U.S. FOOD & DRUG ADMINISTRATION
CENTER FOR FOOD SAFETY & APPLIED NUTRITION

Development of New *Vibrio* Lab Methods

Madison McGough
USFDA, Gulf Coast Seafood Laboratory, Dauphin Island, AL
ISS & GSASSC
11/7/2023

Background on *Vibrio*

- The organism
  - Naturally occurring marine/estuarine bacteria
  - Highly seasonal

- *V. parahaemolyticus*
- *V. vulnificus*

- Vibriosis
  - CDC estimates 52,000 food-borne illnesses annually in U.S.
  - Generally, self-limiting gastroenteritis
  - Occasionally, septicemia

NSSP *Vibrio* Methods

Approved Methods for Vibrio Enumeration

	Vibrio Type:	Application: PHP Sample Type: Shucked	Application: Reopening
EIA[1]	***Vibrio vulnificus (V.v.)***	X	
MPN[2]	***Vibrio vulnificus (V.v.)***	X	
SYBR Green 1 QPCR-MPN[5]	***Vibrio vulnificus (V.v.)***	X	
MPN[3]	***Vibrio parahaemolyticus (V.p.)***	X	
PCR[4]	***Vibrio parahaemolyticus (V.p.)***	X	
MPN-Real Time PCR[6]	***tdh+ and trh+ Vibrio parahaemolyticus (V.p.)***	X	X
MPN-Real Time PCR[7]	***Vibrio parahaemolyticus (V.p.)***	X	X
Direct Plating Method[8]	***Vibrio parahaemolyticus (V.p.)***		X
MPN-Real Time PCR[9]	***Vibrio vulnificus (V.v.)***	X	

Footnotes:

[1] EIA procedure of Tamplin, et al, as described in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, 1992.

[2] MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, followed by confirmation using biochemical analyses or by the DNA -alkaline phosphatase gene probe for vvhA as described by Wright et al., or a method that a State can demonstrate is equivalent.

[3] MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, followed by confirmation using biochemical analyses or the DNA-alkaline phosphatase gene probe for tlh as described by McCarthy et al., or a method that a State can demonstrate is equivalent.

[4] MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, and as described in the "Direct Plating Procedure for the Enumeration of Total and Pathogenic *Vibrio parahaemolyticus* in Oyster Meats" developed by FDA, Gulf Coast Seafood Laboratory, or a method that a State can demonstrate is equivalent.

[5] *Vibrio vulnificus*, ISSC Summary of Actions 2009. Proposal 09-113, Page 123.

[6] MPN-Real Time PCR Method for the tdh and trh Genes for Total *V. parahaemolyticus* as described in Kinsey et al., 2015. ISSC 2015 Summary of Actions Proposal 15-111, Page 397.

[7] MPN-Real Time PCR Method for the *tlh* gene for total *V. parahaemolyticus* as described in Kinsey et al., 2015. ISSC 2015 Summary of Actions Proposal 15-113, Page 418

[8] Direct Plating Procedure in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, and as described in the 'Direct Plating Procedure for the Enumeration of Total and Pathogenic *Vibrio parahaemolyticus* in Oyster Meats' developed by FDA, Gulf Coast Seafood Laboratory.

[9] MPN-Real Time PCR Method for the vvh gene for total *V. vulnificus* as described in Kinsey et al., 2015.

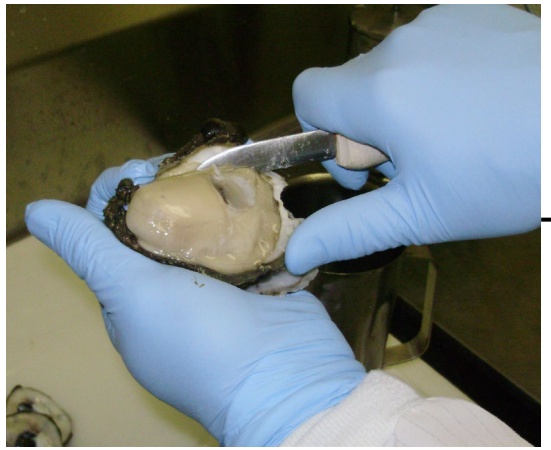
NSSP *Vibrio* Methods

Approved Methods for Vibrio Enumeration

	Vibrio Type:	Application: PHP Sample Type: Shucked	Application: Reopening
EIA[1]	*Vibrio vulnificus (V.v.)*	X	
MPN[2]	*Vibrio vulnificus (V.v.)*	X	
SYBR Green 1 QPCR-MPN[5]	*Vibrio vulnificus (V.v.)*	X	
MPN[3]	*Vibrio parahaemolyticus (V.p.)*	X	
PCR[4]	*Vibrio parahaemolyticus (V.p.)*	X	
MPN-Real Time PCR[6]	*tdh+ and trh+ Vibrio parahaemolyticus (V.p.)*	X	X
MPN-Real Time PCR[7]	*Vibrio parahaemolyticus (V.p.)*	X	X
Direct Plating Method[8]	*Vibrio parahaemolyticus (V.p.)*		X
MPN-Real Time PCR[9]	*Vibrio vulnificus (V.v.)*	X	

