IDEXX Summary #:5BW

Topic: Colilert®, Colilert®-18, Colisure®, Quanti-Tray® and Quanti-Tray®-2000 Approval in Slovakia for water testing

Title: Water Quality enumeration of coliform bacteria and *Escherichia coli* by the defined substrate method

Source: Slovak Technical Standard

Date: September 2007

Highlights:

- Colilert®, Colilert®-18, Colisure®, Quanti-Tray® and Quanti-Tray®-2000 are approved methods for the determination of coliform and *E coli* in all water based on Slovak Technical Standard: STN 75 7841

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STN	Water quality Enumeration of coliform bacteria and *Escherichia coli* by the defined substrate method	STN 75 7841

Wasserqualitätt. Bestimmung von Coliform- und *Escherichia coli*-Bakterien mit Anwendung der Methode des definierten Substrates
Qualité de l'eau. Dosage des bactéries coliformes et *Eschenchia coli* par méthode du substrat défini

National foreword

Cited standards

STN EN ISO 3696:2000 Water for Analytical Laboratory Use - Specification and Test Methods (ISO 3696:1987) (68 4051)

STN ISO 8199:1995 Water Quality - General Guidance on the Enumeration of Microorganisms by Culture (75 7810)

STN EN ISO 19458:2007 Water Quality. Sampling for Microbiological Analysis (ISO 19458:2006) (75 7770)

STN 75 0170:1986 Water Resources Management - Water Quality Nomenclature

Related standards

STN EN 45020:2000 Standardization and Related Activities - General Vocabulary (ISO/IEC Guide 2: 1996) (01 0100)

STN ISO 9998:1998 Water Quality - Practices for Evaluating and Controlling Microbiological Colony Count Media Used in Water Quality Tests (75 7813)

Preparation of the standard

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Introduction

The presence and degree of fecal pollution are important factors for the assessment of water quality and risk of infection for human health. The defined substrate technology method enables the simultaneous enumeration of coliform bacteria and *Escherichia coli* in water samples. The presence of *Escherichia coli*, which is normally found in the human gastrointestinal tract and gastrointestinal tract of other warm-blooded animals, indicates the fecal pollution of water. The evaluation of coliform bacteria can be more complicated, because some coliform bacteria live in soil and surface freshwater and are not always of intestinal origin. Thus, the presence of coliform bacteria may indicate an error during water treatment and distribution. Identification of isolated strains can sometimes help with the determination of their origin.

1 Object of the standard

This standard describes a method for the enumeration of coliform bacteria and presumptive *Escherichia coli* in water based on proof of specific enzymes by means of chromogenic and fluorogenic substrates without the need for further confirmation.

2 Field of application

The method can be applied to all types of water except for samples with a high content of mineral salts or toxic substances, which could affect the growth of bacteria.

Water samples containing humic substances or other compounds may be colored. In that case, a control vial with a sample without substrate must be prepared, for comparison with the sample intended for culture. The salt content in samples with a high calcium content may cause sedimentation; however, this does not inhibit the reaction.

NOTE: Regarding the enumeration of coliform bacteria, this standard is applicable mainly to disinfected and pure water.

3 Terms and definitions

This standard uses the terms and definitions listed in STN 75 0170, as well as the following:

3.1 **Coliform bacteria: Bacteria** (genera of the Enterobacteriaceae family) capable of producing the β-D galactosidase enzyme, which splits the chromogenic substrate, resulting in a change of color.

3.2 ***Escherichia coli:*** A bacterium that is positive as coliform bacterium and, at the same time, produces the β-D-glucuronidase enzyme, which splits the fluorogenic substrate, resulting in fluorescence.

4 Basis of the test

The enumeration is carried out in a liquid culture medium, and the tested sample or sample portion is mixed with a precisely defined amount of substrate. The substrate needed for the enzyme testing is commercially available.[1] Systems can be used for qualitative proof of the monitored bacteria groups (presence/absence). The quantitative determination is based on the calculation of the most probable number (MPN). It is possible to use a system where test tubes are used for the water sample culture, using one of the inoculation systems referred to in STN

ISO 8199 and calculating the probable number of CFUs in the sample based on the calculation and statistical tables in STN ISO 8199. Use of multi-well plates,[2] which are commercially available, is another possibility, in which case the number of CFUs per a given sample volume is calculated based on positive wells and the statistical tables supplied by the manufacturer.

4.1 Enumeration of coliform bacteria

The chromogenic substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) is used for coliform bacteria group enumeration. Chlorophenolred-β-D-galactopyranoside (CPRG) is used for β-D-galactosidase enzyme detection. The enzyme is characteristic for coliform bacteria. It hydrolyzes the substrate and produces a color change that indicates a positive reaction over 18 or 24 hours for ONPG, depending on the system used, and 24 hours for CPRG.

