ENUMERATION OF
STAPHYLOCOCCUS AUREUS

F 12
Issued by Standards Unit, Evaluations and Standards Laboratory
Specialist and Reference Microbiology Division
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INTRODUCTION

Scope

The method described is applicable to the enumeration of *S. aureus* in all food types and dairy products using a surface colony count technique with incubation at 37°C.

Background

The presence of *S. aureus* in ready-to-eat food is generally considered to be unsatisfactory if the count is equal to or greater than $10^2$ colony forming units per gram (cfu/g). Low numbers indicate poor handling whereas high counts may be associated with toxin production and food poisoning. This method allows for the enumeration of *S. aureus* at counts of 20 cfu/g and above. The method is based on BS 5763: Part 7: 1983 and is also described in Practical Food Microbiology.
1.0 PRINCIPLE

The enumeration of *S. aureus* by this method involves inoculation of the surface of the selective agar medium with a specified volume of a $10^{-1}$ and other appropriate decimal dilutions of the test sample and incubation at 37°C for 48 hours. Calculation of the number of *S. aureus* cfu per gram or mL of sample is made from the number of typical and/or atypical colonies obtained on the selective medium and subsequently confirmed by DNase and coagulase tests.

2.0 DEFINITIONS

For the purpose of this method *Staphylococcus aureus* is defined as a micro-organism which forms typical and/or atypical colonies on the surface of the selective agar medium described in this method and which shows positive reactions in confirmatory tests.

3.0 SAFETY CONSIDERATIONS

Normal microbiology laboratory precautions apply. In addition, safety spectacles and chemical resistant gloves must be worn when handling hydrochloric acid used for the DNAse test.

4.0 EQUIPMENT

Usual laboratory equipment and in addition:

- Top pan balance capable of weighing to 0.1g
- Gravimetric diluter (optional)
- Stomacher
- Vortex mixer
- Spiral plater (optional)
- Incubator: 37°C ± 1°C
- Colony counter (optional)
- Stomacher bags (sterile)
- Automatic pipettors and associated sterile plugged pipette tips capable of delivering up to 10 mL and 1 mL amounts (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)
- Spreaders - sterile, disposable.

5.0 CULTURE MEDIA

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

*Peptone saline diluent (Maximum recovery diluent)*

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<tr>
<td>Peptone</td>
<td>1.0 g</td>
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<tr>
<td>Sodium chloride</td>
<td>8.5 g</td>
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<tr>
<td>Water</td>
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pH 7.0 ± 0.2 at 25°C
**Buffered peptone water**

Peptone 10.0 g  
Sodium chloride 5.0 g  
Disodium hydrogen phosphate 9.0 g  
Potassium dihydrogen phosphate 1.5 g  
Water 1 L  
\[ \text{pH 7.2 ± 0.2 at 25°C} \]

**Baird-Parker agar**

Tryptone 10.0 g  
Meat extract 5.0 g  
Yeast extract 1.0 g  
Sodium pyruvate 10.0 g  
Glycine 12.0 g  
Lithium chloride 5.0 g  
Agar 20.0 g  
Egg yolk emulsion 50 mL  
Potassium tellurite 0.1 g  
Water 1 L  
\[ \text{pH 6.8 ± 0.2 at 25°C} \]

**Blood agar**

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

**DNase agar**

Tryptose 20.0 g  
Deoxyribonucleic acid 2.0 g  
Sodium chloride 5.0 g  
Agar 12.0 g  
Water 1 L  
\[ \text{pH 7.3 ± 0.2 at 25°C} \]

**Toluidine blue solution (optional)**

Toluidine blue 0.1 g  
Water 100 mL  

**Hydrochloric acid (1N) (optional)**

**Coagulase reagent.**

Rabbit plasma or any commercial alternative test kit proven to be comparable to the coagulase test.

### 6.0 SAMPLE PROCESSING

#### 6.1 Sample preparation and dilutions

Following the procedure described in Standard Method F 2 - Preparation of Samples and Diluents prepare a 10⁻¹ homogenate in either peptone saline diluent (PSD) or buffered peptone water (BPW) and further decimal dilutions as required in PSD. For dairy products follow the procedures described in Standard Method D 1 - Preparation of Samples and Decimal Dilutions.
6.2 Inoculation and incubation

Starting with the highest dilution inoculate 0.5 mL of each dilution onto the centre of a dried Baird-Parker (BP) plate. Using a sterile spreader and starting with the highest dilution, spread the inoculum carefully over the surface of each plate as soon as possible taking care not to touch the sides of the plate. A spiral plater (Standard Method F 11) may be used if high counts are expected. Leave the plates on the bench for approximately 15 minutes to allow absorption of the inoculum into the agar. Invert the plates and place in an incubator at 37°C for 48 ± 2 hours.

