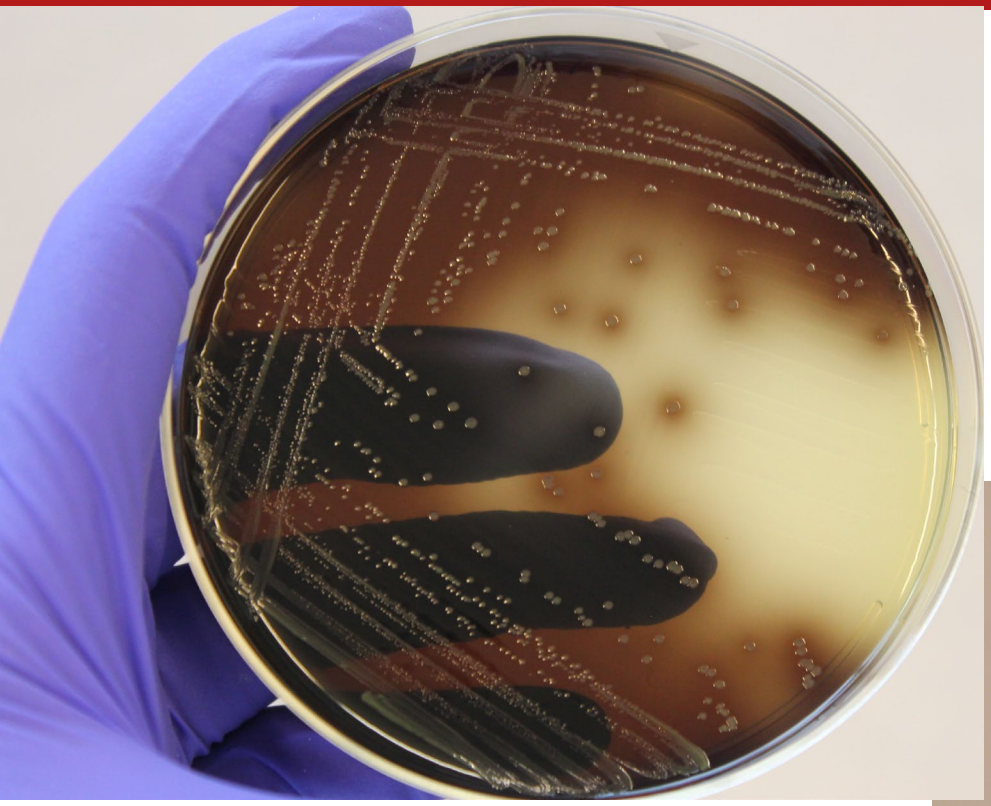
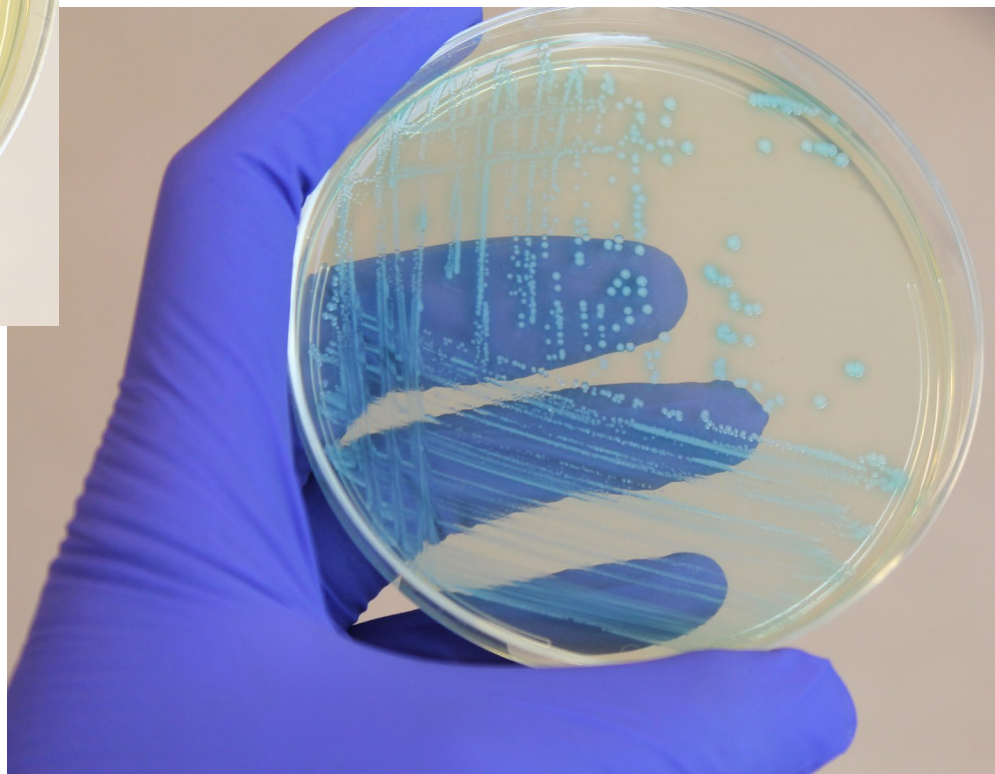
Traditional detection methods