Methods that use the discontinued AP probes

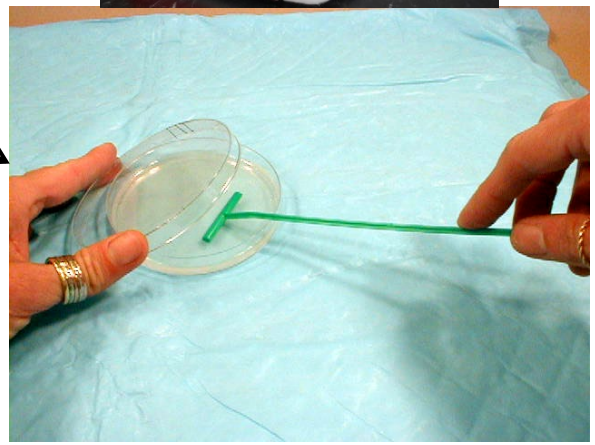
NSSP *Vibrio* Methods – Current Approaches



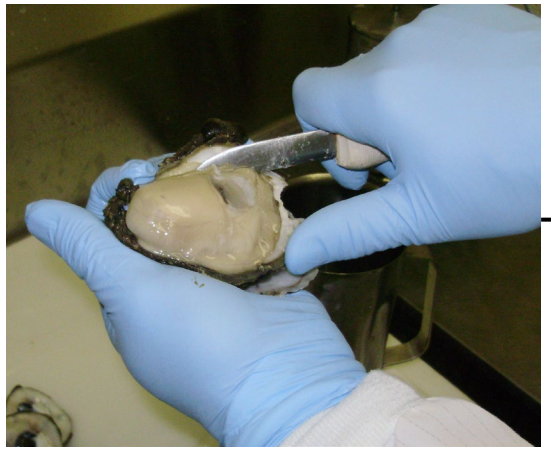
Homogenize

MPN

Direct Plating



NSSP *Vibrio* Methods – Current Approaches

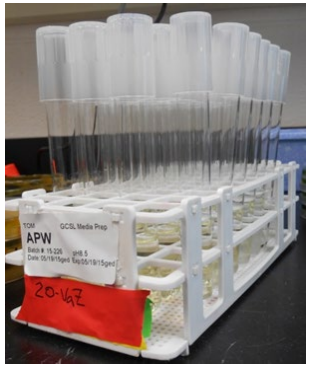


Homogenize

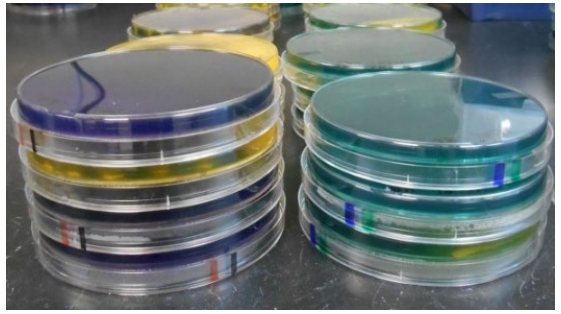
MPN

Direct Plating

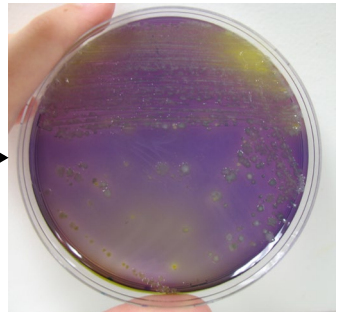
NSSP *Vibrio* Methods – Current Approaches