NOTE - Bacteria that do not belong to the coliform group, such as *Aeromonas* and *Pseudomonas*, can produce a small amount of β-D-galactosidase. Growth of these bacteria is suppressed, and they normally do not produce a positive reaction when present in up to 10^4 CFU/ml.

4.2 Enumeration of Escherichia coli

The fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) is used for enumeration of *Escherichia coli* to detect the β-D-glucuronidase enzyme, which produces *E. coli*. β-D-glucuronidase hydrolyzes the substrate and splits off a fluorescing product visible under the UV light with a wavelength of 365 nm. The presence of fluorescence indicates a positive reaction for *E. coli*.

5 Basic supplies

5.1. Culture substrate

A culture substrate containing chromogenic and fluorogenic elements is commercially available. The substrate unit packs are for 10 ml, 50 ml, 100 ml and 250 ml of sample volume.

5.2 Dilution solution

Dilution can only be performed with sterile distilled water or de-ionized water without elements inhibiting bacteria growth in the testing conditions, in compliance with STN EN ISO 3696.

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[1] IDEXX Laboratories, Inc, Westbrook, ME offers Colilert® and Colilert 18® systems for test tube tests and P/A tests, Colilert MW® for test tube formats and Colisure™ for test tube test and P/A test substrates in unit packs for different sample volumes. This information is provided for STN users and does not imply that these products must be used exclusively. Equivalent products can be used with the same results.

[2)] Quanti-Tray®, Quanti-Tray®/2000 for quantification in multi-well plates.

5.3 Auxiliary supplies
An antifoaming preparation when using Colilert substrate.

6 Instruments and devices

6.1 Plastic supplies
Sterile transparent sample cases with different volumes according to the enumeration purpose, multi-well plates with 51 or 97 wells, or sterile test tubes. The material used for the sample culture must not show positive fluorescence.

6.2 Color scheme
Color scheme for the selected type of substrate (supplied by the manufacturer of the substrate used).

6.3 Sealing machine for the multi-well plates used

6.4 UV lamp with a wavelength of 365 nm

6.5 Air-heated sterilizer for glassware sterilization
Instruments, devices and laboratory glassware that are not supplied sterile must be sterilized as directed by STN ISO 8199.

6.6 Water bath and/or thermostat, thermostatically controlled at 36.0 °C ± 2.0 °C.

7 Sampling and sample handling
Samples are taken and transferred to a laboratory as directed by STN EN ISO 19458. The test begins as soon as the samples are received by the laboratory. If it is not possible to start testing immediately, the recommended storage life is 8 hours. Under rare circumstances, in compliance with STN EN ISO 19458, the permissible storage life is 18 hours from sampling. The storage temperature for samples is 5 °C ± 3 °C. If the sample is not taken just prior to the start of the microbiological analysis and is stored in the icebox, it should be allowed to temperate at laboratory temperature (at least 20 minutes before using the sealing machine for multi-well plates). The procedure is the same for cases where the sample was taken at an exceptionally low temperature.

8 Workflow

8.1 Sample preparation

For enumeration with multi-well plates, provided the sample contamination is higher than the upper enumeration limit (200 for multi-well plates with 51 wells and 2,419 CFU/ml for multi-well plates with 97 wells), the sample must be diluted. Dilution can only be done with sterile distilled water.

8.2 Sample inoculation

8.2.1 Determination of presence/absence

The enumeration of microorganisms is carried out directly in the water sample. The volume of undiluted or, where necessary, diluted sample to be tested is measured into a sterile, transparent, non-fluorescing sample case. The substrate is added to the sample aseptically, so as not to contaminate the sample. The appropriate substrate unit pack for the inoculated sample volume is used. The substrate is stirred in the water sample until it is fully dissolved.

8.2.2 Quantitative enumeration of MPN in test tubes

An appropriate number of sterile test tubes for the sample culture are selected. The water sample is pipetted into these test tubes, the appropriate amount of substrate is added for the volume used and a series of dilutions is prepared as directed by STN ISO 8199. Disposable test tubes supplied by the manufacturer with pre-weighed amounts of substrate can also be used. A series of dilutions is prepared according to the instructions. The necessary sample volume is added to each test tube, observing good microbiological laboratory practice.

8.2.3 Quantitative enumeration of MPN in multi-well plates

For MPN enumeration, multi-well plates, such as the Colilert®/Quanti-Tray[3] system, can be used. The multi-well plates of the Colilert®/Quanti-Tray system are intended for 100 ml samples, and two types of multi-well plates are available: 51-well plates, where the maximum number range for monitored bacteria groups with an undiluted sample is 200 CFU/100 ml of sample; and 97-well plates, available in two sizes, where the maximum number range for monitored bacteria groups with an undiluted sample is 2,419 CFU/100 ml of sample. The type of plate is selected according to the estimated sample contamination. The sample is prepared as directed in point 8.2.1 above and transferred to the multi-well plate. The plate is sealed using the sealing machine.

