ENUMERATION OF *ESCHERICHIA COLI* IN BIVALVE MOLLUSCAN SHELLFISH

NZFSA Guidelines

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HISTORY OF PROCEDURE

Controlled document title: Enumeration of *Escherichia coli* in Bivalve Molluscan Shellfish – NZFSA Guidelines

Version Number	Date Issued	Sections involved	Reason for change
1	10 December 2004	All	First issue
2	1 November 2005	All	Reviewed
3	3 August 2006	2.0 Scope	Revision
4	21 August 2006	8.0	Applicable to cooked as well as raw shellfish
5	3 October 2006	History of Procedure	Up-dated History
6	17 November 2006	1.0, 2.0, 4.0, 8.1, 8.2, 8.3, 8.4, 8.5	Clarification of use for other than raw shellfish

1.0 INTRODUCTION

Infectious human diseases acquired from the consumption of bivalve molluscan shellfish are internationally recognised. These health hazards are largely due to the phenomenon of filter feeding where-by bivalve molluscs concentrate and retain bacterial and viral pathogens often derived from sewage contamination of their growing waters. The risks of exposure to infectious agents are compounded by the traditional consumption of bivalve shellfish raw, or only lightly cooked. Historically, enteric bacteria, such as faecal coliforms, have been adopted as surrogate indicator organisms to assess the quality of shellfish flesh, and, consequently, to predict the risk of exposure to enteric pathogenic viruses.

European Directive 91/492/EEC (Anon 1991) stipulates conditions for the production and placing on the market of live bivalve molluscs. It sets out an end product standard, and categorises shellfish harvesting areas according to the degree of contamination with faecal indicator bacteria present in the shellfish flesh. In the United Kingdom *Escherichia coli* is used as an indicator of faecal contamination of bivalve molluscan shellfish. The European Community Reference Laboratory's (CRL) reference method for testing shellfish for *Escherichia coli* is ISO/TS 16649-3: 2005 (E).

The New Zealand Animal Products (Specifications for Bivalve Molluscan Shellfish) Notice 2006, requires that live raw bivalve molluscan shellfish be tested for *E. coli*. The method described in this document for enumeration of *E. coli* taken directly from ISO/TS 16649-3. ISO/TS 16649-3 refers to another ISO standard, ISO 6887-3:2003, for sample preparation but NZFSA has chosen to retain sample preparation from the Recommended Procedures for the Examination of Sea Water and Shellfish, 4th Edition, American Public Health Association, 1970, at least for live raw molluscan shellfish.

The requirements of ISO 6887-3:2003 are followed for sample preparation of processed and frozen molluscs.

The ISO/TS 16649-3 procedure is also suitable for fish and crustaceans. For preparation of these sample types see ISO 6887-3:2003.

2.0 SCOPE

This procedure describes the method for preparation of samples and enumeration of *E. coli* in live bivalve molluscan shellfish.

This procedure also describes sample preparation and enumeration of *E. coli* in processed* and frozen molluscan shellfish.

The theoretical limit of detection is a most probable number (MPN) of 20 *E. coli* per 100g of shellfish flesh. In the context of this test *E. coli* produces acid from lactose at 37°C and expresses β-glucuronidase activity at 44°C.

**For the purposes of this method, "processed" means any production process that could be applied to molluscan shellfish, and includes any combination of the following:*
Shucked, dried, smoked, marinated, salted, pickled, breaded, cooked.

3.0 REFERENCES

ISO/TS 16649-3:2005. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of β-glucuronidase-positive Escherichia coli –
Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.

ISO 6887-3:2003. Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination–
Part 3: Specific rules for the preparation of fish and fishery products.

TJ Donovan, S Gallacher, NJ Andrews, MH Greenwood, J Graham, JE Russel, D Roberts, R Lee. 1998. Modification of the standard method used in the United Kingdom for counting *Escherichia coli* in live bivalve molluscs. Communicable Disease and Public Health 1: 188-96.

CEFAS Standard Operating Procedure – Enumeration of Escherichia coli in Molluscan Bivalve Shellfish*, MFS SOP05i4.*

Recommended Procedures for the Examination of Sea Water and Shellfish, 4th Edition, American Public Health Association, 1970.

