STATUS OF NATIONAL STANDARD METHODS

National Standard Methods, which include standard operating procedures (SOPs), algorithms and guidance notes, promote high quality practices and help to assure the comparability of diagnostic information obtained in different laboratories. This in turn facilitates standardisation of surveillance underpinned by research, development and audit and promotes public health and patient confidence in their healthcare services. The methods are well referenced and represent a good minimum standard for clinical and public health microbiology. However, in using National Standard Methods, laboratories should take account of local requirements and may need to undertake additional investigations. The methods also provide a reference point for method development.

National Standard Methods are developed, reviewed and updated through an open and wide consultation process where the views of all participants are considered and the resulting documents reflect the majority agreement of contributors.

Representatives of several professional organisations, including those whose logos appear on the front cover, are members of the working groups which develop National Standard Methods. Inclusion of an organisation’s logo on the front cover implies support for the objectives and process of preparing standard methods. The representatives participate in the development of the National Standard Methods but their views are not necessarily those of the entire organisation of which they are a member. The current list of participating organisations can be obtained by emailing standards@hpa.org.uk.

The performance of standard methods depends on the quality of reagents, equipment, commercial and in-house test procedures. Laboratories should ensure that these have been validated and shown to be fit for purpose. Internal and external quality assurance procedures should also be in place.

Whereas every care has been taken in the preparation of this publication, the Health Protection Agency or any supporting organisation cannot be responsible for the accuracy of any statement or representation made or the consequences arising from the use of or alteration to any information contained in it. These procedures are intended solely as a general resource for practising professionals in the field, operating in the UK, and specialist advice should be obtained where necessary. If you make any changes to this publication, it must be made clear where changes have been made to the original document. The Health Protection Agency (HPA) should at all times be acknowledged.

The HPA is an independent organisation dedicated to protecting people’s health. It brings together the expertise formerly in a number of official organisations. More information about the HPA can be found at www.hpa.org.uk.

The HPA aims to be a fully Caldicott compliant organisation. It seeks to take every possible precaution to prevent unauthorised disclosure of patient details and to ensure that patient-related records are kept under secure conditions.

More details can be found on the website at www.evaluations-standards.org.uk. Contributions to the development of the documents can be made by contacting standards@hpa.org.uk.

Suggested citation for this document:

Please note the references are now formatted using Reference Manager software. If you alter or delete text without Reference Manager installed on your computer, the references will not be updated automatically.

DETECTION AND ENUMERATION OF ENTEROBACTERIACEAE
Issue no: 1 Issue date: 14.12.05 Issued by Standards Unit, Evaluations and Standards Laboratory in conjunction with the Regional Food, Water and Environmental Coordinators Forum Page 2 of 13
Reference no: F 18i
This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency www.evaluations-standards.org.uk
Email: standards@hpa.org.uk
AMENDMENT PROCEDURE

<table>
<thead>
<tr>
<th>Controlled document reference</th>
<th>F 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlled document title</td>
<td>Standard Operating Procedure for Detection and Enumeration of Enterobacteriaceae</td>
</tr>
</tbody>
</table>

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

<table>
<thead>
<tr>
<th>Amendment Number/ Date</th>
<th>Issue no. Discarded</th>
<th>Insert Issue no.</th>
<th>Page</th>
<th>Section(s) involved</th>
<th>Amendment</th>
</tr>
</thead>
</table>

DETECTION AND ENUMERATION OF ENTEROBACTERIACEAE

Issue no: 1 Issue date: 14.12.05 Issued by Standards Unit, Evaluations and Standards Laboratory in conjunction with the Regional Food, Water and Environmental Coordinators Forum

Reference no: F 18i3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

www.evaluations-standards.org.uk
Email: standards@hpa.org.uk
INTRODUCTION

Scope

The method described is applicable to the detection and enumeration of Enterobacteriaceae in all food types, including milk and dairy products.