8.3 Culture

The culture is carried out at 36.0 °C ± 2.0 °C. The culture time depends on the system used. It varies from 18 hours to 28 hours, and the reading time specified by the manufacturer must be thoroughly observed.

NOTE - Extending the culture time beyond the maximum permitted time entails a risk of false positive results.

[3)] Colilert/Quanti-Tray®, Colilert 18®/Quanti-Tray®, and/or Colisure®/Quanti-Tray® are the trade names of products supplied by the company IDEXX. This information is provided for STN users and does not imply that these products must be used exclusively. Eequivalent products can be used with the same results.

9 Evaluation

After the culture, the enumeration, performed in accordance with point 8.2.1 above, is followed by an evaluation of appearance (color and fluorescence) in accordance with Table 1.

Table 1

Appearance	**Result**
less color change than with the color scheme	coliform bacteria and *E. coli* negative
yellow color, where the ONPG substrate is used, or red color, where the CRPG substrate is used	coliform bacteria positive
color change and fluorescence under UV light	*E. coli* positive

NOTE: If the original sample is colored, the sample cultured using the defined substrate method must be compared to a control sample to which no substrate has been added.

As for the quantitative test described in point 8.2.2, and regarding the coliform bacteria count, the positive test tubes with yellow coloring are added up and evaluated according to the statistical tables for the chosen inoculation scheme, which are listed in STN ISO 8199. As for the number of *Escherichia coli*, yellow test tubes displaying fluorescence are counted.

Regarding the quantitative test described in point 8.2.3, yellow wells are counted in the multi-well plate first. Every well is marked. Next, wells displaying fluorescence are marked and subsequently counted, then evaluated according to the statistical tables supplied by the manufacturer.

NOTE 1 - Fluorescence verification: In a dark room using a 6 W UV lamp with a wavelength of 365 nm with a maximum distance of 8-12 cm from the sample.

NOTE 2 - Reading can be done using the color scheme supplied by the manufacturer for comparison. This determines the lowest intensity of yellow coloring and fluorescence. A typical positive test result is usually much more intense.

NOTE 3 - If the sample color is not as intense as the comparison sample after the specified culture time, or if the result is dubious, the sample can be further cultured for a maximum of 4 hours with the ONPG medium or 4-24 hours with the CRPG medium. If there is no increase in intensity, the sample is considered negative.

10 Formulation of results

10.1 Determination of presence/absence

Coliform bacteria and *Escherichia coli* are present/absent in a given sample with a sample volume of X (e.g. present/absent in 100 ml).

10.2 Quantitative enumeration

The number of coliform bacteria and *Escherichia coli* is calculated based on the test tubes with positive reactions in accordance with the appropriate statistical tables, e.g. as CFU/100 ml.

11 Reference strain test

When testing a water sample with a particular commercially available system, the suitability of the substrate is tested using reference materials. The procedure can be as follows: Three sterile sample cases (as per point 6.1 above) filled with 100 ml of distilled water, to which substrate has been added, and containing the following strains (the maximum concentration of the inoculated reference strains must be 100 CFU/100 ml of sample) are inoculated:

A. *Escherichia coli* (CCM 3954, ATCC 25922, ATCC 11775)
B. *Klebsiella pneumoniae* (ATCC 31488) or *Enterobacter cloacae* (CCM 1903)
C. *Pseudomonas aeruginosa* (CCM 3955, ATCC 10145, ATCC 27853)

The samples obtained this way are cultured according to the procedure for the given substrate used. Sample A tests positive for coliform bacteria and *E. coli*, sample B tests positive for the presence of coliform bacteria, and sample C must remain negative.

NOTE: The test should use reference materials from the microorganisms collection of a renowned national source of reference microorganisms.

12 Test protocol

The test protocol must contain the following information:

a) A link to this standard;

b) All details needed for complete sample identification;

c) The method and culture media used;

d) Formulation of results as directed in point 9;

e) Any strange phenomena observed over the course of the test and any activities not specified in this method that might affect the result.

13 Quality assurance

The tests must be performed in a laboratory with a clearly defined quality assurance system to ensure that the materials and methods are suitable for the test.

Appendix A (informative)
Further microbiological information on coliform bacteria

The coliform bacteria group consists of several genera of bacteria belonging to the *Enterobacteriaceae* family. The historical definition of this group of microorganisms is based on the method (lactose fermentation) used for their detection, rather than on the principles of systematic bacteriology. Standard tests for the coliform bacteria group are based on the principle of using cultures in liquid culture media, the determination of presence/absence, the enumeration of the most probable number (MPN), membrane filtration and subsequent cultures on solid selective medium, as well as on the principle of defined substrates. In the case of the defined substrate method, the enzymatic activity of certain enzymes produced by the target bacteria group is monitored. Each of these methods is applicable within certain limiting specifications and in keeping with the objective of the test.

References

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[7] *Standard Methods for the Examination of Water and Wastewater.* [Štandardné metódy na skúšanie vody a odpadovej vody] 21th ed. Washington: American Public Health Association, 2005.