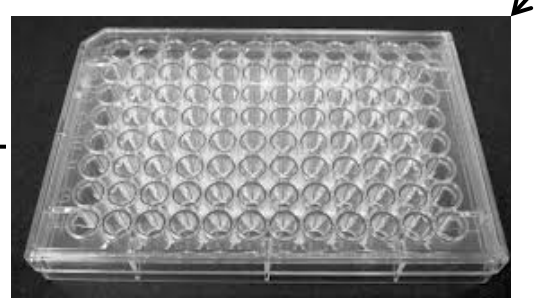
Streak selective media



Incubate overnight



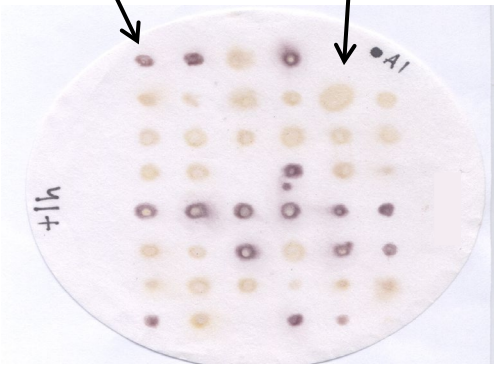
Pick typical colonies



Replica plate

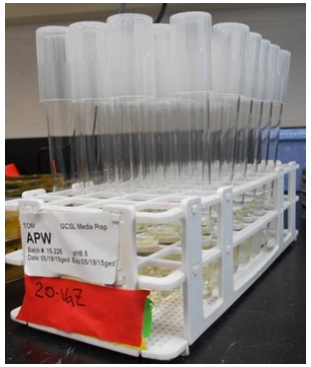
Incubate overnight

Lifts/ hybridization

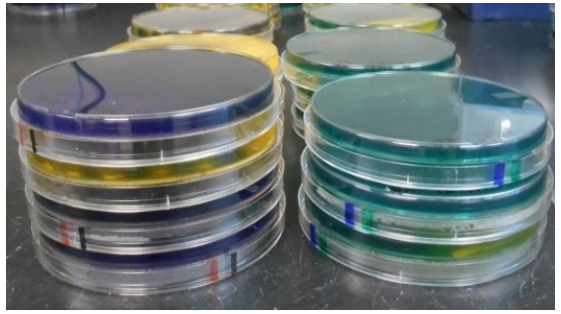


Same general procedure for all MPN-culture methods.

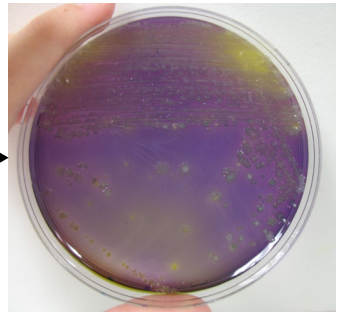
NSSP *Vibrio* Methods – Current Approaches



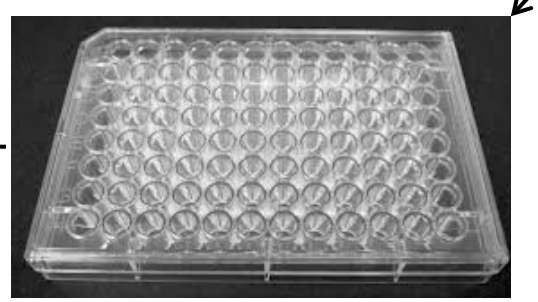
Streak selective media



Incubate overnight



Pick typical colonies



Replica plate

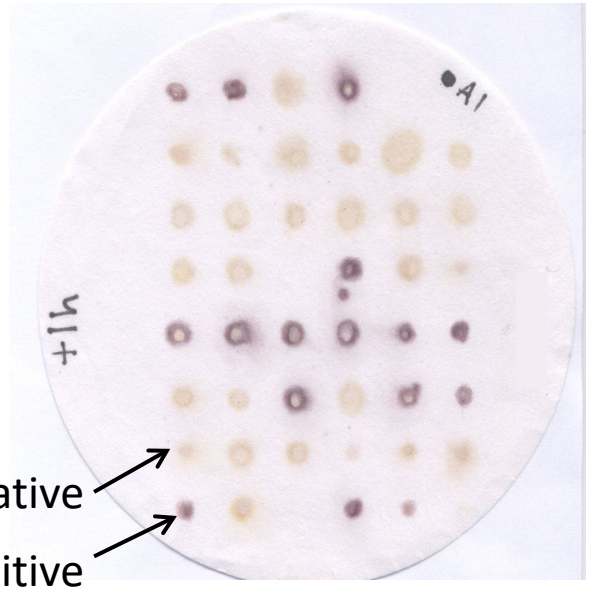
Incubate overnight

Lifts/ hybridization

Same general procedure for all MPN-culture methods.