- Traditional microbiological methods use enrichment and plating, followed by confirmation methods to detect foodborne pathogens
  - Time consuming
  - False negatives as an issue
  - Cross-reactors (false positives): Citrobacter can be mistaken as *Salmonella*
- Some enhancements have been made to these methods
  - Colorimetric media



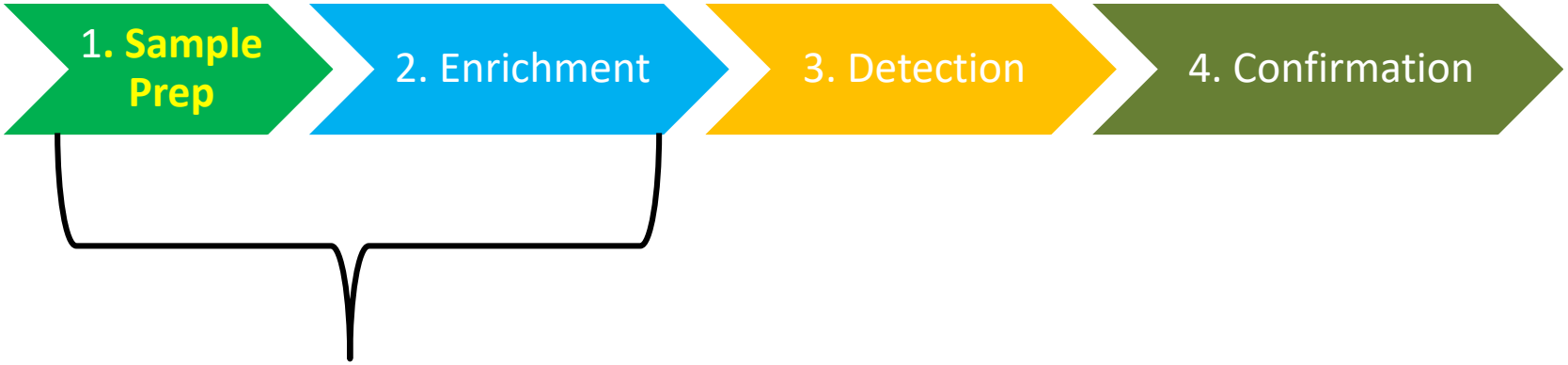


Rapid detection methods

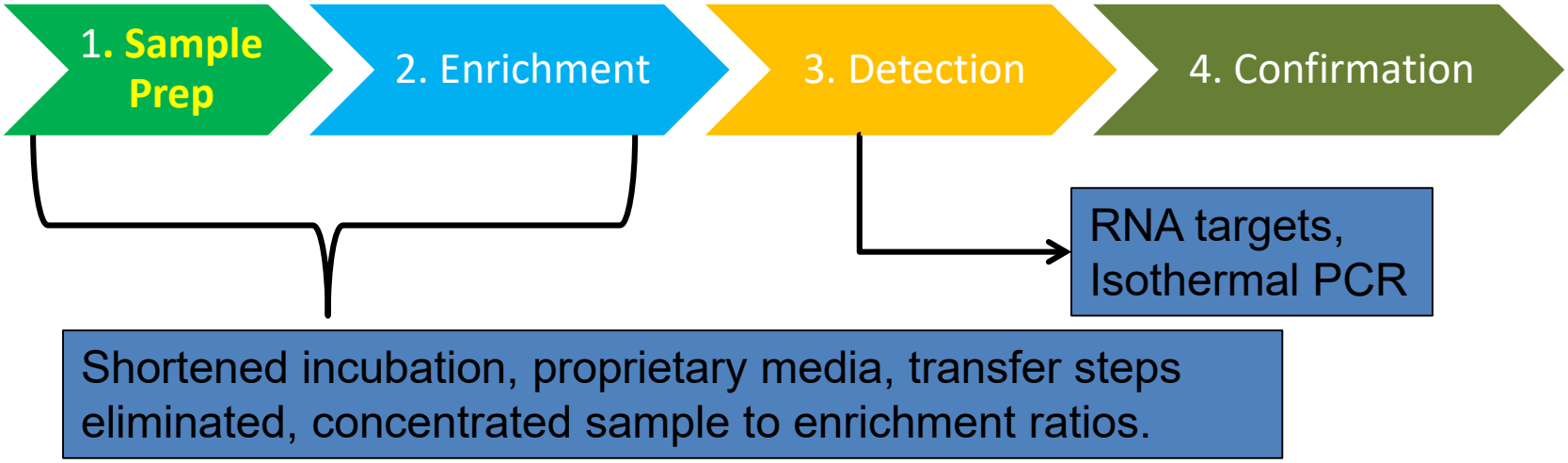
- Immuno-based detection methods (e.g., Vidas)
  - Uses antibodies
  - Sometimes issues with “cross-reactors”
- DNA-based assays
  - Typically PCR-based
  - Can detect dead cells, but typically only if found at high levels
- RNA-based assays
  - The urban myth??
  - Most target rRNA, some may target mRNA
  - rRNA is stable and may still yield detection of dead cells
- Phage-based assays (e.g., Sample6)