4.0 PRINCIPLE

The method used to enumerate *E. coli* is a two-stage, five-tube three-dilution most probable number (MPN) method. The first stage of the method is a resuscitation step requiring inoculation of minerals modified glutamate broth (MMGB) with a series of diluted sample homogenates and incubation at 37±1°C for 24±2 hours. The presence of *E. coli* is subsequently confirmed by subculturing acid producing tubes onto agar containing 5-bromo-4-chloro-3-indolyl-β-D glucuronide and detecting β-glucuronidase activity after incubation at 44±1°C for 24±2 hours.
[Note: ISO 16649-3:2005 (E) specifies a 5-tube MPN for live shellfish. For processed shellfish a 3-tube MPN is acceptable.]

3.0 SAFETY PRECAUTIONS

Standard microbiology safety precautions should be applied throughout. Risks of cuts and minor physical injury exist when performing this procedure, particularly when using sharp knifes to open shellfish. Appropriate measures to reduce these risks should be taken.

4.0 EQUIPMENT

- Waring blender and jars
- Peristaltic homogeniser (processed samples only)
- Refrigerator at 1 - 4°C
- Sterile glassware
- Shucking knife
- Safety/electric Bunsen system
- Latex gloves
- Safety gloves
- Incubator at 37±1°C
- Incubator at 44±1°C
- Loops - sterile, 1-μL and10-μL
- Pipette - automatic or manual for use with 1-mL and 10-mL open ended pipette tips

6.0 MEDIA AND REAGENTS

- Ethanol
- 0.1% Peptone water, pH 7.2 ± 0.2
- Minerals modified glutamate broth (MMGB) - Single strength
    -
    - Sodium glutamate 6.35g
    - Lactose 10.0g
    - Sodium formate 0.25g
    - L-Cystine 0.02g
    - L(-)-Aspartic acid 0.024g
    - L(+)-Arginine 0.02g
    - Thiamine 0.001g
    - Nicotinic acid 0.001g
    - Pantothenic acid 0.001g
    - Magnesium sulphate septahydrate 0.1g
    - Ferric ammonium citrate 0.01g
    - Calcium chloride dehydrate 0.01g

- Dipotassium hydrogen phosphate 0.9g
- Bromocresol purple 0.01g
- Ammonium chloride 2.5g
- Water 1,000mL
- Mix to dissolve completely, adjust pH, if necessary, so that the pH after autoclaving is 6.7 ± 0.1. Dispense in 10mL volumes into tubes. Sterilise in the autoclave for 10 minutes at 116°C.
- Equivalent commercial media maybe used. Prepare according to the manufacturers directions.
- Double-strength media may be prepared by adding twice the concentration of dry ingredients per litre of water.
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- 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) agar
  - Tryptone 20.0g
  - Bile salts No. 3 1.5g
  - BCIG (as cyclohexylammonium salt) 0.075g
  - Dimethyl sulfoxide* 3mL
  - Agar 15g
  - Water 1,000mL
  - Dissolve the BCIG in the dimethyl sulfoxide. Dissolve all ingredients in the water and heat to boiling. Adjust the pH, if necessary so that after sterilisation it is 7.2±0.2 at 25°C. Sterilise in the autoclave for 15 minutes at 121°C.
  - Equivalent commercial media maybe used.
    - Eg. Tryptone bile glucuronide agar (TBGA)
    - Eg. TBX agar
    - Prepare according to manufacturers instructions.

7.0 MICROBIOLOGICAL CONTROL CULTURES

Medium	Control strains	Criteria	Characteristic reactions
MMGM	*E. coli* ATCC 25922 or 8739	Acid production	Colour change to yellow
	E. faecalis ATCC 29212 or 19433	No growth	_
BCIG agar	*E. coli* ATCC 25922 or	Good growth	Blue to blue-green

	8739		colonies
	E. coli NCTC 13216 (weakly β-glucuronidase positive)	Good growth	Blue to blue-green colonies
	E. faecalis ATCC 29212 or 19433	No growth	_

8.0 PROCEDURE

8.1 SAMPLE RECEIPT

Live Raw Molluscs:

Samples must be received in an intact food grade plastic bag and properly packed in a cool box with ice packs – packed in this manner they should reach a temperature near 4°C (certainly <10°C). No more than 24 hour should elapse between sample collection and start of testing. Samples from harvesting areas should have been rinsed, but not immersed, and drained at the time of sampling. They should be regarded as unsatisfactory if they are received in the laboratory and the sample container is leaking, the shellfish are covered in mud or immersed in water or mud/sand.

Processed and Frozen Molluscs:

Samples of processed shellfish must be received in an intact food grade plastic bag or other suitable container and properly packed in a cool box with ice packs in such a manner that they are received at <4°C.