Background

Regulation (EC) 852/2004\(^2\) on the hygiene of foodstuffs and regulation (EC) 853/2004\(^3\) laying down specific rules for food of animal origin will be enacted in English law on 6.1.06 as the Food Hygiene Regulations 2005\(^4\). Associated legislation\(^5\) specifies microbiological criteria and methods for testing for a number of food commodities. Included in the hygiene criteria are specifications for Enterobacteriaceae, to be applied at the end of the manufacturing process of egg products, milk and other dairy products, dried infant formulae and dried dietary foods for special medical purposes, and to meat carcases after dressing but before chilling. The legislation also specifies the methods to be used, which for pasteurised milk, liquid dairy products, milk and whey powder, infant formulae and dried dietary foods is the horizontal method ISO 21528-1\(^6\), a Most Probable Number method. The method described below is based on ISO 21528-1. It allows detection of Enterobacteriaceae and enumeration by the most probable number (MPN) technique after incubation at 30°C (for milk and dairy products) or 37°C (for other food products). It is particularly suitable for use when levels are likely to be low (less than 100/g or 10/mL), and for recovery from products (dried, frozen etc) when the target organism is likely to be stressed.
1.0 PRINCIPLE

The enumeration of Enterobacteriaceae by the MPN technique involves six stages:

- Inoculation of three tubes of buffered peptone water per dilution of the test sample, using those dilutions appropriate to obtaining the required detection parameters for that product
- Incubation of those tubes at 30°C ± 1°C or 37°C ± 1°C for 24 hours
- Subculture to Enterobacteriaceae Enrichment (EE) broth with incubation at 30°C ± 1°C or 37°C ± 1°C for 24 hours
- Subculture of each tube to Violet Red Bile Glucose Agar (VRBGA) and incubation at 30°C or 37°C for 24 hours
- Confirmation of Enterobacteriaceae presence from tubes producing red-purple colonies on VRBGA by glucose fermentation and oxidase tests
- Determination of the MPN index from the number of positive tubes of selected dilutions using an MPN table and calculation of the Enterobacteriaceae count per gram or millilitre of sample

2.0 DEFINITIONS

For the purpose of this method, the following definitions apply:

Enterobacteriaceae
Bacteria which at 30°C ± 1°C or 37°C ± 1°C are capable of forming characteristic colonies on violet red bile glucose agar (VRBGA) and that ferment glucose and show a negative oxidase reaction under the test conditions specified.

Detection of Enterobacteriaceae
Determination of the presence or absence of these microorganisms in a defined weight or volume of food.

Enumeration of Enterobacteriaceae
Determination of the most probable number per gram or millilitre of these microorganisms.

3.0 SAFETY CONSIDERATIONS

Normal microbiology precautions apply. In addition, members of the Enterobacteriaceae family may be pathogenic to man and therefore isolation and identification must be performed by trained laboratory personnel in a properly equipped laboratory and under the supervision of a trained microbiologist. Care must be taken in the disposal and sterilisation of all test materials. Procedures involving subculturing from pre-enrichment and enrichment broths and handling of Enterobacteriaceae cultures must be performed in a designated area of the laboratory.

4.0 EQUIPMENT

Usual laboratory equipment and in addition:

Top pan balance capable of weighing 0.1 g
Gravimetric diluter (optional)
Stomacher
Vortex mixer
Incubator at 30°C ± 1°C or 37°C ± 1°C
Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
Pipettes (total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)
Stomacher bags (sterile)

5.0 CULTURE MEDIA AND REAGENTS

Equivalent commercial dehydrated media may be used; follow the manufacturer’s instructions.

Peptone saline diluent (Maximum recovery diluent)
Peptone 1.0 g
Sodium chloride 8.5 g
Water 1 L
pH 7.0 ± 0.2 at 25°C

Buffered peptone water (BPW)

<table>
<thead>
<tr>
<th></th>
<th>Single strength</th>
<th>Double strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate dodecahydrate</td>
<td>9.0 g</td>
<td>18.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.5 g</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
<td>1 L</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.2 at 25°C</td>
<td></td>
</tr>
</tbody>
</table>

Dispense 10 mL volumes of single and double strength broth into tubes or bottles for enumeration or 90 mL volumes of single strength broth for detection

Enterobacteriaceae Enrichment broth (EE)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>6.45 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Ox bile</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Brilliant green</td>
<td>0.0135 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.2 at 25°C</td>
</tr>
</tbody>
</table>

Dispense in 10 mL volumes in tubes or bottles
Violet Red Bile Glucose Agar (VRBGA)

Yeast extract 3.0 g  
Pepitone 7.0 g  
Sodium chloride 5.0 g  
Bile salts No.3 1.5 g  
Glucose 10.0 g  
Neutral red 3 mg  
Crystal violet 2 mg  
Agar 12 g  
Water 1 L  
pH 7.4 ± 0.2 at 25°C

Glucose agar

Tryptone 10.0 g  
Yeast extract 1.5 g  
Glucose 10.0 g  
Sodium chloride 5.0 g  
Bromocresol purple 15 mg  
Agar 12.0 g  
Water 1 L  
pH 7.0 ± 0.2 at 25°C

Dispense in 10 mL amounts in test tubes or 15 ml amounts in universal containers.