6.3 Counting of colonies

Examine the plates for typical colonies of *S. aureus* on plates containing up to 150 colonies. Typical colonies appear as black, shiny, convex colonies up to 3mm in diameter, with a narrow zone of opacity surrounded by a zone of clearing. Count and record the number of typical colonies. For foods of bovine origin, including dairy products, atypical colonies of *S. aureus* may occur but do not show opacity or clearing. For foods of this type also count and record atypical colonies.

6.4 Confirmatory tests

Subculture five colonies of each type (or all colonies if less than five) for confirmatory testing using DNase and coagulase production. Inoculate each colony onto a DNase agar plate and plate out onto a segment of a blood agar plate. Transfer the plates to an incubator at 37°C for 18-24 hours.

Examine the blood agar plates for purity and colonial morphology consistent with *S. aureus*; cream or golden coloured colonies up to 3mm in diameter.

*DNase production*

Flood the DNase plate with toluidine blue solution (TBS) or normal hydrochloric acid (HCl). After about 30 seconds, discard the excess reagent (TBS or HCl) into a chemical waste container.

Positive reactions are as follows: -

- Toluidine blue solution: colonies surrounded by a pink zone against a blue background
- Hydrochloric acid: colonies showing a defined zone of clearing.

*Coagulase production*

Using the growth on blood agar (BA), perform a slide coagulase test on the strains giving a positive DNase test. Allow the rabbit plasma to equilibrate at room temperature (10-15 minutes) before use and perform the test as follows: -

Place two drops of water on a microscope slide and make a creamy suspension in each drop from a colony on BA. Mix a loopful of undiluted coagulase reagent into one of the suspensions. The presence of bound coagulase is demonstrated by the appearance of microscopic clumping within 5-10 seconds and the absence of autoagglutination in the second suspension.

For commercially produced test kits follow the manufacturers instructions.

Colony types are confirmed as *S. aureus* if they show typical colonial morphology on blood agar and give positive reactions in the DNase and coagulase tests.

*Control cultures*

Positive and negative controls must be used for confirmatory tests.

Positive control: - *S. aureus* (Oxford strain)  NCTC 6571

Negative control: - *S. epidermidis*  NCTC 11047
7.0 CALCULATION OF RESULTS

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

Calculate the count of *S. aureus* per gram or mL as follows:

\[
\text{Count of } S.\text{ aureus per gram} = \frac{\text{Number of typical colonies confirmed} \times \text{Number of colonies counted}}{\text{Number of typical colonies tested}} \times \frac{\text{Volume tested} \times \text{dilution}}{\text{Volume tested} \times \text{dilution}} + \frac{\text{Number of atypical colonies confirmed} \times \text{Number of colonies counted}}{\text{Number of atypical colonies tested}}
\]

8.0 REPORTING OF RESULTS

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

- Less than 20 cfu/g or mL

If the test organism is detected with counts between 20 and 99 per gram report in the form of:

- \(a\) cfu/g or mL

where \(a\) is a number between 20 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 or mL

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/g or mL reported as 1.9 \(\times 10^3\) cfu/g or mL
- 235,000 cfu/g or mL reported as 2.4 \(\times 10^5\) cfu/g or mL
9.0 REFERENCE FACILITIES

In certain circumstances, for example outbreaks, non-compliance with dairy product hygiene regulations\(^4\) or potentially hazardous levels in other foods\(^1\), it may be necessary to investigate isolates further.

Phage typing of isolates and examination of the food for the presence of enterotoxin may also be appropriate.

Reference facilities are available at the following national reference laboratories:

*Phage typing.*
Staphylococcus Reference Unit, Laboratory of Healthcare Associated Infection, SRMD, Colindale

*Enterotoxin Testing.*
Food Safety Microbiology Laboratory, SRMD, Colindale
Appendix: Flowchart showing the process for performing the enumeration of *Staphylococcus aureus*

Prepare a $10^{-1}$ dilution of sample

↓

Homogenise by stomaching

↓

Prepare any further dilutions in peptone saline diluent if required

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Starting with the highest dilution inoculate 0.5 mL of each dilution onto the centre of a Baird Parker plate

↓

Spread the inoculum across each plate using a sterile spreader and leave to dry

↓

Incubate at $37^\circ C$ for 48 hours in aerobic conditions

↓

Count typical colonies (and atypical colonies for foods of bovine origin)

↓

Subculture five suspect colonies onto DNase agar and blood agar and incubate at $37^\circ C$ for 18-24 hours

↓

Perform slide coagulase test on DNase positive isolates

↓

Calculate the total *S. aureus* count per gram or mL
REFERENCES