Samples that have been previously frozen should be received frozen.

8.2 SAMPLE STORAGE

Upon receipt in the laboratory the condition of the samples is to be checked and the temperature of the samples recorded. Records of corrective actions relating to non-conforming samples must be maintained, including notifications.

Chilled samples should be examined immediately or stored at 2-4°C until start of testing. Samples of live raw shellfish for analysis for *E. coli* must not be frozen.

Samples of frozen products that will not be tested on the day of receipt must be maintained in the frozen state (unless they are to be tested the following day, in which case they should be stored at 2-4°C overnight for a maximum of 24h).

8.3 SAMPLE SELECTION

Live Raw Molluscs only:

Choose shellfish that are alive according to the following points:

- If any flesh is exposed and reacts to touch using a sterile shucking knife with movement of any kind.
- If the shellfish are open and then close of their own accord.
- If a tap on the shell causes closing or movement.
- Tightly closed shellfish.

Discard all dead shellfish and those with obvious signs of damage.

The weight of shellfish flesh and liquor must be at least 200g. Sufficient shellfish must be opened to achieve this minimum weight of flesh and liquor, with the proviso that a minimum of ten animals should be used. In general, the more shellfish that are included in the initial homogenate, the less the final result will be influenced by the inherent animal-to-animal variation in *E. coli* concentration.

8.4 SAMPLE PREPARATION

Live Raw Molluscs:

Shucking knives, scrub brushes, and blender jars are (autoclave) sterilised for 30 minutes prior to use.

Blades of shucking knives are not corroded.

Prior to scrubbing and rinsing debris off shellstock, the hands of the analyst are thoroughly washed with soap and water.

The tap used to provide the potable water for rinsing the shellstock does not contain an aerator.

Shellstock are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality.

Shellstock are allowed to drain in a clean container or on clean towels prior to opening.

Prior to opening, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.

Shellstock are not shucked directly through the hinge.

Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.

When opening shellfish ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

The weight of shellfish flesh and liquor must be at least 200g.

Calculate the weight of shellfish flesh. Add an equal weight of sterile peptone diluent. Blend at high speed for 60-120 seconds.

Processed and Frozen Molluscs

Products stored frozen should be brought to a consistency that allows sampling, ie. by storing at 18-27°c (laboratory temperature) for up to 3h, or 2-4°C for up to 24h. Samples must be tested as quickly as possible after this. (Ref. ISO

Take 75-100g of processed shellfish flesh and add twice the weight of sterile peptone diluent. Blend with a rotary homogeniser for approximately 30s to 2 minutes. (A peristaltic homogeniser may be used but precautions may be necessary to prevent bag puncture from shell splinters, eg. double or triple bagging).

Proceed as per 8.5 below.

8.5 DILUTIONS

Live Raw Molluscs

Add 20±0.5 mL of blended shellfish homogenate to 80±1 mL of 0.1% peptone diluent using a 10 mL open-ended pipette to make a master 10^{-1} dilution. Thoroughly mix by vigorous shaking of the bottle. Make further dilutions to 10^{-2} in 0.1% peptone water, or, if samples are expected to be heavily polluted, additional decimal dilutions as necessary.

Processed and Frozen Molluscs

As for live raw molluscs above except add 30±0.5mL of blended shellfish homogenate to 70±1 mL of 0.1% peptone diluent to make the master 10^{-1} dilution.

[Note: ISO 6887-3:2003(E) utilises a smaller sample size (at least 6 animals ≈100g) and adds twice the weight of diluent prior to blending. 30mL of the homogenate is then added to 70mL diluent to prepare the 10^{-1} suspension. This NZFSA procedure requires a more representative sample of 10-12 animals and 200g. The larger size of some New Zealand shellfish means that the weight of 10 animals may be in excess of 300g. The addition of an equal the weight of diluent for blending minimises the prospect of excessive spillage during blending, and matches more closely previously used sample preparation procedures. The required 10^{-1} final concentration of shellfish is achieved in both procedures.]

8.6 INOCULATION AND INCUBATION OF PRIMARY BROTH

Live Raw Molluscs

Inoculate five tubes containing double strength MMGB with 10±0.2 mL of the 10^{-1} homogenate. Each tube contains the equivalent of 1 g of tissue.

Inoculate five tubes of single strength MMGB with 1±0.1 mL of the 10^{-1} homogenate. Each tube contains the equivalent of 0.1 g of tissue.

Inoculate five tubes of single strength MMGB with 1±0.1 mL of the 10^{-2} homogenate. Each tube contains the equivalent of 0.01 g of tissue.

