

NATIONAL STANDARD METHOD

ENUMERATION OF *AEROMONAS* SPECIES BY MEMBRANE FILTRATION

W 9

Issued by Standards Unit, Evaluations and Standards Laboratory
Specialist and Reference Microbiology Division

ENUMERATION OF *AEROMONAS* SPECIES BY MEMBRANE FILTRATION

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AMENDMENT PROCEDURE

Controlled document reference	W 9
Controlled document title	Standard Operating Procedure for Enumeration of <i>Aeromonas</i> species 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
3/ 03.05.05	1.2	1.3	1	Front page	Redesigned
			2	Status of document	Reworded
			4	Amendment page	Redesigned

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STANDARD OPERATING PROCEDURE FOR THE ENUMERATION OF *AEROMONAS* SPECIES BY MEMBRANE FILTRATION

INTRODUCTION

Scope

The method described is applicable to the determination of *Aeromonas* species in samples of potable water, pool waters and surface waters except those with high turbidity which may block the membrane.

Background

The taxonomy of the genus *Aeromonas* is still the subject of considerable research. At least 13 species can be identified by molecular techniques. Using simple biochemical tests it is not possible to identify these species accurately but most isolates can be broadly divided into three groups: *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas veronii* biotype *sobria*. As all *Aeromonas* species may be considered significant, it is not necessary to identify them beyond genus level.

A variety of media have been developed for the isolation of *Aeromonas* species, many of which contain ampicillin as the selective agent. Ampicillin-dextrin agar (ADA)¹ is recommended for use in this SOP. It is essentially the same as mA agar² except that it has been made more specific by the substitution of dextrin for trehalose. Some *Vibrio* species can also grow on ADA and produce similar reactions to *Aeromonas* species. If samples are likely to contain *Vibrio* species eg estuarine waters, then ADA can be made more selective by including 50 mg/L of O129 phosphate.

The method described is based on that in the Microbiology of Drinking Water document 2002³.

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1.0 DEFINITIONS

For the purpose of this method, strains are identified as *Aeromonas* if they are oxidase-positive, resistant to ampicillin (10 mg/L) and ferment dextrin and mannitol. In addition they are Gram-negative, fermentative in the Hugh and Leifson (H/L) fermentation test⁴, able to grow in 1% tryptone water containing 0% but not 6% sodium chloride, resistant to O129 (2,4-diamino-6,7-diisopropyl pteridine) phosphate (50 mg/L) and hydrolyse arginine⁵.

2.0 PRINCIPLE

The enumeration of *Aeromonas* species involves filtering a volume of test water through a membrane, and isolation of the organisms by placing the membrane on an agar medium containing ampicillin as a selective agent, dextrin as the fermentable carbohydrate and bromothymol blue as indicator of acidity. Presumptive isolates are sub-cultured and their identity confirmed using the tests listed in section 1, and described in section 6.3.1.

3.0 SAFETY CONSIDERATIONS⁶⁻¹⁵

Normal microbiology laboratory precautions apply.

3.1 Specimen collection

N/A

3.2 Specimen transport and storage

Compliance with current postal and transportation regulations is essential.

3.3 Specimen processing

- Care must be taken when removing objects from boiling water after disinfection

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

4.0 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 (capacity >5L) or equivalent plastic pressure vessel
- Vacuum pump with moisture trap and protective filter, or alternative vacuum source
- Stainless steel flat-tipped forceps
- Boiling waterbath (instrument steriliser)
- Incubator at 30°C±1°C
- Petri dishes
- Membrane filters – gridded, cellulose ester, 0.45 µm, 47 mm diameter
- Automatic pipettors and sterile pipette tips capable of delivering 1-10 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)

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5.0 CULTURE MEDIA

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

Quarter strength Ringer's solution

Sodium chloride	2.25 g
Potassium chloride	0.11 g
Calcium chloride, anhydrous	0.12 g
Sodium bicarbonate	0.05 g
Water	1 L

Ampicillin dextrin agar (ADA) (freshly prepared)

