IDEXX Summary

**Topic**  
Italian method approval for Colilert-18® /Quanti-Tray®, Filta-Max and Invitrogen products for drinking water and swimming pools

**Title**  
Analytical reference methods for water intended for human consumption according to Legislative Decree 31/2001 Microbiological methods

**Source**  
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**Letter Highlights:**

- A positive equivalency assessment was demonstrated by several Italian labs according to the criteria of ISO 17994

- Colilert-18/Quanti-Tray demonstrated method equivalence with ISO 9308-1 for detection and quantification of both coliforms and *E. coli* and can be used as an official reference method

- Filta-Max is an approved method for the detection of *Giardia* and *Cryptosporidium* in both drinking water and in swimming pools

- Additional approved products for Giardia and cryptosporidium separation and detection include:
  - Sample Mixer MX-1
  - MPC-1 and MPC-S magnetic devices
  - L10 test tubes
  - Spot-on slides (IDEXX SingleSpot slides)
Analytical reference methods for water intended for human consumption according to Legislative Decree 31/2001
Micro-biological methods

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DETECTION OF ESCHERICHIA COLI

0. General and definitions

The summary set out below refers to the Escherichia coli parameter detected with the ISS A methods [001A rev. 00; 001B rev. 00; 001C rev. 00]. Escherichia coli was described for the first time in 1885 by Theodor Escherich with the name of Bacterium coli. The micro-organism, rod gram-negative, facultative aerobe and anaerobe, non-sporogenous, is part of the family of Enterobacteriaceae and is included in the group of the coliforms. According to the traditional classification, the species produces indole in tryptophan media and is lactose fermenting distinguishing itself from the non-thermotolerant coliforms by the rise in temperature of 44°C. In the context of the coliform group, Escherichia coli is widely represented and in an exclusive relationship with the gastro-intestinal tract in humans and warm-blooded animals, as opposed to micro-organisms of a not necessarily fecal origin, belonging to the Enterobacter, Klebsiella and Citrobacter genera and to the number of psychrotrophic coliforms that are characterised by a marked potential for re-growth once exposed to the environment.

For more than a decade the World Health Organisation has recognised the species E. coli as a primary indicator of fecal contamination of water. The studies of the US EPA have also contributed to confirming the need to replace the fecal coliform parameter with that of Escherichia coli for the evaluation of the quality of water. The choice, motivated by the net predominance of E. coli over the other coliforms in the fecal material and by the lower sensitivity of the micro-organism to the disinfection procedures with respect to most of the enteric pathogenic bacteria, has now been accepted by the entire international scientific community. However the more classical methods used to detect it are inadequate due to their laborious nature and time required as they are not designed to select it but to detect an entire group of coliforms, and take a long time to achieve a result. Also for the coliforms it has been confirmed that part of the biotypes of E. coli present in the waters are not even able to ferment the lactose, nor to produce gas in the traditional culture media. Moreover some are not even thermo-tolerant, and do not produce indole in media containing tryptophan. On the other hand there is considerable evidence that a high percentage of E. coli, around 98%, and with the exception of the O157:H7 serotypes, possesses the enzyme β-D-glucuronidase.

In the last few years therefore substrates have been formed, in ever increasing numbers, to search directly for Escherichia coli all based no longer on the traditional reaction of the fermentation of the lactose, but on the detection of the enzyme activity of the β-D-glucuronidase, shown by the hydrolysis of chromogenic and fluorogenic β-glucuronidi with the release of coloured or fluorescent compounds. The introduction of analytical methods that make use of this specific characteristic, often eliminating the need to carry out confirmation tests, enables results to be obtained in much shorter times and to detect the micro-organism with greater accuracy.