If further dilutions have been prepared, inoculate 1±0.1 mL aliquots of each dilution to each of five tubes containing 10 mL of single strength MMGB.

Processed and Frozen Molluscs

As for raw live molluscs above except that a 3-tube MPN may be used instead of the 5-tube MPN.

Control cultures

Inoculate one tube of single strength MMGB with *Escherichia coli* using a 10µl loop.

Inoculate one tube of single strength MMGB with *E. faecalis* using a 10µl loop.

Incubate inoculated tubes of MMGB at 37±1°C for 24±2 hours.

8.7 CONFIRMATION OF *E. COLI*

After incubation examine the MMGB for the presence of acid. Acid production is denoted by yellow coloration throughout the medium but can be any change of colour from the negative. Absence of acid denotes a negative result for *E. coli*.

Confirm the presence of *E. coli* in all acid-positive tubes by subculturing onto a section of BCIG agar, streaking to obtain single colonies.

Divide Petri dishes into up to five equal sections using a marker pen to mark the base. (One plate can accommodate inoculations from five tubes at each dilution.) Label each section with the sample number, dilution, and tube number.

Subculture those tubes showing a change in colour or appearance when compared to the negative control tube.

Use a 1 µL inoculating loop. Remove excess liquid and streak an appropriately labelled section of the Petri dish to give single colonies.

Subculture positive and negative controls onto separate plates of BCIG agar.

Incubate BCIG agar at 44±1°C for 22± 2 hrs.

After the incubation period examine the BCIG agar plates for the presence of blue or blue-green colonies, which signify the presence of β-glucuronidase-positive *Escherichia coli*. Tubes of MMGB that grow blue or blue-green colonies are deemed to contain *E. coli*.
Absence of blue or blue-green colonies denotes a negative result for *E. coli*.

Control cultures

E. coli produces blue or blue-green colonies (β-glucuronidase positive)

E. faecalis does not produce blue or blue-green colonies (β-glucuronidase negative)

8.8 CALCULATION OF *E.COLI* MOST PROBABLE NUMBER AND REPORTING

Determine the number of confirmed MMGB tubes positive for *E. coli* for each dilution tested. This provides a three-figure number, which is required for the MPN table.

From the three figure number derived from the combination of positive results look up the MPN result using the MPN tables:

5-tube MPN – see appendix 1

3-tube MPN – see ISO 7218

For greater dilutions use the MPN Table and multiply the result by the extra number of dilution factors.

Results should be reported as the most probable number per 100g of shellfish. Negative samples should be reported as MPN <20 /100g.

9.0 APPENDIX 1

***E. COLI* MOST PROBABLE NUMBER (MPN) TABLES** *(From Donovan, et al, 1998)*

MPN table for multiple tube methods using **5 × 1g, 5 × 0.1g, 5 × 0.01g.**

1g	0.1g	0.01g	MPN/100g	
0	0	0	<20	
0	0	1	20	
0	1	0	20	
1	0	0	20	
1	0	1	40	
1	1	0	40	
1	2	0	50	
2	0	0	40	
2	0	1	50	
2	1	0	50	
2	1	1	70	
2	2	0	70	
2	3	0	110	
3	0	0	70	
3	0	1	90	
3	1	0	90	
3	1	1	130	
3	2	0	130	
3	2	1	160	
3	3	0	160	
4	0	0	110	
4	0	1	140	
4	1	0	160	
4	1	1	200	
4	2	0	200	Category A
5	0	0	220	(<230 *E. coli*)
4	2	1	250	
4	3	0	250	Category B
4	3	1	310	(>230 *E. coli*)
4	4	0	320	(<4600 *E. coli*)
4	4	1	380	
5	0	1	290	
5	0	2	410	
5	1	0	310	
5	1	1	430	
5	1	2	600	
5	1	3	850	
5	2	0	500	
5	2	1	700	
5	2	2	950	
5	2	3	1200	
5	3	0	750	
5	3	1	1100	
5	3	2	1400	
5	3	3	1750	
5	3	4	2100	
5	4	0	1300	
5	4	1	1700	
5	4	2	2200	

5	4	3	2800	
5	4	4	3450	
5	5	0	2400	Category B
5	5	1	3500	(<4600 *E. coli*)
5	5	2	5400	Category C
5	5	3	9100	(>4600 *E. coli*)
5	5	4	16000	(<46000 *E. coli*)
5	5	5	>18000	