Everyone wants a faster test – is there a free lunch??

- Historically rapid methods followed reference method sample prep and enrichment protocols
  - Detection step (3) is the only difference from the reference method: rapid screen replaces streaking all enrichments to agar plates.



- Today, companies are competing for faster time to results
  - Often this means the development of optimized enrichment approaches coupled with new detection technologies



- What does this mean for Rapid Method applications?
  - Validation approval very specific to the matrices and analytical weight tested as part of the validation study

Evaluation of new (rapid) detection methods

- New methods are typically evaluated by certain evaluation schemes (for example AOAC, AFNOR, ISO, etc.)
  - Includes exclusivity and inclusivity testing
    - Inclusivity testing is typically done with different matrices (typically only 1 strain/matrix)
- Standard evaluations do not necessarily stringently evaluate matrix and strain effects
  - Specific matrices (e.g., certain types of cheese) and specific types of competitive microflora may affect assay sensitivity and specificity
  - Certain strains or strain-matrix combinations may be problematic

Inclusivity testing

- Tests the ability of a method to detect all members of the target groups
  - Does the rapid method detect all Salmonella serotypes, including weird stuff like *Salmonella bongori*
  - Typically with 100 Salmonella or 50 isolates for other pathogens
- For some validation schemes (e.g., AOAC), test kit manufacturer gets to choose the 50 (or 100) strains they test
  - Should we worry about that?
- Concentration of strains used to assess (100 times LOD as a target…)
  - A method may detect all 100 *Salmonella* at 10,000 CFU/g, but what about 100 CFU/g
- 100% inclusivity is the norm
  - Martin's comment: *You can give me virtually any method and I will give you 100% inclusivity*

Table 31 – Inclusivity Results – BAC*Gene Salmonella* spp. Method after enrichment in BPW 16 h at 37°C or BPW 18h at 41.5°C (1)

N°	Strain		Reference	Origin	BPW 16 h at 37°C			BPW 18 h at 41.5°C		
					Inoculation level (CFU/225ml)	BAC*Gene Salmonella* spp. CFX96 Touch™ (Cq)	BAC*Gene Salmonella* spp. AriaMx (Cq)	Inoculation level (CFU/225ml)	BAC*Gene Salmonella* spp. CFX96 Touch™ (Cq)	BAC*Gene Salmonella* spp. AriaMx (Cq)
1	*Salmonella*	Aberdeen	CIP 105618	Human	2	+(20.46)	+(19.05)	12	+(17.97)	+(18.51)
2	*Salmonella*	Abony	CIP 8039	Unknown	3	+(19.41)	+(19.67)	15	+(19.02)	+(17.94)
3	*Salmonella*	Agona	A00V038	Feed for pork	5	+(20.64)	+(18.82)	17	+(18.02)	+(18.73)
4	*Salmonella*	Anatum	A00E007	Dusts	8	+(21.06)	+(19.41)	6	+(18.21)	+(18.18)
5	*Salmonella*	*arizonae* (IIIa) *50:z4,z23*	CIP 5526	Egg powder	1	+(21.92)	+(20.40)	5	+(21.65)	+(22.14)
6	*Salmonella*	*arizonae* (IIIa) *51:z4,z23*	CIP 5523	Turkey meat	12	+(21.25)	+(19.99)	23	+(19.06)	+(19.86)
7	*Salmonella*	Bareilly	Ad 1687	Chocolate industry	4	+(20.27)	+(19.22)	15	+(18.23)	+(19.20)
8	*Salmonella*	Bardo	Adria 569	Meat for sausage	4	+(21.18)	+(19.98)	7	+(28.84)	+(18.40)
9	*Salmonella*	Berta	CIP 105682	Unknown	9	+(18.38)	+(19.68)	18	+(17.23)	+(17.53)
10	*Salmonella*	Blockley	Ad 923	Poultry environment	2	+(18.31)	+(18.69)	22	+(17.96)	+(18.32)
11	*Salmonella*	*bongori* (V) 66 :z35	Ad 599	Environmental sample	9	+(30.82)	+(36.53)	35	+(30.37)	+(29.81)
12	*Salmonella*	Bovismorbificans	Adria 6629	Sausage	5	+(18.86)	+(19.39)	24	+(18.44)	+(18.85)
13	*Salmonella*	Brandenburg	Ad 351	Seafood cocktail	5	+(20.63)	+(18.99)	26	+(18.25)	+(19.22)
14	*Salmonella*	Braenderup	Adria 111	Pork meat	7	+(20.77)	+(19.37)	37	+(17.99)	+(18.23)
15	*Salmonella*	Brazzaville	CIP 54141	Unknown	3	+(20.56)	+(19.98)	12	+(19.17)	+(18.96)
16	*Salmonella*	Bredeney	Adria 396	Ground beef	2	+(20.32)	+(20.03)	33	+(18.65)	+(18.78)
17	*Salmonella*	Carrau	CIP 105619	Pork	0*	-	-	8	+(18.53)	+(18.83)
					9	+(20.32)	+(18.67)	-	-	-
18	*Salmonella*	Cerro	Ad 689	Dehydrated poultry proteins	1*	-	-	21	+(18.40)	+(18.16)
					30	+(20.04)	+(19.31)	-	-	-

