SLOVENSKI STANDARD

SIST-TP CEN/TR 15215-2:2006

01-julij-2006

Karakterizacija blata ï Ugotavljanje prisotnosti in ġtevila Salmonela sp. v blatu, zemljini, izboljġevalcih tal, rastnih substratih in bio-odpadkih - 2. del: Metoda s tekoļ o obogatitvijo v selenit-cisteinskem mediju/gojiġļ u, ki mu sledi metoda po Vassiliadisu za semikvantitativno doloļ evanje najverjetnejġega ġtevila (MPN)

Characterization of sludges - Detection and enumeration of Salmonella spp. in sludges, soils, soil improvers, growing media and biowastes - Part 2: Liquid enrichment method in selenite-cystine medium followed by Rapport-Vassiliadis for semi-quantitative Most Probable Number (MPN) determination

Charakterisierung von Schlämmen - Quantitativer Nachweis von Salmonella spp. in Schlämmen, Böden, Bodenverbesserungsmitteln, Kultursubstraten sowie Bioabfällen - Teil 2: Flüssiganreicherungsverfahren in Selenit-Cystein-Medium in Kombination mit Rappaport-Vassiliadis zur semiquantitativen Bestimmung der höchstwahrscheinlichen Keimzahl (MPN)

Caractérisation des boues - Détection et dénombrement de Salmonella spp. dans les boues, les sols, les amendements du sol, les supports de culture et biodéchets - Partie 2 : Méthode par enrichissement en milieu liquide sélénite-cystine puis en milieu de Rapport-Vassiliadis pour la détermination semi-quantitative par la méthode du Nombre le Plus Probable (NPP)

Ta slovenski standard je istoveten z: **CEN/TR 15215-2:2006**

ICS:

13.030.20

13.080.30

65.080

SIST-TP CEN/TR 15215-2:2006 **en**

TECHNICAL REPORT

RAPPORT TECHNIQUE

TECHNISCHER BERICHT

CEN/TR 15215-2

January 2006

ICS 07.100.99

English Version

Characterization of sludges - Detection and enumeration of Salmonella spp. in sludges, soils, soil improvers, growing media and biowastes - Part 2: Liquid enrichment method in selenite-cystine medium followed by Rapport-Vassiliadis for semi-quantitative Most Probable Number (MPN) determination

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Quantitativer Nachweis von Salmonella spp. in Schlämmen, Böden, Düngemitteln und Bodenverbesserern, Kultursubstraten sowie Bioabfällen - Teil 2: Flüssiganreicherungsverfahren in Selenit-Cystein-Medium gefolgt durch Rapport-Vassiliadis zur semiquantitativen Bestimmung der höchstwahrscheinlichen Keimzahl (MPN)

This Technical Report was approved by CEN on 3 September 2005. It has been drawn up by the Technical Committee CEN/TC 308.

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Ref. No. CEN/TR 15215-2:2006: E

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Foreword

This Technical Report (CEN/TR 15215-2:2006) has been prepared by Technical Committee CEN/TC 308 “Characterization of sludges”, the secretariat of which is held by AFNOR.

This Technical Report does not replace any existing CEN standard.

This Standard is divided in three parts:

- part 1 gives a membrane filtration method

- part 2 is a liquid enrichment method and determination MPN and

- part 3 is a presence/absence method by liquid enrichment.

Introduction

Sludges, soils, soil improvers, growing media and biowastes can contain pathogenic micro-organisms such as *Salmonella* spp. which occur mainly in the intestinal tract of humans and animals and are transmitted through faecal contamination. The use of such pathogen-contaminated materials in agriculture can cause outbreaks of infection due to the production of contaminated food or animal feedstocks and may also be transmitted to wild animals, consequently, there is a need to monitor rates to land.

Examination for *Salmonellae* should only be carried out in laboratories competent for carrying out work involving pathogens. Suitable quality control procedures, at least those described in ISO 8199, have to be applied.

WARNING — "Waste and sludge samples can contain hazardous and inflammable substances. They can contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which can be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided wherever possible. National regulations should be followed with respect to microbiological hazards associated with this method".

1 Scope

This part of the CEN Technical Report method describes a method to detect and semi-quantitatively determine *Salmonellae* in sludges, soils, soil improvers, growing media and biowastes in accordance with the requirements of the European Sewage Sludge Regulation Revision of Directive 86/278/EEC (3rd Draft, CEN/TC 308 – doc525).

The fully defined scope will be determined after the proposed validation trials have been agreed and carried out. The method has a limit of detection of approximately 1cfu/g wet weight sample.

NOTE The objective is to cover untreated and treated sludges, soils, soil improvers, growing media, biowastes and associated materials.