Nutrient agar

Meat extract 10.0 g  
Pepitone 10.0 g  
Sodium chloride 5.0 g  
Agar 15.0 g  
Water 1 L  
pH 7.5 ± 0.2 at 25°C

Plate Count agar

Yeast extract 2.5 g  
Enzymatic digest of casein 5.0 g  
Glucose 1.0 g  
Agar 12.0 g  
Water 1 L  
pH 7.0 ± 0.2 at 25°C

Oxidase reagent (prepare fresh as required or use commercially available equivalent)

6.0 SAMPLE PROCESSING

6.1 Preparation of the sample

6.1.1 Detection

Weigh x g (usually 10 g) of sample, add 9x mL (usually 90 mL) of single strength buffered peptone water and homogenise. Incubate as described in section 6.2 and continue as described in subsequent sections.
6.1.2 Enumeration

Prepare the test portion, the $10^{-1}$ initial suspension and further decimal dilutions as described in Standard Method D1: Milk and Dairy Products - Preparation of Samples and Decimal Dilutions or Standard method F2: Food products – Preparation of Samples and Dilutions. Use a separate pipette for each dilution.

6.2 Pre-enrichment

If a low level of detection is required (<10/g or mL), add 10 mL of test sample if liquid, or 10 mL of the $10^{-1}$ suspension, to each of three tubes containing double strength buffered peptone water (BPW). Add 1 mL of the test sample if liquid, or 1 mL of the $10^{-1}$ suspension, to each of three tubes containing single strength BPW. Add 1 mL of each further dilution, as required, to each of three tubes containing single strength BPW. Carefully mix the inoculum and the medium. Incubate all inoculated tubes at 30°C (if milk or dairy product) or 37°C (other food products) for 24 ± 2 hours.

Positive control: Inoculate a tube of single strength medium with *Escherichia coli* NCTC 9001.

Negative control: Inoculate a tube of single strength medium with *Enterococcus faecalis* NCTC 775.

6.3 Enrichment

At the end of incubation transfer 1 mL from each tube to 10 mL of Enterobacteriaceae Enrichment broth (EE). Incubate EE tubes at 30°C (milk and dairy products) or 37°C (other food products) for 24 ± 2 hours.

6.4 Isolation

At the end of incubation subculture each tube to a third section of a VRBGA plate. Incubate the plates at 30°C (milk and dairy products) or 37°C (other food products) for 24 ± 2 hours. Examine the plates for the presence of purple-red colonies with a diameter of 0.5 mm or greater, with or without a red zone of precipitated bile. Record the results.

6.5 Confirmatory tests

From each section of VRBGA showing presence of typical colonies select a well isolated suspect Enterobacteriaceae colony to perform further tests.

**Fermentation test**

Prior to use, steam or boil the glucose agar for 10 minutes and allow to set. Inoculate by a deep stab inoculation of tubes of glucose agar, then use the same inoculum to streak onto a non-selective medium such as Nutrient Agar (NA) or Plate Count Agar (PCA). Place the media in an incubator at 37°C for 24 ± 2 hours. Enterobacteriaceae produce a yellow colour throughout the glucose medium. Also examine the plate count agar to check for purity.

**Oxidase test**

Use the growth on PCA to perform an oxidase test according to Standard Method BSOP TP 26. Development of a purple colour within 10 seconds denotes a positive reaction. Members of the Enterobacteriaceae are oxidase negative.
7.0 CALCULATION OF RESULTS

Count the number of tubes containing confirmed Enterobacteriaceae at each dilution and use the table (Appendix 1) to obtain the MPN/g or mL. This table assumes the use of $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions. If 10 mL of the $10^{-1}$ dilution have been used as the first dilution (e.g., milk and dairy products), divide the MPN index by 10. If 10 mL of the undiluted liquid sample has been used, divide the MPN index by 100. If more than three dilutions have been used, select the three consecutive dilutions that give a category 1 MPN index and multiply the MPN index by the appropriate power of 10. If no combination with category 1 is available, use the combination with category 2; if more than one combination with category 2 is obtained, use the one with the highest number of positive tubes.

8.0 REPORTING RESULTS

8.1 Detection

Report the result as Detected or Not detected in the weight examined.