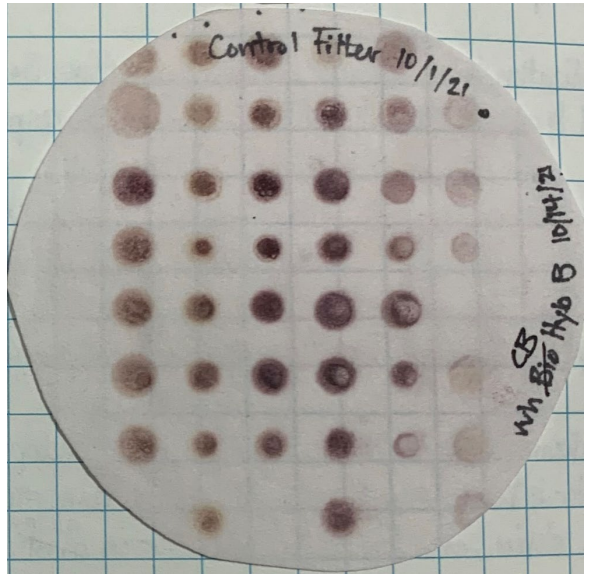
Evaluation of DNA Probe Options

Ex. discontinued AP probe

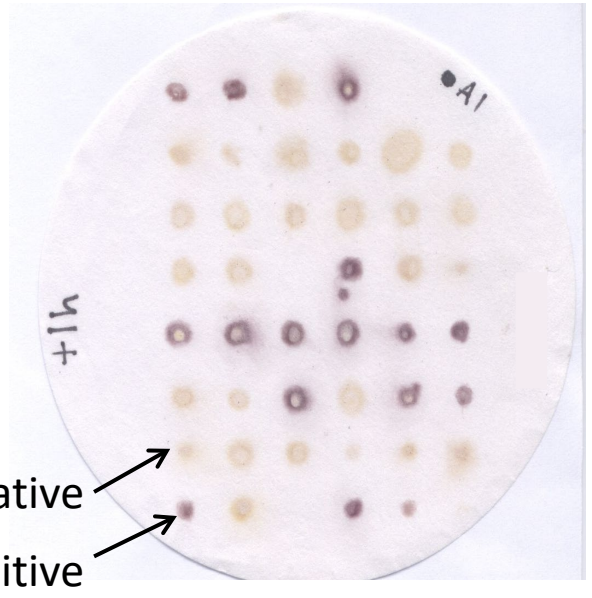


Evaluation of DNA Probe Options

Ex. commercially available AP probe

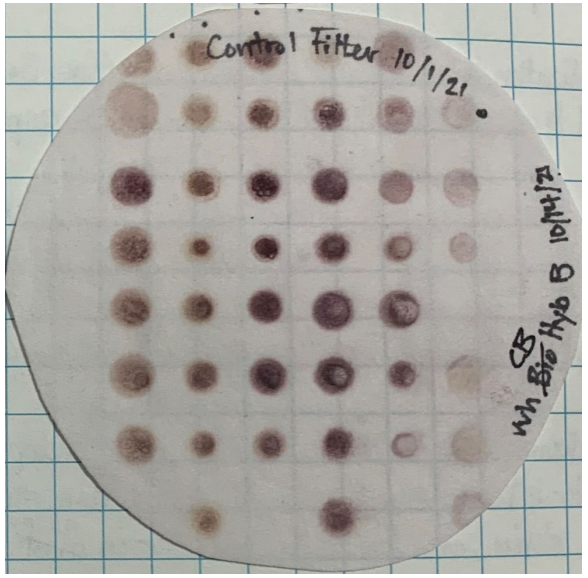


Ex. discontinued AP probe

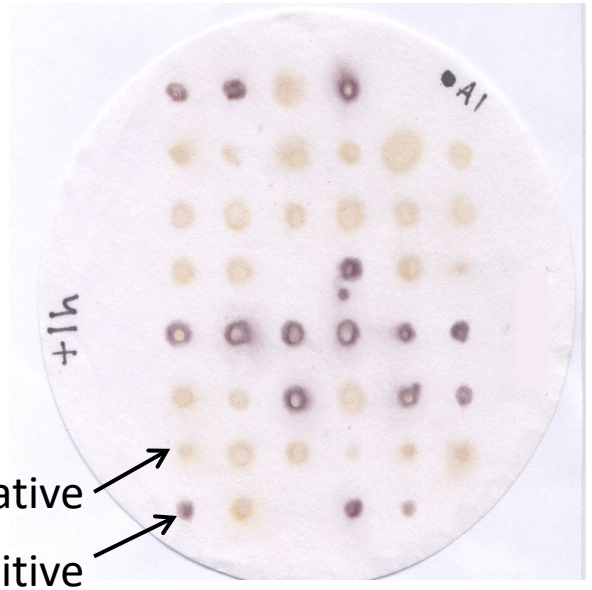


Evaluation of DNA Probe Options

Ex. commercially available AP probe



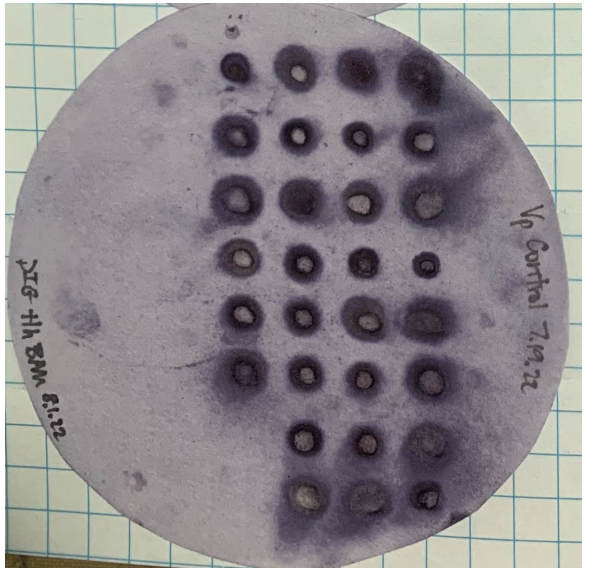
Ex. discontinued AP probe



negative

positive

Ex. DIG probe



Loop-mediated isothermal AMPlification

- Loop-mediated

- Isothermal - carried out at a constant temperature - does not *require* a thermal cycler

- AMPlification – amplifying specific sections of DNA sequence based on sets of primers

