Proposal Subject	Method to determine the presence of Male Specific Coliphage in shellfish meats and the Microbiology
Specific NSSP Guide Reference	NSSP Guidance Documents Chapter II. Growing Areas .10 Approved Laboratory Tests
Key Words	Microbiology Method Isolation of Male-specific Coliphage, MSC
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.

Enumeration of Male- specific bacteriophage in water and shellfish tissue

William Burkhardt III, Ph.D.
U.S. Food and Drug Administration,
Gulf Coast Seafood Laboratory
Dauphin Island, Alabama

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°C 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°C 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 ÷ 2.5 x 33 g supernatant x $\frac{100 \text{ grams}}{50 \text{ g homogenate}}$ = 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**	**3.81**	**3.13[b]**	**2.90[b]**
35°C	**-**	**3.81**	**3.73**	**2.90[b]**
35°C	**Log**	**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