Tryptose	5.0 g
Dextrin	10.0 g
Yeast extract	2.0 g
Sodium chloride	3.0 g
Potassium chloride	2.0 g
Magnesium sulphate heptahydrate	0.2 g
Ferric chloride	0.1 g
Bromothymol blue	0.08 g
Sodium desoxycholate	0.1 g
Ampicillin	0.01 g
2,4-diamino 6,7 di- isopropyl pteridine (O129) phosphate (optional)	50.0 mg
Agar	15.0 g
Water	1 L

pH 8.0 ± 0.2 at 25°C

Nutrient agar (or equivalent)

Meat extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1 L

pH 7.5 ± 0.2 at 25°C

Oxidase reagent (prepare fresh or use commercial equivalent)

Tetramethyl-p-Paraphenylenediamine hydrochloride	0.1 g
Water	10 mL

Carbohydrate utilisation medium

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Peptone	10.0 g
Beef extract (optional)	1.0 g
Sodium chloride	5.0 g
Phenol red	0.018 g
Mannitol (filter sterilised)	1.0 g
Distilled water	1 L
pH 7.4 ± 0.1	

Hugh and Leifson's H/L medium

Peptone	2.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	0.3 g
Bromothymol blue	0.03 g
Glucose	10 g
Agar	3.0 g
Water	1 L
pH 7.1 ± 0.2 at 25°C	

1% tryptone water

Tryptone	10.0 g
Distilled water	1 L

1% tryptone water with 6% sodium chloride

Tryptone	10.0 g
Sodium chloride	60.0 g
Distilled water	1 L

Vibriostatic agent (optional)

2,4-diamino 6,7 di-isopropyl pteridine (O129) phosphate	50.0 mg
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Arginine test medium

Peptone	0.1 g
Sodium chloride	0.5 g
K ₂ HPO ₄	0.03 g
Agar	0.3 g
Phenol red	0.001 g
L(+)-Arginine HCl	1.0 g
Distilled water	100 ml
pH 7.2 ± 0.1	

Commercial biochemical test kits (optional)

6.0 METHOD

6.1 Sample preparation

Water samples should be received and handled as described in SOP W 1 section 5. The nature of the request and condition of the specimen should be noted on arrival. Protect the samples from direct sunlight and transport in an insulated container or refrigerator at 2°C - 10°C. Samples should be analysed as soon as is practicable on the day of collection. In

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exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours before the commencement of analysis.

Following these procedures, suitable volumes should be selected for analysis and the necessary dilutions prepared.

6.2 Filtration and incubation

Following the procedures outlined in SOP W 1 section 5, filter a measured volume of the test sample through the membrane. For potable quality waters use volumes of 100 mL and for other waters use volumes of 100 mL or less.

Place the membrane onto ampicillin dextrose agar (ADA) and incubate at $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$ for 22 hours \pm 2 hours.

6.3 Counting of colonies

After incubation, count all colonies which are yellow, or yellow with a greenish-yellow periphery. Do not count completely blue or white translucent colonies.

6.3.1 Confirmatory tests

Using the procedure described in SOP W 1 section 5, select a proportion of presumptive *Aeromonas* colonies for confirmation.

Oxidase test

Perform an oxidase test on colonies from the nutrient agar plate. Immerse a swab in oxidase reagent and touch the surface of the colony to be tested. The immediate appearance of a dark purple colour at the point of contact denotes a positive reaction. No colour change, or a purple colour which develops later, are both negative reactions.

Alternatively, moisten a piece of filter paper in a Petri dish with 2 – 3 drops of oxidase reagent. Using a stick, glass rod or platinum (not nichrome) loop, transfer a portion of a colony of the test organism to the filter paper and rub it on the moistened area. A positive reaction is indicated by the appearance of a dark purple colour within 10 seconds.

Presumptive *Aeromonas* species are oxidase positive. However, some *Vibrio* species may give similar reactions.

Mannitol fermentation

Inoculate a tube of carbohydrate utilisation medium with a portion of the test organism. Incubate at $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$ for 24 hours – 7 days. Check for turbidity in liquid media that indicates growth of the test organism. Note the colour of the test solution. A positive fermentation will result in a colour change of the liquid media from red to yellow.