For the micro-biological parameters, as opposed to the chemical parameters, the European Directive 98/83/CE establishes analytical reference methods. However it also provides the Member States with the possibility of additional methods at least equivalent to the reference methods established in Appendix III, part 1, to be used as an alternative to those indicated by the law, identified in compliance with specific procedures and statistical processing of the data. In this context the results of the comparative laboratory study organised by Methods Sub-commission II of the Micro-biological and Biological Methods Group of the Upper Health Institute have shown that, as well as the method indicated in Appendix III (ISO 9308-1), the Colilert Quanti-Tray™ system can also be used as an official reference method for the Escherichia coli parameter.

In water intended for human consumption the obligatory absence of Escherichia coli is prescribed in terms of its role as a primary indicator of fecal contamination. Exceeding the parametric value (E. coli 0 in 100 or 250 ml) constitutes a non-compliance with the value established by Legislative Decree 31 of 2001 (Appendix I, part A) with subsequent alterations and additions.
1. Field of application

The ISS A analytical procedures [001A rev. 00; 001B rev. 00; 001C rev. 00] are used to detect *Escherichia coli* in water intended for human consumption, in swimming pools and in treated water and in the standard dialysate when dealing with water and dialysis solutions.

2. Analysis methods

   **Method – ISS A 001A rev. 00**

   **Principle of the method**

   Multi test tube MPN (*Most Probable Number*) method. The method enables the concentration of *Escherichia coli* to be determined in a particular volume of water. It applies to the analysis of water with low or medium contamination, even disinfected and in swimming pools, and in any case to water containing damaged *Escherichia coli*. The method enables the concentration of *Escherichia coli* and coliforms to be determined simultaneously and directly in samples of water by means of a statistical estimate calculated according to the number of positive and negative test tubes obtained by adding 100 mL of sample to the substrate being grown. The result can be obtained from a suitable pre-prepared table (Table 1). After an incubation period of about 18 hours at 36 ± 1 °C the results can be read. By hydrolysing the 4-methylumbelliferone-β-D-glucuronosi (MUG), *Escherichia coli* produces fluorescence in the yellow test tubes when exposed to an ultra-violet lamp. At the same time the presence of coliforms is shown by the yellow colouring that appears in the test tubes due to hydrolysis of the O-nitrophenyl-β-D-galactopiranoside (ONPG).

   The method has been described in the *Standard Methods for the Examination of Water and Wastewater* Manual, under the title of *Enzyme Substrate Coliform Test*, in the 1996 edition. The US *Environmental Protection Agency* (US EPA) approved the method and its subsequent modifications under the title of MMO-MUG test from 1989. The *Association of Official Analytical Chemists* (AOAC) approved the method with the title of *Defined Substrate Technology* (*Colilert*) from 1995. It is recognised for the analysis of drinking water in most of the countries of the European Union and in dozens of countries in the world and is described in the *Manual of the Analytical Methods for Water* of 2003.

   **Instrumentation and glassware**

   As well as the normal laboratory equipment (Appendix) the following are needed to carry out the analysis:
   - Quanti-Tray™ multi test tube envelopes
   - Quanti-Tray™ reference comparator
   - Plastic bottles with anti-foam (100 mL) or glass bottles with Schott screw tops that can be sterilised in sterile autoclaves
   - Wood lamp for observation at 366 nm
   - Quanti-Tray™ automatic heat sealer

   **Volume to be analysed**

   The volume of sample to be analysed is equal to 100 mL, whether the sample is neat or diluted. This is to be determined however according to the type and quantity of water to be examined.
2.1.5. Procedure

2.1.5.1 Mixing the sample

Add the dry medium to a volume of 100 mL of the sample to be analysed. Mix carefully and, after the powder is completely dissolved, wait for a few minutes. Pour the solution obtained into a Quanti-Tray™ multi test tube envelope. Seal the envelope by inserting it into the Quanti-Tray™ automatic heat sealer. The envelope is sealed in 15 seconds. Incubate at 36 ± 1 °C for 18 hours (up to a maximum of 22 hours). Confirmation tests are not required.

