NATIONAL STANDARD METHOD

ENUMERATION OF CLOSTRIDIUM PERFRINGENS

F 14

Issued by Standards Unit, Evaluations and Standards Laboratory
Specialist and Reference Microbiology Division
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Email: standards@hpa.org.uk
AMENDMENT PROCEDURE

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On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.
STANDARD OPERATING PROCEDURE FOR THE ENUMERATION OF CLOSTRIDIUM PERFRINGENS

INTRODUCTION

Scope

The method described is applicable to the enumeration of Clostridium perfringens in all types of food products.

Background

Foods contaminated with large numbers of vegetative cells of Clostridium perfringens can give rise to illness characterised by diarrhoea and abdominal pain which may be accompanied by vomiting. C. perfringens produces spores which can survive normal cooking processes and cause a hazard in meat products that are left at ambient temperatures for a long time after cooking. Other sulphite-reducing clostridia are implicated in food spoilage, especially of poorly processed canned food.

The presence of C. perfringens is considered to be unsatisfactory in ready-to-eat foods if counts of greater than or equal to $10^3$ colony forming units per gram (cfu/g) are found\(^1\).

This method is based on BS 5763: Part 9 1986\(^2\) and is described in Practical Food Microbiology\(^3\).
1.0 PRINCIPLE

Specified volumes of dilutions of the sample are mixed with a tempered molten selective culture medium in a sterile Petri dish. An overlay of the same medium is added and the plates are incubated anaerobically at 37°C for 20 hours. The number of black colonies is counted and the number of \textit{C. perfringens} colonies determined following results of confirmation tests.

2.0 DEFINITIONS

For the purpose of this method, the following definition applies:

\textit{Clostridium perfringens}

Micro organisms which produce black colonies in the specified selective agar, are non motile, reduce nitrates to nitrites, ferment lactose and liquefy gelatin.

3.0 SAFETY CONSIDERATIONS

Normal microbiology laboratory precautions apply. In addition, care must be taken of the boiling waterbath when melting agars or boiling confirmatory test media prior to inoculation. Use heat and water resistant gloves when removing containers and avoid leaning over the bath.

Chemical resistant gloves must be worn when making up nitrate reagents A and B.

4.0 EQUIPMENT

Usual laboratory equipment and in addition:

- Top pan balance capable of weighing to 0.1 g
- Stomacher
- Gravimetric diluter (optional)
- Vortex mixer
- Boiling waterbath for melting agar
- Waterbath at 45°C ± 1°C
- Facilities for anaerobic incubation
- Incubator at 37°C ± 1°C
- Colony counter (optional)
- Stomacher bags (sterile)
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)

5.0 CULTURE MEDIA

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

\textit{Peptone saline diluent (Maximum recovery diluent)}

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>8.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
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</table>

**ENUMERATION OF CLOSTRIDIUM PERFRINGENS**

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pH 7.0 ± 0.2 at 25°C

**Buffered peptone water**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
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<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>9.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
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pH 7.2 ± 0.2 at 25°C

**Tryptose sulphite cycloserine agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Soya peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium metabisulphite</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>D - cycloserine</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
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</table>

pH 7.6 ± 0.2 at 25°C

**Note:**

The complete tryptose sulphite cycloserine agar medium (TSCA) should be prepared on the day of use by adding cycloserine supplement to melted and cooled (45°C) perfringens agar base.

**Motility nitrate medium**

<table>
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<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Meat extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Galactose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Di–sodium hydrogen orthophosphate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
</tbody>
</table>

pH 7.3 ± 0.2 at 25°C

**Lactose gelatine medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Gelatine</td>
<td>120.0 g</td>
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<tr>
<td>Phenol red</td>
<td>50 mg</td>
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<td>Water</td>
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pH 7.5 ± 0.2 at 25°C

**Blood agar**

Columbia agar or any other suitable base with 5% horse blood

**Nitrite reagent A**

5-amino-2-naphthalene-sulfonic acid (0.1% solution in 15% by volume acetic acid)

**Nitrite reagent B**

Sulfanilic acid (0.4% solution in 15% by volume acetic acid)

**Zinc dust**
6.0 SAMPLE PROCESSING

6.1 Sample preparation and dilutions

Following the procedure described in Standard Method F2 - Preparation of Samples and Dilutions prepare a 10⁻¹ homogenate in either peptone saline diluent (PSD) or buffered peptone water (BPW) and further decimal dilutions as required in PSD.

6.2 Inoculation and incubation

Starting with the highest dilution transfer 1 mL of each dilution to a sterile Petri dish. Pour 15 to 20 mL of tryptose sulphite cycloserine agar (TSCA), tempered to 45°C, into each Petri dish. Mix well with the inoculum, rotating each dish five times clockwise, anti-clockwise and sideways in each direction. Allow the plates to set, add a further 10 mL of TSCA as an overlay and allow to solidify.

Incubate the inoculated TSCA under anaerobic conditions in an incubator at 37°C for 20 ± 2 hours. Longer incubation may result in excess blackening along the bottom rim of the plates.

6.3 Counting of colonies

Examine the plates for black colonies. Using plates containing up to 150 colonies count and record the number of typical colonies.