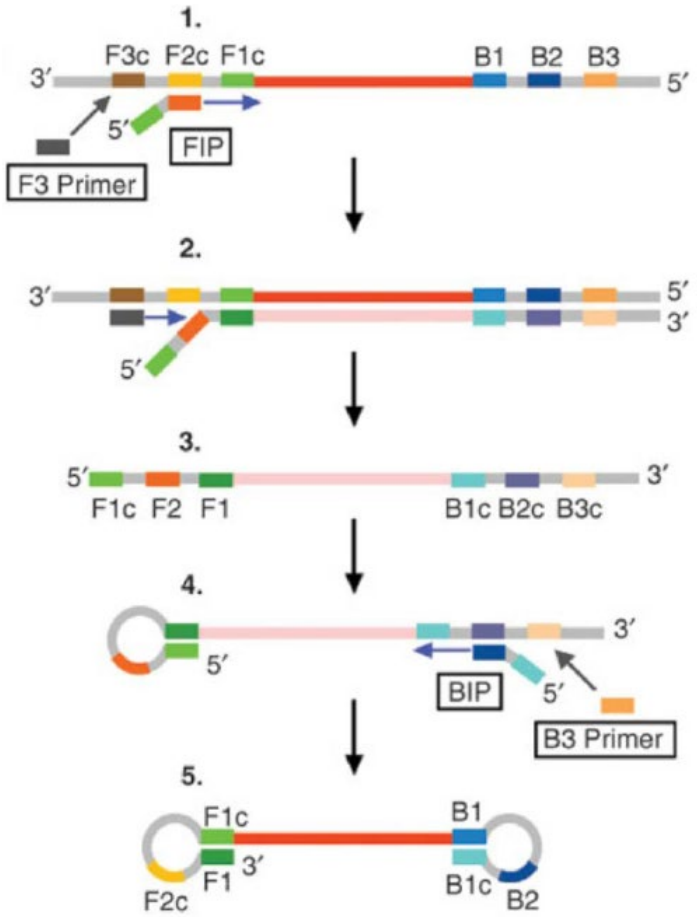
Loop-mediated isothermal AMPlification

- Amplification can be detected by turbidity or fluorescence - possible to visualize using the naked eye
- Not as sensitive to PCR inhibitors - may not need highly purified DNA templates
- Simple and low-cost
- Publications
  - Total *V. parahaemolyticus* (*tox*R) – Chen and Ge, 2010. BMC Microbiol
  - *V. vulnificus* (*vvh*A) – Han and Ge, 2008. Foodborne Pathogens and Disease

LAMP Assay Set-Up

- Reaction components:
  - buffer (Mg++, etc.)
  - *Bst* DNA polymerase
  - dNTPs (nucleotides)
  - LAMP primers
  - target template
- Reaction conditions:
  - run LAMP reactions (63°C for 1h)
  - stop reaction at 80°C for 2min
  - Add SYBR Green, read and record results

