INTERNATIONAL STANDARD

ISO 6888-2

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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —

Part 2:
Technique using rabbit plasma fibrinogen agar medium

*Microbiologie des aliments — Méthode horizontale pour le dénombrement des staphylocoques à coagulase positive (*Staphylococcus aureus *et autres espèces) —*

Partie 2: Technique utilisant le milieu gélosé au plasma de lapin et au fibrinogène

Reference number
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Contents

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 6888-1 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 9, *Microbiology*.

This first edition of ISO 6888-2, together with ISO 6888-1, cancels and replaces ISO 6888:1983, which has been technically revised.

ISO 6888 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species)*:

— *Part 1: Technique using Baird-Parker agar medium*

— *Part 2: Technique using rabbit plasma fibrinogen agar medium*

0 Introduction

0.1 Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this part of ISO 6888 is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this part of ISO 6888 so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

0.2 ISO 6888 describes two horizontal methods (part 1 and part 2) for the enumeration of coagulase-positive staphylococci among which enterotoxinogenic strains are encountered. It is mainly concerned with *Staphylococcus aureus*, but also with *S. intermedius* and certain strains of *S. hyicus.*

In the general case, use part 1 of ISO 6888. However, it is preferable to use the procedure described in part 2 only for foodstuffs (such as cheeses made from raw milk and certain raw meat products) likely to be contaminated by:

— staphylococci forming atypical colonies on a Baird-Parker agar medium;

— background flora which can obscure the colonies being sought.

0.3 For the purposes of this part of ISO 6888, the characterization of staphylococci is based on a positive-coagulase reaction, but it is recognized that some strains of *Staphylococcus aureus* give weakly positive coagulase reactions. These latter strains may be confused with other bacteria but they may be distinguished from such other bacteria by the use of additional tests not included in this part of ISO 6888, such as the sensitivity to lysostaphin, the production of haemolysin, of thermostable nuclease and of acid from mannitol (see reference [1]).

Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —

Part 2:
Technique using rabbit plasma fibrinogen agar medium

1 Scope

This part of ISO 6888 describes a horizontal method for the enumeration of coagulase-positive staphylococci in products intended for human consumption or feeding of animals by counting of colonies obtained on a solid medium (rabbit plasma fibrinogen medium) after aerobic incubation at 35 °C or 37 °C (see reference [2]).

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 6888. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 6888 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Rules for the preparation of the test samples, of initial suspension and of decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and of decimal dilutions*.

ISO 6888-1:1999, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase positive staphylococci (Staphylococcus aureus and other species) — Part 1: Technique using Baird-Parker agar medium*.

ISO 7218, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*.

3 Terms and definitions

For the purposes of this part of ISO 6888, the following terms and definitions apply.

3.1
coagulase-positive staphylococci
bacteria which form typical colonies in a rabbit plasma fibrinogen selective agar medium when the test is carried out according to the method specified in this part of ISO 6888

3.2
enumeration of the coagulase-positive staphylococci
determination of the number of coagulase-positive staphylococci found per millilitre or per gram of sample when the test is carried out according to the method specified in this part of ISO 6888

4 Principle

4.1 Preparation of duplicate poured plates of the rabbit plasma fibrinogen agar medium, with a specified quantity of the test sample if the product is liquid or with a specified quantity of the initial suspension in the case of other products.

Inoculation, under the same conditions, using decimal dilutions of the test sample or of the initial suspension, with two plates per dilution.

4.2 Incubation of the plates at 35 °C or 37 °C [1)] for 18 h to 24 h, and a further 24 h if necessary.

4.3 From the number of typical colonies per Petri dish, calculation of the number of coagulase-positive staphylococci per millilitre or per gram of test sample.

5 Diluent and culture media

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Diluent

See ISO 6887-1 and the specific standard dealing with the product to be examined.

5.3 Rabbit plasma fibrinogen agar medium (see references [3] and [4]).

NOTE Commercially available media, in accordance with this part of ISO 6888, can be used. Nevertheless, considering the experienced variability of manufactured lots of the supplement, it is recommended that each batch of bovine fibrinogen/rabbit plasma solution be tested before use, by running positive and negative controls.

5.3.1 Base medium

Prepare the base medium as stated in ISO 6888-1:1999, 5.3.1, with the exception of the distribution of the base medium, in quantities of 90 ml per flask or bottle.

5.3.2 Solutions

5.3.2.1 Potassium tellurite solution

Prepare the potassium tellurite solution as indicated in ISO 6888-1:1999, 5.3.2.1.

5.3.2.2 Bovine fibrinogen solution

5.3.2.2.1 Composition

Bovine fibrinogen	5 g to 7 g [1)]
Sterile water	100 ml
1) Depending on the purity of the bovine fibrinogen.	

5.3.2.2.2 Preparation

Under aseptic conditions, dissolve the bovine fibrinogen in the water just prior to use.

1) The temperature is agreed between the interested parties and is indicated in the test report.

5.3.2.3 Rabbit plasma and trypsin inhibitor solution

5.3.2.3.1 Composition

Rabbit plasma with EDTA for coagulase (EDTA coagulase plasma)	30 ml
Trypsin inhibitor	30 mg

5.3.2.3.2 Preparation

Operating under aseptic conditions, dissolve the components in the water, just prior to use.