Exclusivity testing

- Tests the ability of a method to yield negative results with non-target bacteria
  - Does a rapid method that is designed to detect Salmonella serotypes yield negative results with other bacteria (focus in closely related bacteria)
  - Typically tested with 50 non-target bacteria; Target level is growth limit of the organism
- For some validation schemes (e.g., AOAC) to test kit manufacturer gets to choose the strains they test
- 100% exclusivity is the norm

Matrix Evaluation

- 30 Samples each for Rapid Method and Reference Method for each food matrix
  - 5 Uninoculated
  - 20 Sub-fractional Inoculation: 0.2-2.0 cfu
  - 5 High Inoculation: 5.0 cfu
- Organisms are stressed according to the product type
  - Cold, Heat, Drying
- Following inoculation, samples must be held prior to enrichment
  - 2 weeks for ambient, frozen matrices
  - 48-72h for refrigerated
- Pass/Fail overview of criteria
  - All high pass
  - Sub-fractional between 25-75% positive
  - Statistically perform as good or better than the reference method.

AOAC OMA – Example of Validation Claim

Salmonella in Selected Foods	
Method Type	Qualitative Microbiology
Kit Name	BAX system Start-Up Package
Company Info	DuPont Qualicon, Wilmington, DE 19810, USA
Analyte	Bacteria/Salmonella
Analytical Technique	BAX® Automated System
Equipment	Polymerase Chain Reaction Technology (PCR)
Matrices	Hot Dogs and Frankfurters/Frankfurters, Beef/Raw Ground Beef, Chicken/Raw Ground Chicken, Fish/Raw Frozen Tilapia Fish, Fruit Juices/Orange Juice, Cheese/Mozzarella Cheese
Approved By	AOAC
Method Number	2003.09

Validation claim lists the matrix types included in the validation of a Food Category.

AOAC Validation information can be found on: www.aoac.org

Should you trust AOAC validated methods?

Regulatory References

- FDA-BAM, Appendix 3: Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed. "**either a verification process or additional validation studies will be required before any given validated method can be used to test a food not included in the original method validation.**"

- USDA FSIS Directive 5100.1 Rev. 3 Attachment 1, Section 1.a.i. states, "**The establishment is ultimately responsible for ensuring that the sampling and testing method meet the needs of its food safety program.**"

- FSMA **requires food manufacturers use validated methods that have been shown to be "fit for purpose".**