2 Normative references

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

EN 12880:2000, *Characterisation of sludges — Determination of dry residue and water content*

ISO 8199, *Water quality — General guide to the enumeration of micro-organisms by culture.*

3 Terms and definitions

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

3.1
***Salmonella* spp.**
member of the family of *Enterobacteriaceae*, these are Gram-negative, non-sporulating, rod-shaped bacteria, most of which are motile. They can be distinguished from other genera of the *Enterobacteriaceae* family by biochemical methods and serologically identified by their somatic or flagellar antigens (O and H-antigens)

3.2
method definition
Salmonella spp. capable of being enriched in selenite cystine broth at (36 ± 2) °C followed by growth in Rappaport-Vassiliadis medium at (42 ± 1) °C followed by characteristic growth on SMID/Rambach agar or XLD agar at (36 ± 2) °C (see also 4 and 8.5)

NOTE Some *Salmonella* (e.g. *S. typhi* and *S. paratyphi*) will not be detected.

3.3
cfu, colony forming unit
growth of individual bacterial cells into visible colonies on agar media, including on membrane filters overlaying the agar media

3.4
vegetative bacteria
those bacteria which are capable of normal growth in broth or on agar media without pre-culture resuscitation

3.5
sub-lethally damaged bacteria
those bacteria which have been stressed but not killed in treatment processes or storage

3.6
resuscitation
stimulation to vegetative growth of sub-lethally damaged bacteria previously incapable of growth on agar media

3.7
quantitative resuscitation
stimulation to vegetative growth of sub-lethally damaged bacteria recovered discretely on a membrane filter, prior to transfer to chromogenic medium for growth of individual colonies

3.8
presumptive positives
isolates which are believed to be *Salmonella* spp., but not yet confirmed

3.9
dry residue
the dry mass portion of the sludge obtained after the specified drying process. It is expressed as percent or in grams per kilogram (see EN 12880:2000, 3.1)

4 Principle

The steps involved in this method have been made as close as possible to those involved in the ISO CD 6340-2:2000. The main differences are the following:

— sample preparation suitable for a solid matrix;

— a selective pre-enrichment step according to the possible high contamination of the sludge with interfering bacteria.

Three series of three tubes containing serial dilutions of the sludge suspension should be used for the Most Probable Number enumeration method.

The detection of *Salmonella* spp. requires four stages:

a) culturing of bacteria in a primary selective medium;

b) enrichment in a secondary selective medium which inhibits the growth of other micro-organisms but promotes that of *Salmonellae* (selective enrichment);

c) preparation of pure cultures by inoculating special solid media with subcultures;

d) biochemical and serological identification tests.

5 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilised in accordance with the instructions given in ISO 8199.

Usual microbiological laboratory equipment and in particular:

5.1 Wide-mouth glass flasks or beakers for example, 125 ml, 200 ml, 500 ml and 2 000 ml

5.2 Thermostatic incubator regulated at (36 ± 2) °C and (42 ± 1) °C

5.3 Autoclave (steam sterilizer)

5.4 Refrigerator

5.5 Sterile plastics culture dishes, with lid of about 90 mm in diameter

5.6 Sterile graduated pipettes, glass or disposable plastic ware, capable of dispersing 0,1 ml, 1 ml and 10 ml

5.7 Inoculating loop (10µl) (e.g. platinum-iridium wire), loop diameter approximately 3 mm

5.8 Apparatus for shaking the culture tubes

5.9 Culture tubes, 25 ml capacity, or equivalent containers

5.10 Vortex mixer suitable for 25 ml culture tubes or equivalent containers

5.11 pH meter, with temperature compensation and pH-measuring cell

5.12 Homogeniser (e.g. Stomacher ®, Seward Laboratories or equivalent)

5.13 Filter membrane, for media sterilisation (0,2 µm cellulose nitrate 47 mm diameter)

5.14 Boiling water bath

6 Sampling and hazards

6.1 Introduction

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible (within 24 hours). In order to prevent propagation or inactivation of *Salmonella* during transport to the laboratory and subsequent storage, the necessary precautions depending upon the matrix shall be taken.

NOTE Generally chilling the sample to (5 ± 3) °C is recommended.

6.2 General

Samples are liable to ferment and can contain pathogenic micro-organisms. It is essential to keep them away from any food or drink, and to protect any cuts. When transporting and handling samples, it is essential that national and international regulations relating to biohazardous samples are followed.

See also the Warning note in the introduction.

6.3 Storage

It is not advisable to store samples in the open laboratory. If samples are to be stored, store them at (5 ± 3) °C for a maximum period of 36 hours.

6.4 Handling

Cleanliness when working is essential. When handling sludge samples, it is necessary to wear gloves, a face and eye protection, and ensure adequate protection against bottles bursting. The gas evolved is flammable.