How LAMP works



FIP extends

FOP displaces

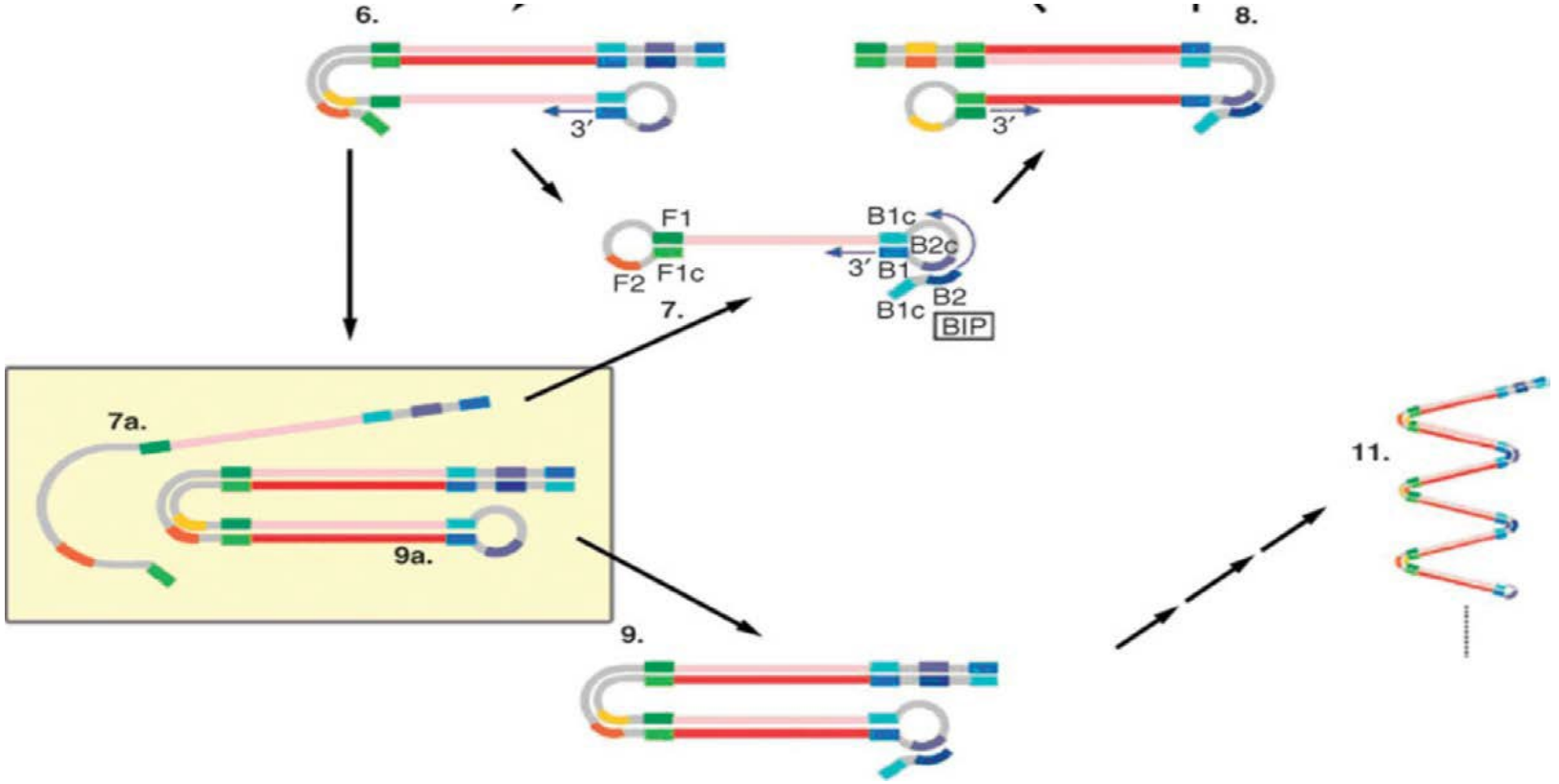
Displaced strand forms loop

BIP extends and BOP displaces

Dumbbell structure forms

Eiken Genome Site (2021).

How LAMP works



Eiken Genome Site (2021).

DNA Prep Method

- Compared DNA prep methods on panel of 185 isolates
- Total *V. parahaemolyticus* (*toxR*)

- Boiled plate
- WSB plate

- Boil prep
- WSB

2X

DNA Prep Method Results - *toxR*

	Boiled Plate	WSB Plate	Boil Prep (tube)	WSB (tube)
Species	**% detected**			
Vibrio parahaemolyticus, n=100	100%	99%	100%	100%
Vibrio vulnificus, n=50	2%	23%	11%	34%
Vibrio cholerae, n=5	50%	10%	60%	20%
Vibrio fluvialis, n=10	10%	30%	10%	35%
Vibrio alginolyticus, n=6	0%	58%	8%	58%
Pseudomonas aeruginosa, n=1	0%	50%	0%	50%
Photobacterium damselae, n=3	0%	33%	50%	33%
Enterobacter aerogenes, n=1	50%	50%	0%	0%
Escherichia coli, n=1	100%	100%	0%	100%
Grimontia hollisae, n=5	100%	0%	40%	20%
Staphylococcus aureus, n=1	0%	0%	0%	100%
Vibrio metschnikovii, n=1	0%	0%	0%	50%
Klebsiella pneumoniae, n=1	100%	50%	0%	50%
false positives	6%	12%	7%	16%
false negatives	0%	1%	0%	0%

DNA Prep Method Results - *toxR*

	Boiled Plate	WSB Plate	Boil Prep (tube)	WSB (tube)
Species	**% detected**			
Vibrio parahaemolyticus, n=100	100%	99%	100%	100%
Vibrio vulnificus, n=50	2%	23%	11%	34%
Vibrio cholerae, n=5	50%	10%	60%	20%
Vibrio fluvialis, n=10	10%	30%	10%	35%
Vibrio alginolyticus, n=6	0%	58%	8%	58%
Pseudomonas aeruginosa, n=1	0%	50%	0%	50%
Photobacterium damselae, n=3	0%	33%	50%	33%
Enterobacter aerogenes, n=1	50%	50%	0%	0%
Escherichia coli, n=1	100%	100%	0%	100%
Grimontia hollisae, n=5	100%	0%	40%	20%
Staphylococcus aureus, n=1	0%	0%	0%	100%
Vibrio metschnikovii, n=1	0%	0%	0%	50%
Klebsiella pneumoniae, n=1	100%	50%	0%	50%
false positives	6%	12%	7%	16%
false negatives	0%	1%	0%	0%

Differential and selective media results

Species	Chromagar Vibrio	TCBS
Vibrio parahaemolyticus	purple	green
Vibrio vulnificus	blue	green
Vibrio cholerae	blue	yellow
Vibrio fluvialis	opaque	no growth
Vibrio alginolyticus	opaque	green, yellow, no growth
Pseudomonas aeruginosa	no growth	no growth
Photobacterium damselae	opaque	no growth
Enterobacter aerogenes	no growth	no growth
Escherichia coli	no growth	no growth
Grimontia hollisae	no growth	no growth
Staphylococcus aureus	no growth	no growth
Vibrio metschnikovii	opaque	yellow
Klebsiella pneumoniae	no growth	no growth