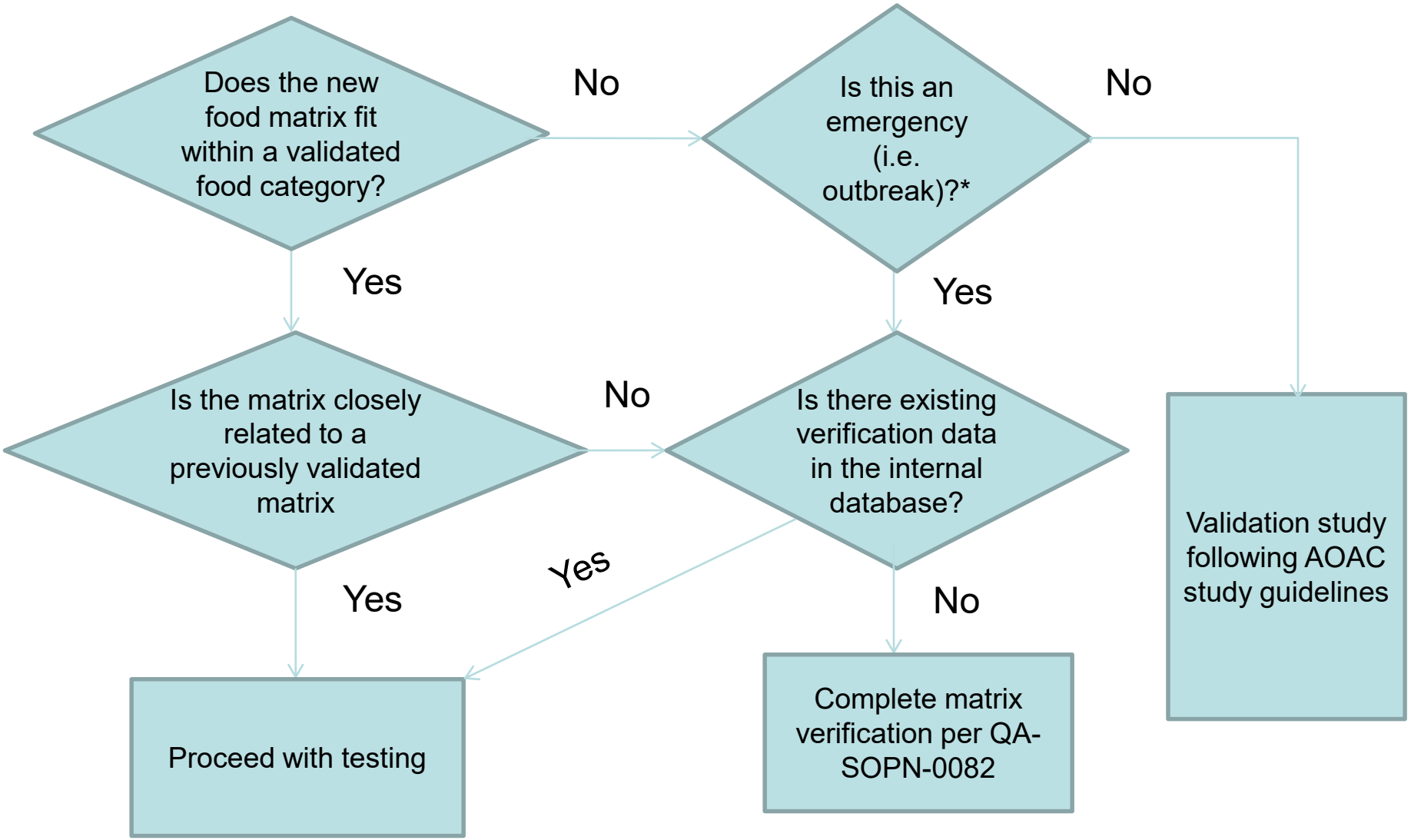
The top reasons why you may not want to "trust" AOAC (etc.) validated methods?

- Your food matrix has not been validated
- You want to use sample sizes/analytical weights the method has not been validated for (375 g versus 25 g)
- Your products have unique characteristics
  - French raw milk blue cheese with high Staph levels
- Your ingredients come from countries and areas which have weird *Salmonella* serotypes
- Companies could "fudge" inclusivity and exclusivity evaluations

Validation and Verification: When Additional Studies are Needed

- For every validated method, there are often requests to test food matrices and/or analytical weights that were not evaluated in the validation study.
- FDA specifies requirements for a matrix extension of an existing validated method.
  - FDA-BAM Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed.
- There is no one size fits all rule, each product needs evaluated on a case by case basis.
  - Determination if validation or verification is needed is based on existing study data, potential inhibitory components, analytical weight requested

Validation and Verification: Overview



****In an outbreak situation, a verification is acceptable. However, once the emergency passes, a Validation study is required.***

Validation Protocol

- Required when a new food matrix does not fit within the validated food categories for the Rapid method in question.
  - Study design and data evaluation follows AOAC PTM guidelines.
    - 5 control samples, 5 high level inoculated, 20 low level.
    - Pass/Fail is based on statistical comparison to the reference method.
- Protocol Review: Culture preparation → inoculation of the matrix →inoculated matrix stored to allow for stabilization of the organism →Testing and cultural confirmation → statistical review of the results by calculating the probability of detection (POD).
- Testing and timeline generally looking at 1-2 months.

Verification Protocol

- Required when a new matrix falls within a validated category, but is not closely related to what was tested in the validation.
- Protocol Review: 7 samples are inoculated at 30 cfu + matrix control + APC to evaluate background
- Verification studies are only for the Rapid Method, no comparison to a reference method.
- All 7 samples must recover the target for the verification to pass.
- If verification study fails, then a validation may be necessary. Often alternate enrichment ratios may be tested in a repeated study.
- Typical timeline is 1 week.

