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विशिष्ट नियम

**Microbiology of Food and
Animal Feed — Preparation of
Test Samples, Initial Suspension
and Decimal Dilutions for
Microbiological Examination —
Specific Rules for the Preparation
of Samples Taken at the Primary
Production Stage**

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NATIONAL FOREWORD

This Indian Standard which is identical with ISO 6887-6 : 2013 'Microbiology of food and animal feed — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of samples taken at the primary production stage' issued by the International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on recommendation of the Food Hygiene, Safety Management and Other Systems Sectional Committee and approval of the Food and Agriculture Division Council.

The text of ISO Standard has been approved as suitable for publication as an Indian Standard without deviations. Certain terminologies and conventions are, however, not identical to those used in Indian Standards. Attention is particularly drawn to the following:

- a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'.
- b) Comma (,) has been used as a decimal marker, while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, reference appears to the following International Standard for which Indian Standard also exists. The corresponding Indian Standard which is to be substituted in its place, is listed below along with their degree of equivalence for the editions indicated:

<i>International Standard</i>	<i>Corresponding Indian Standard</i>	<i>Degree of Equivalence</i>
ISO 7218 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations	IS 16122 : 2013 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations	Identical

The technical committee has reviewed the provisions of the following International Standards referred in this adopted standard and has decided that they are acceptable for use in conjunction with this standard.

<i>International Standard</i>	<i>Title</i>
ISO 6887-1 : 2017	Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions
ISO 13307 : 2013	Microbiology of food and animal feed — Primary production stage — Sampling techniques

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'.

Indian Standard

MICROBIOLOGY OF FOOD AND ANIMAL FEED — PREPARATION OF TEST SAMPLES, INITIAL SUSPENSION AND DECIMAL DILUTIONS FOR MICROBIOLOGICAL EXAMINATION SPECIFIC RULES FOR THE PREPARATION OF SAMPLES TAKEN AT THE PRIMARY PRODUCTION STAGE

1 Scope

This part of ISO 6887 specifies rules for the preparation of samples taken at all stages from the farm to the slaughterhouse and their suspension for microbiological examination when the samples require different preparation from the methods described in ISO 6887-1. ISO 6887-1 defines the general rules for the preparation of the initial suspension and decimal dilutions for microbiological examination.

This part of ISO 6887 excludes the preparation of samples for both enumeration and detection test methods where preparation details are specified in the relevant International Standards.

This part of ISO 6887 is applicable to various samples taken from the hatchery, the farm, from the vehicle or the animals during transportation, or from animals or their carcasses in the slaughterhouse, to indicate the microbiological status of the animals in relation to zoonotic agents. This part of ISO 6887 does not apply to samples taken to assess the hygiene of meat. These are covered by ISO 6887-2.

This part of ISO 6887 does not consider samples taken from the aquatic environment (marine or freshwater) at the primary production stage. These are covered by ISO 6887-3.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

ISO 7218:2007, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 13307, *Microbiology of food and animal feed — Primary production stage — Sampling techniques*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1

laboratory sample

sample prepared for sending to the laboratory and intended for inspection or testing

3.2 test portion

measured (volume or mass) representative sample taken from the *laboratory sample* (3.1) for use in the preparation of the *initial suspension* (3.4)

3.3 pooled sample

composite sample taken from a number of different animals or composites of separate environmental samples

3.4 initial suspension

primary dilution

suspension, solution or emulsion obtained after a weighed or measured quantity of the product under examination (or of a test sample prepared from the product) has been mixed with, normally, a nine-fold quantity of diluent, allowing large particles, if present, to settle

Note 1 to entry: Sometimes higher or lower initial dilutions are prepared, for example 1 in 5 or 1 in 100 ($V_1 \rightarrow V_2$).

3.5 further decimal dilutions

suspension or solution obtained by mixing a measured volume of the *initial suspension* (3.4) with a nine-fold volume of diluent and by repeating this operation with each dilution prepared in this way, until a decimal dilution series, suitable for the inoculation of culture media, is obtained

4 Principle

An initial suspension (3.4) is prepared to obtain as uniform a distribution as possible of the microorganisms contained in the test sample, without reducing their viability.

A suspension for pre-enrichment or enrichment is prepared in the same way, using the medium recommended by the method of analysis concerned, except in the special cases mentioned in each product subclause (section) of this part of ISO 6887.

If necessary, further dilutions (3.5) are prepared in order to reduce the number of microorganisms per unit volume to allow, after incubation, observation of any growth (in the case of liquid media) or enumeration of colonies (in or on agar plates), as stated in each specific standard.

In order to restrict, if required, the range of enumeration to a given interval, or if high numbers of microorganisms are foreseen, it is possible to inoculate only the appropriate dilutions (at least two successive dilutions) according to the calculation described in ISO 7218.

