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Health Protection Agency

NATIONAL STANDARD METHOD

ENUMERATION OF ENTEROCOCCI BY MEMBRANE FILTRATION

W 3

Issued by Standards Unit, Evaluations and Standards Laboratory
Centre for Infections

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AMENDMENT PROCEDURE

Controlled document reference	W 3
Controlled document title	Standard Operating Procedure for Enumeration of Enterococci by Membrane Filtration

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.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
5/ 11.08.06	2.2	3	5	**Background**	Updated
			6	**Definitions**	Updated
			8 & 9	**6.4.1 Aesculin hydrolysis**	Updated in line with SCA 2006 Photographs of aesculin hydolysis on Bile aesculin agar added
			12	**References**	Updated and incorporated into Reference Manager Software

STANDARD OPERATING PROCEDURE FOR THE ENUMERATION OF ENTEROCOCCI BY MEMBRANE FILTRATION

SCOPE OF DOCUMENT

The method gives general guidelines for the enumeration of enterococci in samples from water intended for human consumption by the membrane filtration technique. It is also applicable to environmental and bathing waters. See QSOP 57[2] for examples of samples which can be examined.

INTRODUCTION

Background

Enterococci do not multiply in water. They are usually more resistant than *Escherichia coli* to environmental stress and chlorination, and as a result survive longer. Enterococci normally inhabit the intestine of man and animals, and can provide an indication of past pollution. Examination for enterococci also assists the interpretation of doubtful results from other tests such as the occurrence of large numbers of coliforms in the absence of *E. coli*[3].

For potable waters, enterococci should not be detected in 250 mL samples of bottled water[4], water in containers[5] and natural mineral water[6] or 100 mL of other potable waters[7-9]. Guidelines are given for bathing waters in The Bathing Waters (Classification) Regulations 1991[10] and the revised Bathing Water Directive 2006/07/EC[11]. This method is based on the method described in The Microbiology of Drinking Water, 2006[3] and BS EN ISO 7899-2[12] which is the method specified in the EC Directive[13] and the Water Supply Regulations[14].

The Microbiology of Drinking Water, 2006[3] contains validation data from an inter-laboratory trial for the membrane filter transfer technique for the confirmation of enterococci isolated from various waters. Laboratories should perform an in-house verification demonstrating that the method is fit for purpose.

1 DEFINITIONS

For the purpose of this method the following definitions apply:

Presumptive enterococci
Organisms capable of aerobic growth and of forming characteristic pink to maroon coloured colonies by reduction of tetrazolium chloride to the red dye formazan on a selective isolation medium containing sodium azide and glucose, at 37°C or 44°C within 48 hours. Some strains may produce pale colonies.

Note: Incubating at the higher temperature is a compromise between selectivity and sensitivity. Selectivity is better at 44°C although lower counts of enterococci may be obtained as some strains of enterococci do not grow at 44°C.

Enterococci
Presumptive enterococci that hydrolyse aesculin at 44°C in the presence of bile salts.

2 PRINCIPLE

A measured volume of the sample or a dilution is filtered through a membrane that is capable of retaining enterococci. The membrane is incubated on a selective/differential agar and characteristic colonies are counted. Confirmatory tests are then carried out and the result is calculated as the colony count per 100 mL of sample. For bottled waters, water in containers and natural mineral water the count is calculated as the colony count per 250 mL of sample.

3 SAFETY CONSIDERATIONS[15-24]

Normal microbiology laboratory precautions apply.

3.1 SAMPLE COLLECTION

N/A

3.2 SAMPLE TRANSPORT AND STORAGE

Compliance with current transportation regulations is essential.