Ruggedness - *toxR*

	% detected	
Species	**2 µL template**	**5 µL template**
Vibrio parahaemolyticus, n=16	100%	100%
Vibrio vulnificus, n=14	0%	0%
Vibrio cholerae, n=2	50%	50%
Vibrio fluvialis, n=4	0%	0%
Vibrio alginolyticus, n=1	0%	0%
Grimontia hollisae, n=2	100%	100%
Staphylococcus aureus, n=1	0%	0%

Ruggedness - *toxR*

	% detected	
Species	**2 µL template**	**5 µL template**
Vibrio parahaemolyticus, n=16	100%	100%
Vibrio vulnificus, n=14	0%	0%
Vibrio cholerae, n=2	50%	50%
Vibrio fluvialis, n=4	0%	0%
Vibrio alginolyticus, n=1	0%	0%
Grimontia hollisae, n=2	100%	100%
Staphylococcus aureus, n=1	0%	0%

Ruggedness - *toxR*

	% detected	
Species	**Lot 1**	**Lot 2**
Vibrio parahaemolyticus, n=20	100%	100%
Vibrio vulnificus, n=13	8%	0%
Vibrio cholerae, n=2	50%	50%
Vibrio fluvialis, n=2	0%	0%
Vibrio alginolyticus, n=2	0%	0%
Pseudomonas aeruginosa, n=1	0%	0%
Photobacterium damselae, n=1	0%	0%

Ruggedness - *toxR*

	62℃	63℃	64℃
Species	**% detected**		
Vibrio parahaemolyticus, n=20	100%	100%	100%
Vibrio vulnificus, n=13	8%	0%	4%
Vibrio cholerae, n=2	25%	25%	25%
Vibrio fluvialis, n=2	0%	0%	25%
Vibrio alginolyticus, n=2	0%	0%	0%
Pseudomonas aeruginosa, n=1	0%	0%	0%
Photobacterium damselae, n=1	0%	0%	0%
false postives	4%	1%	4%
accuracy	96%	99%	96%

Ruggedness - *toxR*

	62°C	63°C	64°C
Species		**% detected**	
Vibrio parahaemolyticus, n=20	100%	100%	100%
Vibrio vulnificus, n=13	8%	0%	4%
Vibrio cholerae, n=2	25%	25%	25%
Vibrio fluvialis, n=2	0%	0%	25%
Vibrio alginolyticus, n=2	0%	0%	0%
Pseudomonas aeruginosa, n=1	0%	0%	0%
Photobacterium damselae, n=1	0%	0%	0%
false postives	4%	1%	4%
accuracy	96%	99%	96%

Inclusivity and Exclusivity - *vvhA*

Species	% detected
Vibrio vulnificus, n=50	100%
Vibrio parahaemolyticus, n=100	12%
Vibrio cholerae, n=5	100%
Vibrio fluvialis, n=10	20%
Vibrio alginolyticus, n=6	8%
Pseudomonas aeruginosa, n=1	0%
Photobacterium damselae, n=3	33%
Enterobacter aerogenes, n=1	0%
Escherichia coli, n=1	100%
Grimontia hollisae, n=5	100%
Staphylococcus aureus, n=1	0%
Vibrio metschnikovii, n=1	0%
Klebsiella pneumoniae, n=1	0%

Differential and selective media results

Species	Chromagar Vibrio	mCPC
Vibrio vulnificus	blue	yellow with yellow halo
Vibrio parahaemolyticus	purple	yellow with white border
Vibrio cholerae	blue	green
Vibrio fluvialis	opaque	no growth
Vibrio alginolyticus	opaque	opaque with purple halo
Pseudomonas aeruginosa	no growth	no growth
Photobacterium damselae	opaque	yellow with white border
Enterobacter aerogenes	no growth	no growth
Escherichia coli	no growth	no growth
Grimontia hollisae	no growth	green
Staphylococcus aureus	no growth	clear
Vibrio metschnikovii	opaque	no growth
Klebsiella pneumoniae	no growth	no growth

Ruggedness - *vvhA*

	% detected	
Species	**2 µL template**	**5 µL template**
Vibrio vulnificus, n=12	100%	100%
Vibrio parahaemolyticus, n=23	7%	4%
Vibrio fluvialis, n=3	17%	17%
Vibrio alginolyticus, n=2	0%	25%
Photobacterium damselae, n=1	0%	0%
Grimontia hollisae, n=3	83%	100%
Vibrio metschnikovii, n=1	0%	0%
Klebsiella pneumoniae, n=1	0%	0%