5 Diluents and disinfectants

Diluents for general use are described in ISO 6887-1.

5.1 Diluents for special purposes

5.1.1 Neutralizing agents

Neutralizing liquid, prepared in accordance with ISO 13307, is used if needed at 10 % volume fraction in diluent. Neutralizer is normally added when the sample is taken, before transportation to the laboratory.

NOTE If a high level of formalin is expected in the samples, L-histidine (0.9 %) can be also added to the pre-enrichment broth.

5.2 Disinfectants for use during laboratory examination

Disinfectants are given in ISO 7218:2007, 6.2.4.

6 Apparatus and glassware

Usual microbiological laboratory equipment for general use (see ISO 6887-1 and ISO 7218) and, in particular, the following:

6.1 Homogenizers

6.1.1 Peristaltic homogenizer (stomacher)

6.1.2 Rotary homogenizer (blender)

6.1.3 Vibrational mixer (pulsifier)

6.2 Sterile hammer or plastic mallet

6.3 Sterile sand, pestle and mortar

6.4 Sterile forceps, scissors, scalpels, spatulas, spoons

6.5 Sterile flasks or screw-cap bottles of appropriate capacities

6.6 Sterile total delivery graduated pipettes, pipette tips

7 Types of sample that can be sent to the laboratory

The samples shall be taken and transported in accordance with ISO 7218 and ISO 13307.

The following list includes examples only. More detailed information on samples needed in different situations is given in [Clause 9](#):

- samples taken at the farm:
 - from the environment (e.g. swabs, litter, faeces, dust, water);
 - from animals (e.g. swabs);
- samples taken at slaughter (e.g. rectal or caecal content, mesenteric lymph nodes);
- samples taken at the hatchery (e.g. hatcher basket liners, broken egg-shells);
- samples taken on vehicles, modules and crates for animal transport (e.g. swabs).

8 Preparation of samples

8.1 General

All preparations and manipulations should be carried out using good aseptic techniques and with sterile equipment to prevent microbial contamination of samples from all external sources (see ISO 7218).

If the sample is transferred to another container and the whole laboratory sample is used, ensure that all of the sample is transferred (e.g. when transferring bootsocks from the original container to a new one, take care that no material from the bootsocks is left in the first container).

Indicate in the report which procedure has been used for analysis if it is different from the procedure described in this part of ISO 6887.

8.2 Storage

Samples shall be stored in conditions suitable for the optimal survival of the target microorganism(s) in accordance with ISO 7218.

9 Specific procedures

9.1 Procedures carried out on samples taken at farm

9.1.1 Samples from the environment or live animals

9.1.1.1 Fabric swabs

If possible, add the appropriate quantity/amount of medium directly to the sample in the transport container. The particular quantity to be added depends on the dimensions of the swab and the purpose of the test. Ensure that all parts of the swabs are submerged.

Take account of the effect of the quantity/amount of medium added on the detection limit of the test (see the note to 3.4).

For enumeration, add sufficient quantity/amount of medium to saturate the swab fully while providing the minimum amount of free liquid needed for enumeration, and for detection, ensure a sufficient amount of free liquid is present.

For example for enumeration from a 10 x 10 cm sponge swab add 100 ml of diluent. Squeeze the swab several times (e.g. by hand if the container is a bag) so that the microorganisms are released into the suspension, then shake well.

For detection, the swab is included in the culture.

9.1.1.2 Stick swabs

Transfer the swab to a suitable container. Break (or cut if necessary) the stick using sterile tools, add the appropriate quantity/amount of medium and mix. If the swab is already in a tube or other suitable container, add the medium to the same container, unless the stick swab container includes solid transport medium.

Where appropriate, stick swabs can be pooled, adding a suitable volume of medium.

9.1.1.3 Bootsock swabs, drag swabs, rope swabs

Where possible, add the appropriate quantity/amount of medium directly into the transport container.

Ensure that all parts of the swabs are submerged.

For example for *Salmonella* testing, add at least 225 ml of the appropriate diluent (see Annex D of ISO 6579:2002) per pair of bootsock swabs.

9.1.1.4 Moore's drain swab (tampon swab)

Handle these like bootsocks, (see [9.1.1.3](#)), but because of the accumulation of high numbers of organisms over time, a dilution of 1/20 may be advantageous.

9.1.1.5 Litter samples and naturally pooled faeces

It is important to homogenize the laboratory sample by mixing dry material or by adding the sample to an equal quantity/amount of diluent, which may be in the same container used for transport. Mix to make a slurry. Allow to stand for 10 min to 15 min and mix again. Transfer 50 ml of the suspension (containing 25 g of initial sample) to an appropriate volume of diluent according to the specific standard for the target microorganism.

9.1.1.6 Faecal samples

Individual sample: take a portion or the whole dropping, if small. Mix it gently and add the appropriate quantity/amount of medium according to the specific ISO procedure being followed.