Company or industry specific evaluation of new methods

- Use existing information to evaluate assays using standardized criteria ("paper-based")
- Evaluate assays for use in routine screening, considering company and matrix specific criteria ("lab based"):
    - Inclusivity: ability to detect diversity of target organisms relevant to a given company or industry
        - May include serotypes not tested originally
    - Exclusivity: ability to not yield positives with specific organisms found in products of interest
    - Ability to detect target organisms in specific food matrices and other samples of interest

System to assess information on commercially available detection methods based on standardized criteria

Journal of Food Protection, Vol. 77, No. 4, 2014, Pages 670–690
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Review

Assessment Criteria and Approaches for Rapid Detection Methods To Be Used in the Food Industry

MARTIN WIEDMANN,[1]* SIYUN WANG,[1,2] LAURIE POST,[3] AND KENDRA NIGHTINGALE[4]

Assessment Criteria

- Assessment covers two sets of criteria:
  - Generic criteria (13)
  - Pathogen specific criteria (10)
- Score of 1 to 5 given for each criterion

Examples of Pathogen specific criteria

- Inclusivity: ability to detect different strain
- Exclusivity: ability to not detect non-target strains
- Diagnostic sensitivity: Ability to detect target in naturally contaminated samples; low number of false negatives
- Diagnostic specificity: Ability to specifically detect the target and not yield false positives
  - An assay may have perfect exclusivity but still have a high number of false positives (e.g., if a sample has a lot of dead cells)
- Analytical sensitivity (detection limit)
- Reproducibility and repeatability

Example of criteria and scoring matrix

Key criteria for evaluation	Target	Key factors affecting performance	Quantitative evaluation (scale of 0–5)[b]
Inclusivity: ability to detect different strains and/or subtypes of the target organism, also sometimes referred to as analytical specificity	Should be validated to detect various strains and/or subtypes of the target organism	Target gene must be highly conserved and present in all strains of the target species or serotype; target gene should be required for virulence so that strains that lack the target gene are unlikely to cause disease (bona fide virulence genes may not have been identified for all target pathogens)	0—tested on a small no. of standard laboratory or collection strains and/or no strong scientific support for choice of target gene 3—tested on a diverse strain collection, but diversity does not represent a variety of geographical regions or food matrices 5—(i) tested on isolates from different sources (human, food, environmental), different countries and continents, and a wide variety of subtypes and/or (ii) strong scientific support that target is unique to target organism and linked to virulence

Examples of "generic" criteria

- Ability to differentiate viable and non-viable organisms
- Ruggedness
- Cross-contamination controlled and prevented through multiple barriers
- Throughput
- Speed

Example of System Rankings

	Detection of surface antigen	Real-time PCR, Target DNA	Real-Time PCR, Target DNA	Real-time PCR, Target DNA	Real-Time PCR, Target DNA	Isothermal ampli-fication	Isothermal DNA ampli-fication	Hybridization, Target rRNA
***Salmonella* specific**	30	24	29	24	27	25	22	20
Generic	39	35	37	42	38	42	37	38
Sum	69	59	66	66	65	67	59	58

Lab-based evaluation - example

- Selected four assays for further evaluation based on two criteria:
  - Inclusivity: ability to detect diversity serotypes relevant to chocolate and dry pet food
  - Ability to detect *Salmonella* in matrix of interest (dark chocolate and dry pet food)
  - Selected difficult to detect serotypes, inoculated at low levels ("fractional positives"), adapted to low water activity environment

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Evaluation of Rapid Molecular Detection Assays for *Salmonella* in Challenging Food Matrices at Low Inoculation Levels and Using Difficult-to-Detect Strains

GINA RYAN,[1]† SHERRY ROOF,[1] LAURIE POST,[2] AND MARTIN WIEDMANN[1]*

Inclusivity Study

- Assays were evaluated for ability to detect diverse *Salmonella* serotypes and strains at limits of detection (LOD)
- Approach:
  - Pure cultures for each isolate were tested at 2 levels:
    - Limit of detection (LOD), one log above (LOD +1), and LOD + 3 as needed

Salmonella characteristics

Species	Subspecies	Serotype	Source
enterica	I *enterica*	> 1500	human, animals
	II *salamae*	505	Reptiles
	IIIa *arizonae*	99	Reptiles, fish, poultry, human (rare)
	IIIb *diarizonae*	336	Reptiles, fish, poultry, human (rare)
	IV *houtenae*	73	Reptiles, fish, porcine, human (rare)
	VI *indica*	13	Reptiles, fish, porcine, human (rare)
bongori	V	22	Reptiles, birds, human (rare)
Total (Salmonella genus)		> 2500	

CDC *Salmonella* surveillance data, 2009

Salmonella Strain Selection

- 68 strains and serotypes, including:
  - *S. enterica* subsp. I (63 isolates representing 63 different serotypes)
  - *S. enterica* subspp. (4):
    - II *salamae*
    - IIIa *arizonae*
    - IIIb *diarizonae*
    - IV *houtenae*
  - *S. bongori*
- Source
  - Previously associated with pet food/environment, chocolate and relevant ingredients
  - Globally distributed
    - Virchow and Stanley- endemic to SE Asia (Japan, Indonesia, Cambodia, Thailand, Philippines)
  - Common clinical serotypes in US