Fermentation test

Using a straight wire, stab a representative colony and transfer a portion to a pre-labelled nutrient agar sector. Using the same inoculum, stab the wire into the bottom of a tube containing H/L medium. Return to the previously inoculated nutrient agar sector and spread the inoculum using a loop. This plate will be used for the oxidase test following incubation. Cover the inoculated H/L tube with a layer of liquid paraffin (petrolatum)⁴ and incubate all plates and tubes at $30^{\circ}\text{C}\pm 1^{\circ}\text{C}$ for 22 hours \pm 2 hours.

Observe the H/L tube for a fermentation reaction.

Presumptive *Aeromonas* species will give a fermentative reaction in H/L medium. This is shown by a change of colour from blue to yellow throughout the tube. No change in colour or a colour change just below the paraffin layer is a negative fermentation reaction.

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Growth in 1% tryptone water

Inoculate 10 µl of a 3-5 hour culture of the presumptive *Aeromonas* into a tube of (a) 1% tryptone water containing no added sodium chloride, and (b) 1% tryptone water containing 6% sodium chloride. Incubate tubes at 30°C±1°C and examine after 24 and 48 hours. Growth is demonstrated by turbidity of the tryptone water. *Aeromonas* species will grow in 1% tryptone water containing no added sodium chloride, but not in 1% tryptone water containing 6% sodium chloride.

Resistance to O129

Prepare a lawn of the culture to be tested on a nutrient agar plate. Place a 150 µg O129 disc on the plate, and incubate at 30°C±1°C for 22 hours ± 2 hours. *Aeromonas* species are resistant to 150 µg O129 and will therefore not produce a zone of sensitivity around the disc.

Arginine hydrolysis

Using a straight wire, stab a representative colony and inoculate into the bottom of a tube containing Arginine test medium. Incubate at 30°C±1°C and read daily for a maximum of 5 days. *Aeromonas* species are positive for arginine hydrolysis. A positive reaction is shown by a change in colour of the phenol red indicator.

Commercially available biochemical test kits may also be used for confirmation.

7.0 QUALITY CONTROL

7.1 Membrane filtration

When the membrane filtration technique is used, internal quality control procedures should be carried out at least monthly. 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 should be carried out using suspensions of positive and negative control organisms known to contain less than 100 colony forming units in the volume filtered.

7.2 Control cultures for ADA

Positive control:

Aeromonas hydrophila NCTC 8049

Negative control:

Escherichia coli NCTC 9001

Prepare suspensions of the positive and negative control organisms. Process alongside routine test samples.

In addition, filter 100 mL of sterile distilled water or peptone saline diluent using the same funnel as was used for the positive control following sterilisation.

Incubate all quality control tests with routine tests, proceed with confirmatory tests and determine counts.

7.3 Control cultures for confirmatory tests

Positive control:

Aeromonas hydrophila NCTC 8049

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Negative control:
Vibrio furnissii NCTC 11218

Process alongside routine test samples using the confirmatory tests described in section 6.3.1.

8.0 CALCULATION OF RESULTS

Calculate the presumptive count of *Aeromonas* species per 100 ml as follows:

Presumptive count = $\frac{\text{No. of colonies counted}}{\text{Volume tested}} \times 100$

Calculate the number of confirmed *Aeromonas* species in 100 mL of original sample using the procedure described in SOP W 1 section 7.

9.0 REPORTING OF RESULTS

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

If *Aeromonas* species are not detected, report as:

“*Aeromonas* species not detected in 100 mL”

If *Aeromonas* species are detected, report as:

“a in 100 mL”

where **a** is the confirmed count

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Flowchart showing the enumeration of *Aeromonas* species by membrane filtration

Transport to laboratory at 2 – 10°C out of direct sunlight in suitable containers



Samples should be analysed as soon as is practicable on the day of collection, otherwise within 24 hours of collection



Mix sample well and make any necessary dilutions



Filter



Place membrane on ampicillin dextrose agar



Incubate at 30°C±1°C for 22±2 hours. Count yellow colonies (including those with a greenish-yellow periphery)



Perform confirmatory tests



Calculate confirmed count for *Aeromonas* species

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