8.2 Enumeration

Report the result as the most probable number of Enterobacteriaceae per gram or mL. If the result is less than 10, report to the nearest whole number. If the result lies between 10 and 99 report that number. If the result is 100 or more, report as a number between $1.0$ and $9.9 \times 10^{x}$, where $x$ is the appropriate power of 10. If Enterobacteriaceae are detected but are less than 1, report detected, less than 1 per g or mL. If no tubes are positive, report the result as less than the lowest MPN value per gram or mL for the dilutions used, or as not detected in 1 g or 1 mL if the lowest MPN value is 0.3.
### APPENDIX 1: DETERMINATION OF MOST PROBABLE NUMBER

#### Determination Of Most Probable Number

<table>
<thead>
<tr>
<th>Number of positive results</th>
<th>Confidence limits</th>
<th>Category</th>
<th>Lower limit</th>
<th>Upper limit</th>
<th>Lower limit</th>
<th>Upper limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻³ dilution</td>
<td>10⁻² dilution</td>
<td>MPN per g/ml</td>
<td>&gt; 95%</td>
<td>&gt; 99%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>&lt;3</td>
<td>0.1</td>
<td>3</td>
<td>9.4</td>
<td>14</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1.2</td>
<td>6.1</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>3</td>
<td>1.2</td>
<td>6.2</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>9.4</td>
<td>1.2</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>7.2</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>11</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>7.4</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>14</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>20</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>20</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>20</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>20</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>29</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>36</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>23</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>38</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>64</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>43</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>75</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>120</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>160</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>93</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>210</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>290</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>240</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>460</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1100</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>&gt;1100</td>
<td>0.1</td>
<td>3.6</td>
<td>17</td>
<td>25</td>
</tr>
</tbody>
</table>

Adapted from de Man JC, 1983, Eur J Appl Biotechnol. 17, 301-305

**Category 1:** Results have the greatest chance of being obtained. There is only at most 5% chance of obtaining a result that is less likely than the least likely one in this category.

**Category 2:** Results have less chance of being obtained than even the least likely one in category 1, but there is only at most 1% chance of obtaining a result that is less likely than the least likely one in this category.

**Category 3:** Results have less chance of being obtained than even the least likely one in category 2, but there is only at most 0.1% chance of obtaining a result that is less likely than the least likely one in this category.

**Category 0:** The result is one of those that have less chance of being obtained than even the least likely one in category 3. There is only a chance of 0.1% of obtaining a result in this category without anything being wrong.

---

DETECTION AND ENUMERATION OF ENTEROBACTERIACEAE

Issue no: 1 Issue date: 14.12.05 Issued by Standards Unit, Evaluations and Standards Laboratory in conjunction with the Regional Food, Water and Environmental Coordinators Forum

Reference no: F 18i1

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

Email: standards@hpa.org.uk
APPENDIX 2: FLOWCHART SHOWING THE DETECTION AND ENUMERATION OF ENTEROBACTERIACEAE

For detection weigh x g or mL and add 9 x g of BPW; homogenise

\[ \downarrow \]

For enumeration prepare $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions of the sample as required in MRD (adjust pH of $10^{-1}$ if necessary to 6.8 ± 0.2)

\[ \downarrow \]

For milk and most dairy products use 3 tubes of double strength BPW with 10mL of $10^{-1}$ dilution and three tubes of single strength BPW with 1mL of $10^{-1}$ and $10^{-2}$ dilutions (detection limit 0.3/g)

For frozen dairy products and most other foods use three tubes of single strength BPW with 1mL of $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions (detection limit 3/g)

\[ \downarrow \]

Place in an incubator at 30°C ± 1°C (milk and dairy products) or 37°C ± 1°C (other products) for 24 ± 2 h

\[ \downarrow \]

Transfer 1 mL of all tubes to 10 mL of EE broth and place in an incubator at 30°C ± 1°C (milk and dairy products) or 37°C ± 1°C (other products) for 24 ± 2 h

\[ \downarrow \]

Subculture all tubes to a 1/3 segment of VRBGA and incubate at 30°C ± 1°C (milk and dairy products) or 37°C ± 1°C (other products) for 24 ± 2 h

\[ \downarrow \]

Subculture a typical colony from each tube yielding growth of presumptive Enterobacteriaceae on VRBGA to glucose agar and NA or PCA. Incubate media at 37°C ± 1°C for 24 ± 2h

\[ \downarrow \]

Examine glucose agar for the presence of fermentation; examine PCA for purity and oxidase reaction

\[ \downarrow \]

Count tubes which yield oxidase negative cultures that show fermentative growth as positive for Enterobacteriaceae

\[ \downarrow \]

Calculate the count per g or mL and report
REFERENCES