5.3.3 Complete medium

5.3.3.1 Composition

Base medium (5.3.1)	90 ml
Potassium tellurite solution (5.3.2.1)	0,25 ml
Bovine fibrinogen solution (5.3.2.2)	7,5 ml
Rabbit plasma and trypsin inhibitor solution (5.3.2.3)	2,5 ml

5.3.3.2 Preparation

Melt the base medium, then let it cool down to 48 °C ± 1 °C in a water bath (6.3).

Under aseptic conditions, add the three solutions previously warmed to 48 °C ± 1 °C in a water bath. Mix thoroughly after each addition by rotation to minimize foaming.

Use the complete medium **immediately after its preparation**, in order to avoid any precipitation of the plasma.

WARNING If a commercially available solution of bovine fibrinogen/rabbit plasma is used, follow with great care the manufacturer's instructions for the preparation of this solution and of the complete medium (in particular the temperature of the base medium). Otherwise, the medium can completely lose its activity.

5.4 Preparation of agar plates

See ISO 6888-1:1999, 5.3.4.

6 Apparatus and glassware

NOTE Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) and **wet sterilization (autoclave)**

See ISO 7218.

6.2 Incubator, for maintaining the inoculated media, plates and tubes within the temperature range 35 °C ± 1 °C or 37 °C ± 1 °C.

6.3 Water bath, or similiar apparatus, capable of being maintained at 48 °C ± 1 °C.

6.4 Petri dishes, sterile, made of glass or plastic.

6.5 Total-delivery graduated pipettes, of nominal capacities 1 ml, 2 ml and 10 ml, graduated in 0,1 ml, 0,1 ml and 0,5 ml divisions, respectively.

7 Sampling

Sampling is not part of the method specified in this part of ISO 6888. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilution

See ISO 6887-1 and the specific standard appropriate to the product concerned.

9.2 Inoculation and incubation

9.2.1 Take two sterile Petri dishes (6.4). Transfer, by means of a sterile pipette (6.5), 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products, to each of the dishes. Take two other sterile Petri dishes and transfer 1 ml of the first decimal dilution to each of the dishes.

Repeat these operations with successive dilutions using a new sterile pipette for each decimal dilution.

9.2.2 Into each Petri dish (9.2.1), immediately pour freshly prepared complete medium (5.3.3) (do not keep this in a liquid form) to a depth of approximately 3 mm.

Carefully mix the inoculum with the culture medium and leave to solidify by placing the Petri dishes on a cool horizontal surface.

9.2.3 After complete solidification, invert the thus prepared dishes and place them in the incubator (6.2) set at 35 °C or 37 °C [2)] for 18 h to 24 h. If necessary, re-incubate for 18 h to 24 h.

9.3 Counting of colonies

After a sufficient incubation period (see 9.2.3), the staphylococci form black or grey or even white, small colonies surrounded by a halo of precipitation, indicating coagulase activity. *Proteus* colonies may show, at the beginning of incubation, an appearance similar to coagulase-positive staphylococci colonies. However, after 24 h or 48 h of incubation, they may have the appearance of a spreading culture, more or less brownish, which allows them to be distinguished from staphylococci.

Count the typical colonies in each dish.

NOTE As the rabbit plasma fibrinogen agar is based on a coagulase reaction, it is not necessary to confirm this activity.

2) The temperature is agreed between the interested parties and is indicated in the test report.

10 Expression of results

10.1 General case

Select those dishes containing at the maximum 300 colonies, with 100 typical colonies at two successive dilutions. One dish shall contain at least 15 typical colonies.

Calculate the number N of coagulase-positive staphylococci present per millilitre or per gram of product as a weighted mean from two successive dilutions using the following equation:

$$N = \frac{\sum C}{V(n_1 + 0{,}1\,n_2)d}$$

where

$\sum C$ is the sum of the characteristic staphylococcal colonies on all the dishes selected;

V is the volume of inoculum on each dish, in millilitres;

n_1 is the number of dishes selected at the first dilution;

n_2 is the number of dishes selected at the second dilution;

d is the dilution rate corresponding to the first dilution selected (the initial suspension is a dilution).

Round off the calculated results to two significant figures (see ISO 7218).

Take as the result the number of coagulase-positive staphylococci per millilitre (liquid products) or per gram (other products), expressed as a number between 1,0 and 9,9 inclusive, multiplied by 10^x where x is the appropriate power of 10.

EXAMPLE

A count of a product after inoculation with 0,1 ml of product gave the following results:

— for the first dilution selected (10^{-2}): 66 typical colonies and 54 typical colonies;

— for the second dilution selected (10^{-3}): 4 typical colonies and 7 typical colonies.

$$N = \frac{66 + 54 + 4 + 7}{2{,}2 \times 10^{-2}} = 5\,955$$

The result, after rounding off, is $6{,}0 \times 10^3$.

10.2 Estimation of low numbers

10.2.1 If the two dishes, corresponding to the test sample (liquid products) or the initial suspension (other products) each contain less than 15 colonies, report the result as follows.

a) For liquid products, estimated number of coagulase-positive staphylococci per millilitre:

$$N_e = \frac{C}{2}$$

where C is the sum of the colonies of coagulase-positive staphylococci counted (9.3) on the two dishes selected;