Proposal for Task Force Consideration at the 2009 Biennial Meeting Interstate Shellfish Sanitation Conference	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
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Proposal Subject:	Method to Determine the Presence of Male Specific Coliphage in Shellfish Meats and the Microbiology
Specific NSSP Guide Reference:	Section IV Guidance Documents, Chapter II. Growing Areas .10 Approved Laboratory Tests
Text of Proposal/ Requested Action	
Public Health Significance:	FDA is submitting a proposal to ISSC to allow MSC to be used as a re-opening criterion in cases where unexpected, unusual sewage contamination occurs that may have impacted shellfish harvest areas (not for conditional re-openings). The MSC method must be reviewed and adopted prior to use in determining the acceptability of shellfish growing waters for reopening.
Cost Information (if available):	Not available
Action by 2005 Laboratory Methods Review Committee	Recommended referral of Proposal 05-114 to the appropriate committee as determined by the Conference Chairman.
Action by 2005 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 05-114.
Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force I.
Action by USFDA	Concurred with Conference action.
Action by 2007 Laboratory Methods Review Committee	Recommended no action on Proposal 05-114. Rationale – The data necessary to approve the method is not available. The submitter will send data to the Executive Office for Conference approval consistent with Procedure XVI.
Action by 2007 Task Force I	Recommended referral of Proposal 05-114 to an appropriate committee as determined by the Conference Chairman.
Action by 2007 General Assembly	Adopted recommendation of 2007 Task Force I.
Action by USFDA	December 20, 2007 Concurred with Conference action with the following comments and recommendations for

	ISSC consideration. The Conference has made considerable progress in its efforts to recognize new and developing analytical methods for the detection of indicators, pathogens, and marine toxins. Much credit goes to the Laboratory Methods Review Committee and its leadership for ensuring a scientifically defensible process for adopting analytical methods under the NSSP. At the 2007 meeting numerous analytical methods were proposed for ISSC adoption. However, many of these methods were lacking the validation and associated data needed by the Laboratory Methods Review Committee to make a final determination regarding their efficacy for use in the NSSP. As a result the General Assembly voted "No Action" on analytical method Proposals 05-107, 05-108, 05-109, 05-111, 05-113, and 05-114. It is FDA's understanding that the intent of the "No Action" vote was not to remove these Proposals from ISSC deliberation as "No Action" normally suggests, but rather to maintain them before the Conference pending submission of additional data for further consideration. The Voting Delegates, by requesting the Proposal submitters provide additional data to the Executive Office for methods approval consistent with Procedure XVI, clearly recognized the importance and utility of these methods and intended to maintain them before the Conference for possible adoption following additional data submission. FDA requests that the ISSC Executive Board confirm FDA's understanding of this outcome. FDA fully supports such a Conference action and encourages the Executive Office to pursue submission of additional data as necessary to move forward with acceptance of these methods.

Enumeration of Male- specific bacteriophage in water and shellfish tissue

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What are male- specific (f-specific) bacteriophage?

•Lytic viruses of bacteria- (killing of host bacteria)

E. coli and S. typhimurium

(production of E. coli pili*)*

•Requires a piliated host cell for adsorption, they do not attach to cell surface (somatic).

•Requires host cell in log- phase of growth- cells do not produce pili at < 30°C

•Optimal growth temperature: 35- 37°C.

•Plaque size is generally self- limiting

Two Predominant Host Strains

•*E. coli* HS(pFamp)RR

- Resistant to Streptomycin and Ampicillin

•*Salmonella typhimurium* WG49

Result of mating: *E. coli* WG27 (piliated)

x

S. typhimurium WG45

-Resistant to Naladixic acid and Kanamycin

--Pili production in each strain is plasmid mediated

Media Composition
E. coli Famp

Bottom Agar

•Tryptone 10.0 g
•Dextrose 1.0 g
•NaCl 5.0 g
•Agar 15.0 g
••Water 1000 ml

•Autoclave 121℃ 15 min
- temper to 50°C.
•Add 0.05 g Streptomycin sulfate
0.05 g Ampicillin (aseptically)