3.3 SAMPLE PROCESSING

- Care must be taken when using boiling water or a boiling waterbath for disinfection of the filtration apparatus

- Use heat and water resistant protective gloves and do not put the face or hands over the bath when opening to remove objects

- In addition, membrane enterococcus agar (mEA) (Section 5) contains sodium azide. This substance is highly toxic. However this medium contains only 0.04% azide and therefore has low toxicity. Although sodium azide can form spontaneously explosive compounds when it comes into contact with metals such as in copper pipes the concentration is so little that handling and disposal of the prepared medium presents no significant risk

The above guidance should be supplemented with local COSHH and risk assessments

4 EQUIPMENT

Usual laboratory equipment and in addition:

- Membrane filtration manifold
- Filter funnels graduated to 50 mL and 100 mL
- Pyrex vacuum flask with protective jacket or equivalent: large volume eg 5 L
- Vacuum pump with moisture trap or protective filter, or alternative vacuum source
- Stainless steel flat tipped forceps
- Boiling waterbath (for instrument disinfection)
- Cycling incubator: 37°C ± 1.0°C and 44°C ± 0.5°C (optional)
- Incubator: 37°C ± 1.0°C
- Incubator: 44°C ± 0.5°C
- Petri dishes
- Cellulose ester 0.45 μm gridded membrane filters
- Automatic pipettors and associated sterile tips capable of delivering up to 10 mL and 1mL volumes (optional)
- Pipettes, (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)

5 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 (NaCl)	8.5 g
Water	1 L
pH 7.0 ± 0.2 at 25°C	

Quarter strength Ringer's solution

Sodium chloride (NaCl)	2.25 g
Potassium chloride (KCl)	0.11 g
Calcium chloride ($CaCl_2$)	0.12 g
Sodium bicarbonate ($NaHCO_3$)	0.05 g
Water	1 L

Membrane enterococcus agar (mEA)

Tryptose	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.0 g
Sodium azide (NaN_3)	0.4 g
2,3,5 triphenyl tetrazolium chloride (TTC)	0.1 g
Agar	12.0 g
Water	1 L
pH 7.2 ± 0.2 at 25°C	

Bile aesculin agar (BAA)

Bile salts	20.0 g
Peptone	8.0 g
Ammonium iron (III) citrate	0.5 g
Aesculin	1.0 g

Agar	12.0 g
Water	1 L
pH 7.1 ± 0.2 at 25°C	

6 SAMPLE PROCESSING

6.1 SAMPLE PREPARATION

Water samples are to be received and handled as described in SOP W 1 Section 5. In brief the nature of the request and condition of the sample is to be noted on arrival. Protect the samples from direct sunlight and transport in an insulated container or refrigerator at 2°C – 8°C. Samples should be analysed as soon as is practicable on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours before the commencement of analysis.

Following the procedures laid down in SOP W 1 Section 5 select suitable volumes for analysis and prepare any necessary dilutions.

6.2 FILTRATION AND INCUBATION

Following the procedures laid down in SOP W 1 Section 5, filter a measured volume of sample through the membrane. For bottled waters, water in containers and natural mineral waters filter 250 mL water, for other potable quality waters use volumes of 100 mL. For surface waters use 100 mL and, if expected to be highly polluted, 10 mL and 1 mL volumes.

Place the membrane onto mEA.

At 37°C ± 1°C for 44 hours ± 4 hours for potable water, if few enterococci are expected such as in drinking water.

At 37°C ± 1°C for 4 hours ± 0.5 hour followed by 40 ± 4 hours at 44°C ± 0.5°C for non-potable water likely to contain a high background count eg river water, sea water.