See also the Warning note in the introduction.

6.5 Toxic chemicals

Extreme care must be taken when handling sodium selenite and its solutions due to their high toxicity.

7 Reagents, diluents and culture media

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare them with fit for purpose demineralised or distilled water free from substances capable of inhibiting growth under the test conditions. (ISO 8199). If the media are not used immediately, preserve them in the dark at (5 ± 3) °C for up to one month in conditions avoiding any alterations in their composition.

NOTE 1 The use of chemicals of other grades is permissible provided that they are shown to be of equivalent performance in the test.

NOTE 2 Ready to use media may also be used for the examination provided their compositions are equivalent to those specified in this sub clause.

7.1 Saline solution

Dissolve 0,85 g of sodium chloride in 100 ml of water and adjust the pH value of the solution to (7,0 ± 0,1) with sodium hydroxide or hydrochloric acid (0,1 mol/l). Pour the solution into suitable glass containers as required and sterilise in an autoclave (5.3) at (121 ± 3) °C for (15 ± 1)min.

7.2 Bromocresol purple solution

Dissolve 1 g of bromocresol purple $C_{21}H_{16}Br_2O_5S$ in 100 ml of water.

7.3 Kovac's reagent (indole reagent)

4-dimethylaminobenzaldehyde, $C_9H_{11}NO$	5 g
Isoamyl alcohol, $C_5H_{12}O$	75 ml
Hydrochloric acid (0,1 mol/ l)	25 ml

Dissolve the 4-dimethylaminobenzaldehyde, $C_9H_{11}NO$, in 75 ml of isoamyl alcohol, $C_5H_{12}O$, and heat in a water bath at 60 °C for 5 min. Then add 25 ml of hydrochloric acid (0,1 mol/l). The reagent will be ready for use after about 6 h to 7 h (indicated by a yellow colour).

Commercially available Kovac's reagent can be used according to the manufacturer's instructions.

7.4 Magnesium chloride solution

Dissolve 36 g of magnesium chloride hexahydrate. $MgCl_2$. $6H_2O$, in 100 ml of water.

7.5 Malachite green solution

Dissolve 0,72 g of malachite green oxalate, $C_{23}H_{25}ClN_2$. C_2O_4, in 100 ml of water.

7.6 Phenol red solution

Dissolve 1g of phenol red in 1,25 ml of sodium hydroxide solution (0,1 mol/l) and make up to 250 ml with water.

7.7 Lactose/peptone solution

Dissolve 20 g of tryptone and 5 g of sodium chloride in 1 000 ml of water in a 2 000 ml flat bottom flask, while heating in a boiling water bath. Adjust the pH value of the solution to (7,2 ± 0,1) using sodium hydroxide solution (0,1 mol/l), then add 10 g of lactose and 2 ml of bromocresol purple solution (7.1.2).

Transfer the solution in 10 ml portions to culture tubes (5.10), and sterilize at (121 ± 3) °C for (15 ± 1) min in an autoclave (5.3) Use within one week.

7.8 Urea medium

7.8.1 Urea basal medium

a) 1,0 g of tryptone;

b) 1,0 g of D-glucose;

c) 5,0 g of sodium chloride;

d) 2,0 g of potassium di-hydrogen phosphate (KH_2PO_4);

e) 0,012 g of phenol red;

f) 12 g of agar.

Mix all the ingredients and heat the mixture in a boiling water bath until its components have dissolved. Adjust the pH value, using sodium hydroxide solution or hydrochloric acid (0,1 mol/l), so that after sterilization it will be (6,8 ± 0,1). Sterilize in an autoclave (5.3) at (121 ± 3) °C for (15 ± 1) min.

7.8.2 Urea solution

Dissolve 400 g of urea, H_2NCONH_2, in water and make up to 1 000 ml. Filter sterilise the solution through a 0,2 µm sterile membrane filter.

7.8.3 Urea complete medium

Melt theUrea basal medium (7.8.1) and cool to 45 °C. Aseptically add 50 ml of urea solution (7.8.2) to 950 ml of Urea basal medium maintained at 45 °C . Distribute the medium into sterile culture tubes (5.10) in volumes of 10 ml portions. Allow the medium to solidify in order that the medium forms a slant in the tube.

7.9 Tryptophan-tryptone broth for the Indole formation test

Dissolve10 g of tryptone, 1 g of DL-tryptophan for biochemical applications, and 5 g of sodium chloride in 1 000 ml of water while heating in a boiling waterbath.

Adjust the pH value using sodium hydroxide solution (0,1 mol/l) so that it will be (7,4 ± 0,1) after sterilization. Transfer 5 ml portions of the solution to culture tubes and sterilize in an autoclave (5.1.3) at (121 ± 3) °C for (15 ± 1) min.