DS Soft Agar

•Tryptone 10.0 g
•Dextrose 1.0 g
•NaCl 5.0 g
•1M $CaCl_2$ 0.5 ml
•Agar 7.0 g
••Water 500 ml
••Boil- Dispense in 2.5 ml aliquots (16 x 100 ml tubes) and freeze (-20°C)
••Autoclave prior to use;
temper to 50- 52° C

Growth broth- same formulation as Bottom Agar w/o agar or antibiotics

Media Composition
S. typhimurium WG49
Bottom Agar

•Trypticase Peptone 10.0 g
•Yeast Extract 1.0 g
•Dextrose 1.0 g
•NaCl 8.0 g
•Agar 15.0 g
••Water 1000 ml

•Autoclave 121℃ 15 min
- temper to 50°C.
•Add 0.10 g Naladixic Acid
0.02 g Kanamycin sulfate (aseptically)

DS Soft Agar

•Trypticase Peptone 10.0 g
•Yeast Extract 1.0 g
•Dextrose 1.0 g
•NaCl 5.0 g
•1M $CaCl_2$ 0.5 ml
•Agar 7.0 g
••Water 500 ml
••Boil- Dispense in 2.5 ml aliquots (16 x 100 ml tubes) and freeze (-20°C)
••Autoclave prior to use;

temper to 50- 52° C

Growth broth- same formulation as Bottom Agar w/o agar or antibiotics

Differentiation of RNA and DNA Bacteriophage

•RNAse Type I-A Sigma # R4875
••Final conc= 100ug/ ml of media
•Stock concentration= 10 mg/ml (100X)
••Dissolve at a conc. of 10 mg/ml in 0.01 M Sodium Acetate (pH 5.2); Boil for 15 min and allow to cool to RT; PH by adding 0.1 vol of 1M Tris HCl (pH 7.4)
••Store @ -20C

Propagation of *E. coli* Famp

Bottom Agar Streak plate-
Transfer preferable < 1 week old.
•Broth Growth medium tempered to 35- 37°C- vortex to aerate.
•Using 10ul loop collect material from of several colonies and transfer to broth medium.
•Shake briefly to mix, then incubate at 35- 37°C for 4-6 hours
(turbidity ≈ 10^7cells/ ml; O.D @540 nm= 0.4)

% RECOVERY OF BACTERIOPHAGE F-2W/ VARIOUS AGES OF FAMP CULTURE

Age of a 10 ml host cell culture(h)	% Recovery (Mean ± SD)3.0
105.4 ± 2.5	
3.5	97.4 ± 2.2
4.0	96.0 ± 2.8
4.5	95.0 ± 3.3
5.0	92.5 ± 1.2
6.0	90.9 ± 1.8

Adapted from DeBartolomeis, 1999

For MSB density determinations in shellfish tissue

1. Homogenize by blending 12 shellfish for 1 min at high speed.
2. Aliquot 30- 50 g from each sample into centrifuge bottle.
3. Centrifuged for 15 min. @ 9,000 x g; 4°C.
4. Collect and weigh supernatant in a sterile container.
5. Allow supernatant to warm to RT (20- 30 min)
6. Combine 2.5 ml aliquot of supernatant, 2.5 ml DS Soft agar (tempered to 52°C) and 0.2 ml of *E. coli* HS(pFamp)RR
7. Overlay onto a tryptone agar plate containing streptomycin/ ampicillin (50 µg/ml final).
8. Plates are inverted and incubated for 18- 24 h @ 35- 37°C

Information needed for

Bacteriophage density determinations:

Plate counts of plaques

g Shellfish homogenate centrifuged
g Shellfish supernatant recovered

Calculations

MSB/ 100 grams=

1) Ave PFU/ plate ÷ number of ml added/ plate= Average PFU/ml

2) Average PFU/ml x grams of supernatant x $\frac{100\text{ g}}{\text{g homogentate}}$ = PFU/ 100 g

Example: Plate counts- 75, 73,80; 2.5 ml/ plate

50 g homogenate; 33 g supernatant

$76 \div 2.5 \times 33\text{ g supernatant} \times \frac{100\text{ grams}}{50\text{ g homogenate}} =$ 2006 PFU/ 100 grams