Ruggedness - *vvhA*

	% detected	
Species	**2 µL template**	**5 µL template**
Vibrio vulnificus, n=12	100%	100%
Vibrio parahaemolyticus, n=23	7%	4%
Vibrio fluvialis, n=3	17%	17%
Vibrio alginolyticus, n=2	0%	25%
Photobacterium damselae, n=1	0%	0%
Grimontia hollisae, n=3	83%	100%
Vibrio metschnikovii, n=1	0%	0%
Klebsiella pneumoniae, n=1	0%	0%

Ruggedness - *vvhA*

	% detected	
Species	**Lot 1**	**Lot 2**
Vibrio vulnificus, n= 14	100%	100%
Vibrio parahaemolyticus, n=21 and n=16	5%	19%
Vibrio cholerae, n=2	100%	50%
Vibrio fluvialis, n=4	75%	0%
Vibrio alginolyticus, n=1	0%	0%
Grimontia hollisae, n=2	100%	0%
Staphylococcus aureus, n=1	0%	0%

Ruggedness - *vvhA*

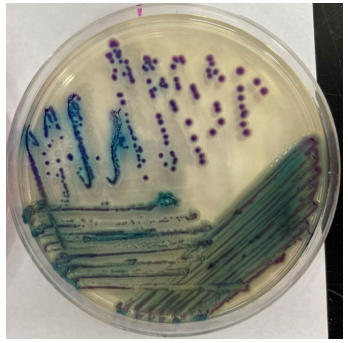
	62°C	63°C	64°C
Species	**% detected**		
Vibrio vulnificus, n=13	100%	100%	96%
Vibrio parahaemolyticus, n=23	9%	7%	2%
Vibrio fluvialis, n=3	0%	0%	0%
Vibrio alginolyticus, n=2	0%	0%	0%
Photobacterium damselae, n=1	0%	0%	0%
Grimontia hollisae, n=3	83%	50%	50%
Vibrio metschnikovii, n=1	0%	50%	0%
Klebsiella pneumoniae, n=1	0%	0%	50%
false positives	10%	7%	5%
accuracy	90%	93%	94%
false negatives	0%	0%	1%

Ruggedness - *vvhA*

	62°C	63°C	64°C
Species		**% detected**	
Vibrio vulnificus, n=13	100%	100%	96%
Vibrio parahaemolyticus, n=23	9%	7%	2%
Vibrio fluvialis, n=3	0%	0%	0%
Vibrio alginolyticus, n=2	0%	0%	0%
Photobacterium damselae, n=1	0%	0%	0%
Grimontia hollisae, n=3	83%	50%	50%
Vibrio metschnikovii, n=1	0%	50%	0%
Klebsiella pneumoniae, n=1	0%	0%	50%
false positives	10%	7%	5%
accuracy	90%	93%	94%
false negatives	0%	0%	1%

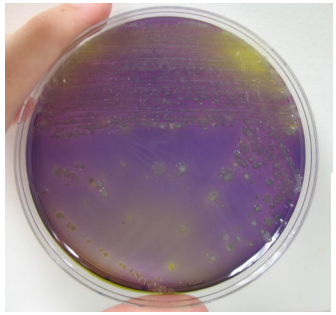
LAMP Method Approach

Streak differential media



Streak selective media

mCPC (*V.v.*)



TCBS (*V.p.*)

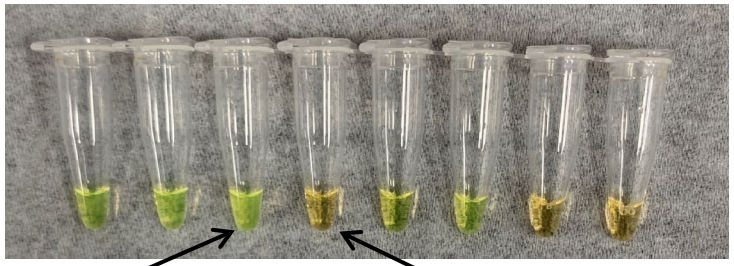
Same general procedure for all LAMP methods.

Pick typical colonies

Boil

LAMP

Add SYBR Green; read results



positive

negative

Comparing to AP Probe

LAMP

- 4-5 days
- Cost - ~$1.50/reaction (as of 1/3/23)
- Thermal cycler or dry heat bath
- Targets – *tox*R, *vvh*A, *tdh*, *trh* 1 and 2
- Post-amplification area required
- Isolate confirmation only

AP Probe

- 4-5 days
- Cost - No longer available
- Water baths
- Targets – *tlh*, *vvh*, *tdh*, *trh*
- All in one area
- Isolate confirmation or direct plating

Next steps

- Complete data analysis and submit validation data for *tox*R and *vvh*A to ISSC
- Complete validation experiments for pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+)

Acknowledgements

- Jessica Jones, Elizabeth Leard, Whitney Neil, and Victoria Pruente
- Bill Burkhardt, Ed Iannuzzi, Ed Jester, Schaeffer Lersch, Sarah May, Marlee Mims, Carolyn Simmons, and Jacquelina Woods


FDA
U.S. FOOD & DRUG
ADMINISTRATION
CENTER FOR FOOD SAFETY & APPLIED NUTRITION