6.3 COUNTING OF COLONIES

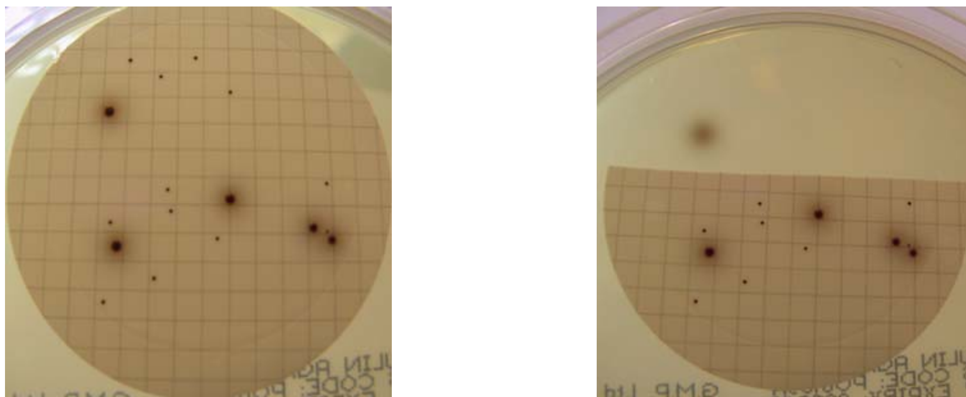
After incubation, enumerate the presumptive enterococci by counting all red, maroon, pink or pale pink colonies.

6.4 CONFIRMATORY TESTS

6.4.1 AESCULIN HYDROLYSIS

Transfer the membrane filter from mEA (membrane filter face up with the colonies uppermost) to a BAA plate pre-warmed to room temperature. Ensure that no air bubbles are trapped between the membrane filter and the medium. Incubate the BAA plate at 44°C ± 0.5°C for 4 hrs ± 5 minutes.

Figure 1 **Colonies on bile aesculin agar (BAA)**



Enterococcus faecalis (large colonies with halo) with *Aerococcus viridans* (small colonies without halo)

Development of colonies that are surrounded by a black or brown halo in the surrounding medium resulting from the aesculin hydrolysis process confirms the presence of enterococci (see Figure 1). The use of a heavy inoculum will increase the speed at which results are obtained, and a positive result may be evident after a few hours incubation.

7 CALCULATION OF RESULTS

Calculate the presumptive count of enterococci as follows:

$$\text{Presumptive count / 100mL} = \frac{\text{Number of colonies counted}}{\text{Volume tested}} \times 100$$

Following the procedures described in SOP W 1 Section 7, calculate the number of confirmed enterococci present in the original sample.

8 REPORTING

Report the results using the procedure described in SOP W 1 Section 9.

If enterococci are not detected, report as:

Not detected in 100 mL

If the test organisms are detected report as:

a in 100 mL

where **a** is the presumptive or confirmed count

For natural mineral waters the result is reported as the count per 250 mL.

9 QUALITY CONTROL

Membrane filtration

When the membrane filtration technique is used internal quality control procedures must be carried out at least once daily depending on the workload of the laboratory. If more than one batch of media is used in a session it is necessary to repeat the quality control test for each batch.

The quantitative internal quality controls are to be carried out using suspensions of positive and negative control organisms known to contain less than 100 colony forming units in the volume filtered.

Positive control:
Enterococcus faecalis NCTC 775

Negative control:
Escherichia coli NCTC 9001

Blank control:
Filter 100mL of sterile distilled water, peptone saline diluent or quarter strength Ringer's solution using the same funnel as used for the positive control following disinfection.

Incubate all tests in parallel with routine tests, proceed with confirmatory tests and determine the count.

Confirmatory tests
Details of the test strains are presented below. Inoculate each batch of medium/reagent used on the day of testing with the control organism. Incubate in parallel with the routine tests.

Bile aesculin agar
Test strains 44°C ± 0.5°C for the same incubation time as the routine tests.