To determine level of sensitivity

3 plates containing 0, 0, 0; 2.5 ml/ plate
50 g Homogenate; 33 g Supernatant
Assume 1 plaque on 1 plate then calculate
1÷ 3 plates ÷ 2.5 ml x 33 x (100 ÷ 50) =
Reported as < 9 pfu/ 100 grams

For MSB density determinations in low contaminated water- Concentration technique

1. Weigh 100 ml of water in a sterile container centrifuge bottle.
2. Allow water to warm to RT (20- 30 min).
3. Add 1g tryptone and 1 g beef extract to water aliquot, shake to dissolve.
4. Add 10 ml of *E. coli* Famp culture- Do not shake
5. Incubate at 35- 37°C for 50 min - rotate at 100 rpm.
6. Centrifuged for 15 min. @ 9,000 x g; 4°C.

For MSB density determinations in highly contaminated water (> 100 pfu/ 100 ml)

1. Allow an aliquot of water to warm to RT (20- 30 min)
2. Combine 2.5 ml aliquot of supernatant, 2.5 ml DS Soft agar (tempered to 52^{o} C), and 0.2 ml of *E. coli* HS(pFamp)RR
3. Overlay onto a tryptone agar plate containing streptomycin/ ampicillin (50µg/ml final).
4. Plates are inverted and incubated for 18- 24 h @ 35- 37°C

Problems that may arise

Multiple layers are formed after centrifugation

Reason- glycogen- lipids associated w/ shellfish physiological state

Sliding pellet- not solid

Reason- waited too long to remove supernatant

Clumping Agar

Reason- sample was too cool

Runny plaques

Reason- wet plates; too much condensation

No plaques/ individual bacterial colonies on agar plates

Reason- no phage present or inadequate amount host cell

Ways of Enhancing Plaque Visibility

Addition of 2,3,5- triphenyl tetrazolium chloride (TTC), 1% solution in ethanol
65 ul / tube of tempered DS soft agar
Assuming: 2.5 ml of DS agar and
2.5 ml sample
or
Grams Safrin 1:100 in water- differentiates lawn from plaque

Storage of *E. coli* Famp

Selective pressure- Streptomycin and Ampicillin

Bottom Agar Streak Plate
Storage: Refrigerator (2-3 weeks)
•Tryptic Soy Agar Deep w/ Mineral oil overlay
Storage: Room temperature in Dark (2-5 years +)
•Addition of glycerol (10% final) into broth culture. Storage: Freeze at - 80°C (Indefinite?)

Source of Bacterial Host Strains

•*E. coli* HS(pFamp)R; ATCC #700891
•*Salmonella choleraesuis* subsp. *choleraesuis* (Smith) Weldin serotype Typhimurium aka WG49; ATCC #700730

Types and Sources of Positive MSB Controls

Bacteriophage MS2; ATCC# 15597-B1

Bacteriophage Fd; ATCC# 15669 -B2

Municipal Wastewater

Bacteriophage Stability in Shellfish Homogenate

		Time (h)		
Temperature	**Addition**	**0**	**4**	**10**
1- 3 °C	**-**	**2.57**	**2.58**	**2.42**
25°C	**-**	**3.81**	**3.64**	
25°C	**Log Famp[a]**	**3.81**	**3.86**	**3.89 (24h)**
35°C		**3.81**	**3.45[b]**	

[a]Famp added at a density of 270 cells/ g
[b]Significant decrease at 95% Confidence limit

Bacteriophage Stability in Shellfish Supernatant

		Time (h)		
Temperature	**Addition**	**0**	**4**	**24**
25°C	**-**	**3.81**	**3.74**	**2.60[b]**
25°C	**Log Famp[a]**	**3.81**	**3.13[b]**	**2.90[b]**
35°C	**-**	**3.81**	**3.73**	**2.90[b]**
35°C	**Log Famp[a]**	**3.81**	**3.56[b]**	**5.51[c]**

[a]Famp added at a density of 270 cells/ g
[b]Significant decrease at 95% Confidence limit
[c]Significant increase at 95% Conifidence limit