Inclusivity Study Results

System (LOD)	LOD Inclusivity	Serotypes NOT detected at LOD	LOD +1 Inclusivity	Serotypes NOT detected at LOD+1
rRNA (10^3 CFU/ml)	100 %		100 %	
DNA (10^4 CFU/ml)	100 %		100 %	
DNA (10^4 CFU/ml)	93 %	II, IIIa, IIIb, IV, *S. bongori*	96 %	IIIb, IV, *S. bongori*
Antigen (10^5 CFU/ml)	82 %	Adelaide, Alachua, 13,22;b-Kentucky, Loubomo, Minnesota, Mississippi Typhi, Virchow	100 %	

Summary - Inclusivity

- **One DNA-based assay and rRNA-based assay**: all isolates detected at LOD and LOD +1
- **Antigen-based assay**: 59/68 isolates detected at LOD; all isolates detected at LOD +1
- **One DNA-based assay**: all *S. enterica* subsp. I serotypes detected at LOD and LOD +1; subsp. IIIb only detected at LOD +3; subsp. IV and *S. bongori* not detected at LOD +3
  - *Not selected for further evaluation*
- Three assays selected for evaluation in "matrix study":

Matrix Study

- Overall Aim: Assess rapid method for ability to detect diverse *Salmonella* in dry pet food and dark chocolate
- Samples inoculated with selected serotypes were evaluated for detection following stabilization at room temperature for two weeks.
  - Contamination Level: 25 g samples inoculated at levels to yield fractional positive results (5 to 15 out of 20 samples)
  - Stabilized target level: 0.5 – 1 cfu/ 25 g sample at 2 weeks
  - Detection by reference method (FDA BAM)

Isolate Selection Procedure

- Each matrix was tested with isolates representing 5 different serotypes
- Criteria for isolate se:
  - Preliminary evaluation of LOD of assays with isolates representing 68 serotypes: selected difficult to detect strains
  - Clinical or food industry significance
    - CDC top 20 clinical isolates (Typhimurium, Kentucky, and Mississippi)
    - Isolates of relevance in dry pet food (Alachua and Minnesota) and chocolate (13,22:b:-)
    - Geographic distribution (Virchow; common in SE Asia)

Example - Evaluation of the antigen-based assay for the Detection of *Salmonella* on Dry Pet Food and Dark Chocolate

Detection of *Salmonella* in Dry Pet Food – FDA BAM and Assay D comparison: unpaired enrichment data

Strain	MPN / 25g	95% CI	N	Assay C Pos, Culture Pos		N	FDA-BAM Positive	dPOD[a]	95% CI[b]
Adelaide	0.52	(0.32, 0.85)	20	11		20	13	-0.10	(-0.36, 0.18)
Alachua	0.94	(0.57, 1.54)	20	14		20	10	0.20	(-0.10, 0.45)
Kentucky	0.62	(0.37, 1.04)	20	5		20	11	-0.30	(-0.54, 0.00)
Minnesota	0.75	(0.45, 1.23)	20	8		20	11	-0.15	(-0.41, 0.15)
Typhimurium	0.74	(0.44, 1.23)	20	8		20	12	-0.20	(-0.46, 0.10)
Total			**100**	**46**		**100**	**57**	**-0.11**	**(-0.24, 0.03)**

[a]**dPOD**: difference between Assay C and FDA BAM method probability of detection (POD) values; POD is calculated as the number of confirmed positive results divided by the total number of samples

[b]**95% CI**: If the confidence interval of dPOD contains a zero, then Assay C is not significantly different from the FDA BAM method at the 5% level.

Detection of *Salmonella* in Dry Pet Food – Assay D culture confirmation data

Strain	MPN / 25g	95% CI	N	Assay C Pos, Culture Pos	Assay C Pos, Culture Neg	Assay C Neg, Culture Pos	Assay C Neg, Culture Neg	Sensi-tivity[a]	False Negative[b]	Specificity[c]	False Positive[d]	Relative Accuracy[e]	P-value[f]
Adelaide	0.52	(0.32, 0.85)	20	11	0	2	7	0.85	0.15	1.00	0.00	0.90	0.500
Alachua	0.94	(0.57, 1.54)	20	14	1	2	3	0.88	0.12	0.75	0.25	0.85	1.000
Kentucky	0.62	(0.37, 1.04)	20	5	0	3	12	0.63	0.37	1.00	0.00	0.85	0.250
Minnesota	0.75	(0.45, 1.23)	20	8	0	7	5	0.53	0.47	1.00	0.00	0.65	0.016
Typhimurium	0.74	(0.44, 1.23)	20	8	0	2	10	0.80	0.20	1.00	0.00	0.90	0.500
Total			**100**	**46**	**1**	**16**	**37**	**0.74**	**0.26**	**0.97**	**0.03**	**0.83**	**< 0.001**

[a]**Sensitivity**: Assay C confirmed positive results divided by total culture positive results
[b]**False negative rate**: 1 – Sensitivity
[c]**Specificity**: Assay C confirmed negative results divided by total culture negative results
[d]**False positive rate**: 1 – Specificity
[e]**Relative Accuracy**: Total Assay C confirmed positive and negative results divided by number of test portions
[f]**p-value determined by McNemar's test:** binomial distribution used to test for the tendency of an assay towards false positive or false negative results.