6.4 Confirmatory tests

Subculture at least five black colonies to two blood agar (BA) plates. Incubate one plate aerobically and the other anaerobically in an incubator at 37°C for 18 - 24 hours. Examine the plates for the presence or absence of growth and for purity. Perform confirmatory tests for *C. perfringens* on colonies that fail to grow aerobically.

Anaerobic cultures with a diffuse spreading morphology are considered motile, and therefore confirmatory tests are not carried out.

Using pure non spreading cultures from the anaerobic blood agar subculture plates inoculate the following media:

Motility-nitrate medium²

Immediately prior to use heat the medium in boiling water for 15 minutes and then cool rapidly to set. Inoculate by stabbing into the medium and incubate under anaerobic conditions at 37°C ± 1°C for 20 - 24 hours. After incubation examine the medium for growth along the stab line. Motility is evident as diffuse growth out into the medium away from the stab line.

Mix equal volumes of nitrite reagent A and B just before use and test for the presence of nitrite by adding 0.2 to 0.5 mL of this mixture to each tube of motility-nitrate medium. The formation of a red colour confirms the reduction of nitrate to nitrite. If a red colour does not develop within 15 minutes add a small amount of zinc dust and allow to stand for 10 minutes. If a red colour develops after the addition of zinc dust no reduction of nitrate has taken place.

Lactose gelatine medium²

Immediately prior to use heat the medium in boiling water for 15 minutes and then cool rapidly. Inoculate the medium and incubate anaerobically at 37°C for 20 - 24 hours. Examine the tubes for the presence of gas and for a yellow colour indicating the production of acid. Chill the tubes for 1 hour at 2 - 8°C and check for gelatine liquefaction. If the medium has solidified reincubate for an additional 24 hours and again check for liquefaction of the gelatine.

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Email: standards@hpa.org.uk
C. perfringens produces black colonies in TSCA, is non-motile, reduces nitrate to nitrite, produces acid and gas from lactose and liquefies gelatine in 48 hours.

Control culture
Clostridium perfringens  NCTC 8237

The control culture must be used for confirmatory tests.

7.0  CALCULATION OF RESULTS

Counts should be calculated, where possible, using dilutions giving 15 to 150 colonies on the plate.

Calculate the number of C. perfringens per gram as follows:

\[
\text{Count per g} = \frac{\text{No. of colonies confirmed}}{\text{No. of colonies tested}} \times \frac{\text{No. of colonies counted}}{\text{Volume tested} \times \text{dilution}}
\]

8.0  REPORTING OF RESULTS

If no colonies of the test organism are present on the 10⁻¹ dilution report as:

Less than 10 cfu/g

If the test organism is detected with counts between 10 and 99 per gram report in the form of:

\( a \) cfu/g

where \( a \) is a number between 10 and 99.

If the test organisms are detected at counts of 100 or higher per gram, report with one figure before and one figure after the decimal point expressed to the power of 10 in the form of:

\( a \times 10^b \) cfu/g

where \( a \) is never less than 1.0 or greater than 9.9 and \( b \) represents the appropriate power of ten. Round counts up if the last figure is 5 or more and down if the last figure is 4 or less: e.g.

1920 cfu per g reported as 1.9 X 10³ cfu per g

235,000 cfu per g reported as 2.4 X 10⁵ cfu per g

9.0  REFERENCE FACILITIES

In certain circumstances, such as food poisoning incidents, it may be necessary to investigate isolates further. Reference facilities for serotyping are available at the Food Safety Microbiology Laboratory, Specialist and Reference Microbiology Division, Colindale, London.
Appendix: Flowchart showing the process for the enumeration of Clostridium perfringens

1. Prepare a $10^1$ dilution of sample
2. Homogenise by stomaching
3. Prepare further dilutions in PSD if required
4. Inoculate 1.0 mL of each dilution into an empty Petri dish and add 15–20 mL of molten tempered tryptose sulphite cycloserine agar (TSCA). Mix by gentle rotation and allow to set
5. Add an overlay of 10 mL of molten tempered TSCA and allow to set
6. Incubate anaerobically at $37^\circ$C for 20 ± 2 hours
7. Count black colonies
8. Subculture 5 colonies to blood agar and incubate aerobically and anaerobically at $37^\circ$C for 18–24 hours
9. Subculture colonies showing only anaerobic growth to motility nitrate medium and lactose gelatine medium for confirmation as *C. perfringens*
10. Calculate the count of *C. perfringens* per gram
REFERENCES

4 Advisory Committee on Dangerous Pathogens. Categorisation of biological agents according to hazard and categories of containment. 4th Edn. HSE Books, Suffolk, 1995 with supplements 1, 1998 and 2, 2000
6 Control of Substances Hazardous to Health Regulations. General ACOP, Carcinogens ACOP and Biological agents ACOP, L5. HSE Books, Suffolk, 1999
7 Health and Safety Executive. 5 steps to risk assessment: a step by step guide to a safer and healthier workplace, IND (G) 163 (L). London: HSE, 1/94
8 Health and Safety Executive. A guide to risk assessment requirements: common provisions in health and safety law, IND (G) 218 (L). London: HSE, 5/96