For samples which are difficult to mix, refer to [9.1.1.5](#).

To pool samples: mix each sample as thoroughly as possible, take an equal quantity of each sample, add the appropriate quantity/amount of medium according to the specific standard for the target microorganism and mix again thoroughly.

It is preferable to pool no more than 20 animal faeces.

9.1.1.7 Dust

This sample is usually relevant for *Salmonella* or other robust organisms, but not, for instance, for *Campylobacter*.

At least 10 g of dust shall be tested. A 1:20 ratio of sample to pre-enrichment medium is advantageous for detection of *Salmonella* in very dry absorbent samples such as dust. Large dust samples may be prepared in the laboratory by mixing 1:4 with diluent, and then taking a subsample, which is subsequently diluted 1:5 for pre-enrichment, making sure that at least 10 g of the original sample is included. Samples of up to 25 g may be cultured without subsampling.

To reduce handling of dust in the laboratory and the consequent risk of cross-contamination, it is advisable to collect the dust to be analysed in sufficiently large bags or vessels so that only the required quantity/amount of medium is added in the laboratory. Free dust should be handled in a laminar flow cabinet.

9.1.1.8 Water

Small volumes of water (such as 100 ml) can be added to an equal volume of double strength medium for culture. Put the water in a container of suitable size, add medium, according to the ratio stated in the specific international procedure and mix.

For larger volumes of water from water systems: filter the sample through a membrane filter of pore size 0,45 µm, (for *Campylobacter*, 0,20 µm), then culture the filter. The larger the volume of water filtered, the more sensitive the detection.

For further details, see ISO 8199.

9.1.2 Animals from the farm

9.1.2.1 General

A separate autopsy room should preferably be used or, if this is not possible, a cabinet or dedicated areas should be used.

9.1.2.2 Birds

Isolating bacteria from bird viscera needs special attention with regard to the risk of cross-contamination and the need for post-mortem dissection according to organs to be analysed. Post mortem dissection

shall be carried out with care to avoid cross-contamination, especially avoiding scattering fluff or feathers. Surfaces, such as post-mortem tables or dissection boards, shall be thoroughly disinfected between batches. Ideally, separate disposable bench covers should also be used.

The following procedures are mainly used for *Salmonella* detection.

9.1.2.2.1 Chicks from one to three days old

Liver and yolk sac are taken aseptically from up to 60 chicks and pooled in sterile plastic bags or containers large enough for further homogenization and dilution of the test portion.

Complete caeca are removed with their contents, and those from up to 30 animals pooled in sterile plastic bags or bowls large enough to allow further homogenization and dilution of the test portion.

Make sure the caecal contents have been expelled before dilution. This can be done, for example, by massaging the stomacher bag from the outside or chopping the intestines with scissors.

9.1.2.2.2 Birds over three days old

Organs or pieces or contents of organs (liver, spleen, ovary, oviduct, caeca) are taken aseptically. Caecal samples from up to 30 birds or other samples from up to 60 birds can be pooled. It is useful to take the upper part of the oviduct with the ovary. Do not mix caecal samples with other organs. Use sterile plastic bags or containers large enough to allow further homogenization and dilution of the test portion.

9.1.2.3 Other animals (pigs, cattle, sheep, goats, horses, etc.)

Whole carcasses or organs and biological materials taken from dead animals at the farm or slaughterhouse can be sent to the laboratory. Whole carcasses should not be accepted unless the laboratory has a dedicated autopsy room.

The organs to be used for analysis vary depending on the microorganism to be detected/enumerated.

9.2 Procedures carried out on samples taken from slaughterhouse

9.2.1 Pigs

9.2.1.1 Caecal sample

Disinfect the surface of the caecum with a suitable disinfectant (as given in 6.2.4 of ISO 7218:2007) or cauterize with a red-hot iron or flame. Using sterile instruments make an incision and remove a sample, usually 10 g to 25 g, of the contents with sterile spoon or spatula. Place in a sterile container. Continue as specified in [9.1.1.5](#).

Up to five individual caecal contents samples can be pooled.

9.2.1.2 Caecal or rectal content

See [9.1.1.6](#).

9.2.1.3 Mesenteric lymph nodes (ileocaecal, caudal, jejunal as well as more proximal mesenteric lymph nodes)

Remove residual fat and connective tissue from the surface of the lymph nodes. Disinfect the surface of each lymph node by careful flaming or immersing in a suitable disinfectant and allow to dry. Using sterile scissors or scalpel and forceps, cut into small pieces, weigh and put in a sterile container. Macerate lymph nodes by hammering a strong sterile plastic bag containing the samples or using sterile sand and a pestle and mortar.

Add 9 ml of suitable diluent per gram of sample.