Overall Comparison of Assay Performance for *Salmonella* Detection in Dry Pet Food and Dark Chocolate

TABLE 3. *Comparison of rapid assay results for detection of* Salmonella *in paired sample enrichment cultures*

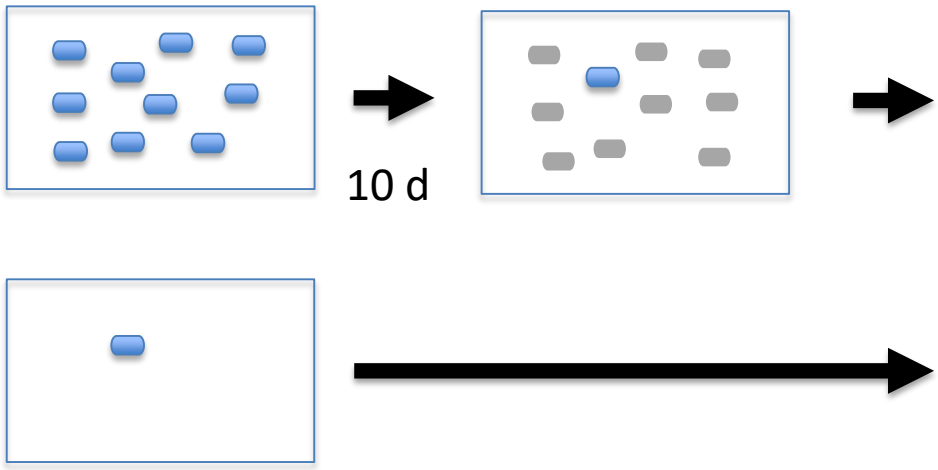
Matrix[a]	Assay	No. of samples[b]				Sensitivity[c]	False negative rate[d]	Specificity[e]	False positive rate[f]	Relative accuracy[g]
		Assay pos, culture pos	Assay pos culture neg	Assay neg, culture pos	Assay neg, culture neg					
Dry pet food	A	51	2	5	42	0.91	0.09	0.95	0.05	0.93
	B	68	3	3	26	0.96	0.04	0.90	0.10	0.94
	D	46	1	16	37	0.74	0.26	0.97	0.03	0.83
Dark chocolate	A	61	1	8	30	0.88	0.12	0.97	0.03	0.91
	B	56	4	11	29	0.84	0.16	0.88	0.12	0.85
	D	55	2	10	33	0.85	0.15	0.94	0.06	0.88

[a] Assays were evaluated with 20 replicate samples for each of the five *Salmonella* serotypes tested per food matrix; thus, a total of 100 samples was tested for each assay for each matrix.
[b] Results from rapid assay sample enrichments were confirmed by secondary enrichment in selective media and isolation on selective agar plates; selected *Salmonella* colonies were confirmed by *invA* PCR assay.
[c] Number of confirmed assay positive results divided by the total number of culture positive results.
[d] Calculated as 1 – sensitivity.
[e] Number of confirmed assay negative results divided by the total number of culture negative results.
[f] Calculated as 1 – specificity.
[g] Number of confirmed rapid assay positive results divided by the number of confirmed rapid assay negative results.

Case study

- Company X started using a new real time method to detect Listeria in their environmental samples 2 years ago
- They had 1 positive since then
- FDA comes and visit….

How are methods validated for testing environmental samples



New 30 sec magic nucleic acid test: **POS**

Summary and Conclusions

- False negative rate under "worst case scenario" (difficult to detect serotypes, challenging matrix, low level inoculation, stressed cells) can be higher than 10%
- Some assays (e.g., assays detecting surface antigens) more likely to have strains that are difficult to detect
- Evaluation of detection assays under end-user relevant conditions will yield important data
- Rapid methods need to be carefully evaluated
  - May not work with all matrices
  - May show reduced sensitivity

Recommendations

- Understand the technology
  - Need to know more than “It’s fast and usually gives you a green light”
- Review validation of the test you use/want to use
  - Matrix? Analytical weights? Strains tested? Strains used in matrix evaluation?
- Engage independent experts
- Ask tough questions