Enterococcus faecalis NCTC 775
positive (black)

Escherichia coli NCTC 9001
negative (colourless)

10 REFERENCE FACILITIES

N/A

11 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the Water Working Group for Standard Methods (http://www.hpa-standardmethods.org.uk/wg_water.asp). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

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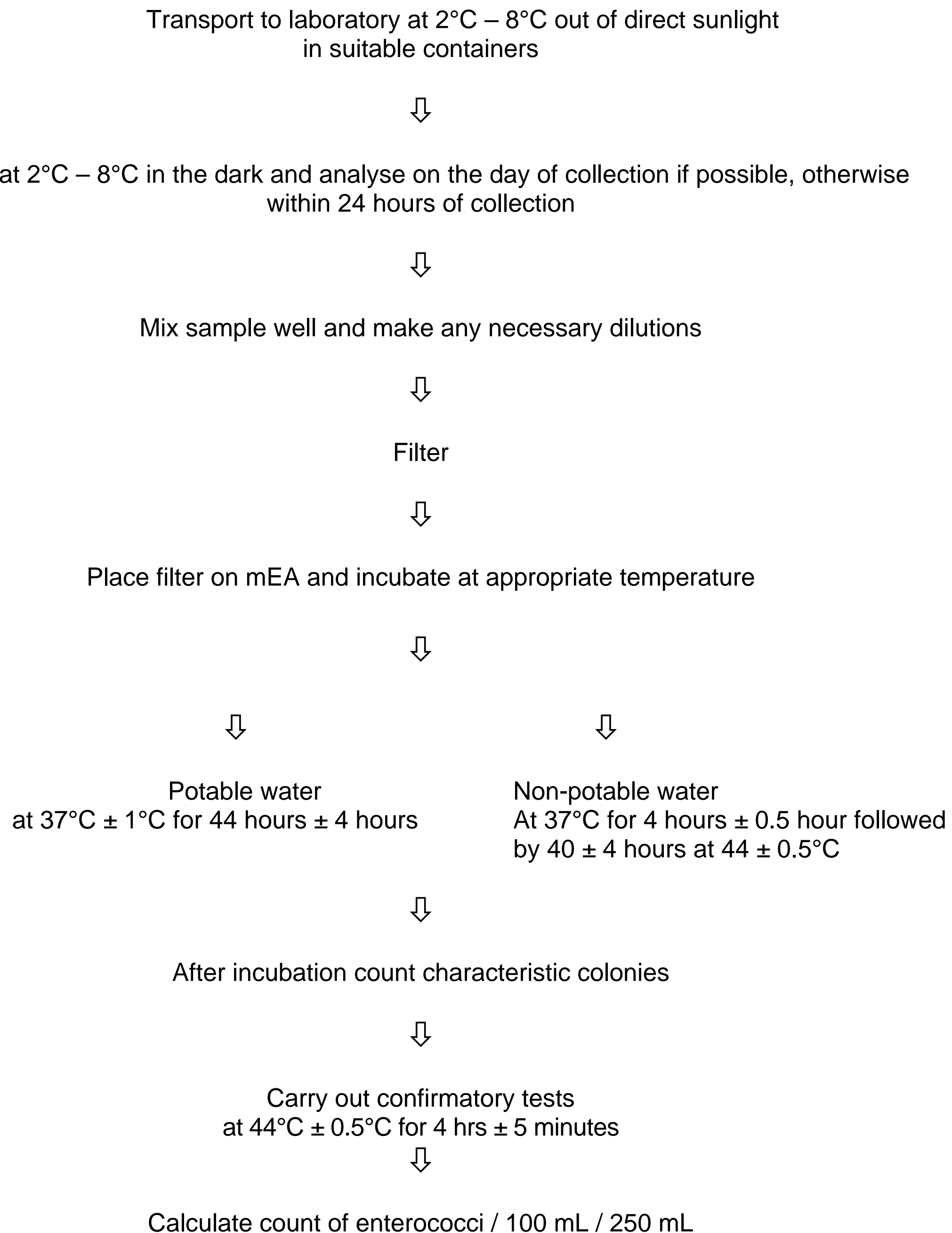
APPENDIX 1: FLOWCHART SHOWING THE ENUMERATION OF ENTEROCOCCI BY MEMBRANE FILTRATION



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