9.2.1.4 Tonsils

Disinfect the surface of the tonsils by immersing briefly in boiling water or in a suitable disinfectant and allow to dry. Using sterile scissors or scalpel and forceps, cut into small pieces, or macerate, weigh and put in a sterile container. Add 9 ml of suitable diluent per gram of sample.

9.2.2 Ruminants, horses, rabbits and other animals raised for meat

9.2.2.1 Caecal or rectal contents

See [9.1.1.6](#).

9.2.2.2 Mesenteric lymph nodes (ileocaecal, caudal, jejunal, as well as more proximal mesenteric lymph nodes)

See [9.2.1.3](#).

9.2.3 Poultry

9.2.3.1 Contents from whole caeca

The contents of up to 30 caeca can be pooled as follows:

The intact caeca are cut open with sterile scissors and the whole content is mixed before a test portion is taken.

Alternatively: cut one intact caecum open per bird with sterile scissors. Using a 10 µl loop or a sterile stick swab, transfer a portion of caecal contents into a tube containing a small volume (up to 5 ml) of diluent. Repeat this procedure for the remaining samples to be pooled. Mix the pooled sample well.

9.2.3.2 Caecum including the ileocaecal junction

This procedure is normally only followed for *Salmonella* in breeders and layers.

Up to 30 caeca from different birds can be pooled. Disinfect the surface of each caecum by flaming, and, cut the caeca into pieces with sterile scissors. Weigh and add 9 ml diluent per gram of sample.

9.3 Procedures for samples taken from poultry at hatchery or when transporting from hatchery to farm

These samples are taken for *Salmonella* only. The specific ISO method prescribed by ISO 6579 may be used.

9.3.1 Hatcher basket liners

Put at least five basket liners per flock, totalling at least 1 m² surface area, in a sterile plastic bag, add 1 l to 2 l of pre-enrichment medium pre-warmed to room temperature (or preferably to 37 °C, due to the large volume of diluent).

9.3.2 Broken eggshells

Crush and mix the sample of eggshells, then add to the pre-enrichment medium with a ten-fold dilution, e.g. take a test portion of 25 g and add 225 ml of pre-enrichment medium.

9.3.3 Hatcher fluff

To avoid handling fluff in the laboratory and the consequent risk of cross-contamination, it is advisable to collect the amount of fluff to be analysed in sufficiently large bags or vessels so that only the required volume or mass of medium is added in the laboratory, or in vessels allowing the whole sample to be transferred without scattering fluff.

9.3.4 Meconium

Usually, the laboratory receives the pooled meconium from 250 to 300 chicks. Add the appropriate quantity/amount of medium in a ratio of 1 to 9.

9.3.5 Swabs from hatcher baskets

Proceed as 9.1.1.1 (fabric swabs)

9.3.6 Macerated hatchery waste sample

Proceed as for broken eggshell samples ([9.3.2](#)), or fabric swab samples (see [9.1.1.1](#)) if used to obtain the sample.

9.3.7 Dead in shell embryos

9.3.7.1 Incubated eggs with an intact shell

The egg content shall be sampled aseptically. The shells are sterilized by immersion in boiling water for 2 s to 5 s, or by immersion in a suitable disinfectant for 1 min to 2 min, ensuring that the eggs and the disinfectant are at ambient temperature to avoid uptake of the disinfectant. After disinfection, allow the eggs to dry, remove the shells and examine the contents.

- If there are differentiated embryos in the eggs, these samples shall be prepared as for one- to three-day old chicks (see [9.1.2.2.1](#)).
- If the embryos are not well differentiated, the contents of up to 30 eggs can be pooled in sterile plastic bags or containers large enough to allow further homogenization and dilution of the test portion.

Some eggs without developed embryos can contain a great number of the target bacteria (*Salmonella*) without secondary contamination or associated flora. The homogenates can be examined either by direct plating and enrichment, or only by enrichment.

When examined by an enrichment method, samples are diluted in a suitable volume of medium (1 to 9).

9.3.7.2 Incubated eggs with a broken shell

These are pipped eggs: the egg shell is not intact, as the chick has failed to hatch but started to break the shell.

External disinfection of the shell is not necessary. The content of the egg is prepared in the same way as one- to three-day old chicks ([9.1.2.2.1](#)).

9.3.8 Cull chicks

See [9.1.2.2.1](#).

10 Further decimal dilutions

Make further decimal dilutions in accordance with ISO 6887-1.

Bibliography

- [1] ISO 6579, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Salmonella spp.*
- [2] ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms by culture*

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Amendments are issued to standards as the need arises on the basis of comments. Standards are also reviewed periodically; a standard along with amendments is reaffirmed when such review indicates that no changes are needed; if the review indicates that changes are needed, it is taken up for revision. Users of Indian Standards should ascertain that they are in possession of the latest amendments or edition by referring to the latest issue of 'BIS Catalogue' and 'Standards: Monthly Additions'.

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