2.1.6. Interpretation of the results

After incubation count the number of yellow test tubes that are fluorescent when exposed the light from the Wood lamp and calculate the MPN value by referring to the relative Table 1. For quality checks it is advisable to use E. coli NCTC 9001 or ATCC 25922 as a positive control and Pseudomonas aeruginosa NCTC 10662 as a negative control; as an alternative however certified reference cultures can be used.

2.1.7. Expressing the results

Express the results obtained as MPN/100 mL: consider dilution if the sample has not been analysed undiluted. In the event that there are no positive test tubes (result obtained based on Table 1: <1/100 mL) the analysis is considered statistically equivalent to a P/A (Presence/Absence) test. In this case express the result therefore as 0/100 mL. If a non-integer value is obtained – a case not considered by the law – it should be considered whether the result should be obtained by rounding to the nearest integer, down when the value of the decimal part is ≤ 0.5, otherwise up.
2.1.8. Performance characteristics of the method

The evaluation of the performance of the method, as part of a trial programme among 4 laboratories, has produced the following results:

- Sensitivity: 99%
- Specificity: 98%
- Recovery (E. coli ATCC 25922): 108%
- Escherichia coli: Repeatability = 0.000; Reproducibility = 0.004
- Intervals of confidence: corresponding to those calculated based on the formula $MPN = N \times \ln \frac{N}{N-X}$ (where $N =$ total number of test tubes with positive results) of Table 1 \(^{(1)}\)

The evaluation of the equivalence of the method (ISO 17994), as part of a programme of tests among 9 laboratories, provided the following results:

- Normality (Rankit plots – Wilk-Shapiro): 99%
- No. of colonies subject to confirmation 2145 (n % of z = 95%); confirmation rate 85%
- Average difference compared to minimum significance of the confirmed counts with respect to UNI EN ISO 9308-1: +8%.
DETECTION OF THE COLIFORM BACTERIA AT 37°C

0. General and definitions

The summary set out below refers to the Coliforms at 37°C parameter detected with the ISS A methods [001A rev. 00; 001B rev. 00; 001C rev. 00].

The coliforms, included in the family of the Enterobacteriaceae, are rod shaped bacteria, gram-negative, facultative aerobe and anaerobe, non-sporogenous. As they are present in the fecal material of human origin with an average density of $10^9$ organisms/gm, they have been considered for decades, together with fecal streptococci, to be indicators of water contamination. However it is now widely recognised in the scientific communities that environmental species able to colonise water, soil and vegetation are included in the group. The wide diffusion in the environment of micro-organisms belonging to the group has therefore redefined their role and significance in the waters and is in net contrast with the specific requirements of a fecal contamination indicator.

The most recent studies divide the micro-organisms included under this term into two main categories that based on the species and no longer the genus separate the coliforms of fecal origin from those of aquatic and telluric origin, naturally present in the water beyond any contamination.

Membership of the coliform group is based, as well as on the systematic characteristics of the different micro-organisms, historically on the method used to detect them that makes use of their capacity to ferment the lactose producing gas and acid at a temperature of 35-37 °C in 48 hours. However in the past few years the notion has been confirmed that of the coliforms present in the water a relatively high percentage can neither ferment the lactose, nor produce gas in the traditional culture media and above all that the more classic tests such as IMVIC (Indole, Red Methyl, Voges Proskauer, Citrate) do not have any discriminatory value for the identification of Klebsiella pneumoniae, Enterobacter cloacae and the many environmental species. On the other hand there is considerable evidence that a high percentage, around 99%, possesses the enzyme β-D-galactosidase. In the last few years new methods have been drawn up based on this principle that can represent an interesting alternative to the classic cultural techniques. In fact they use different substrates from the traditional ones, modified with the addition of chromogenic and fluorogenic compounds, and that are based on the development of this specific enzyme activity.

In Legislative Decree 31/2001 with subsequent alterations and additions, enacting European Directive 98/83/CE, the coliform bacteria parameter at 37°C is described in Part C (indicator parameters) of Appendix 1. In terms of the different meaning attributed to the group, the coliforms are therefore considered to be indicators of the quality and efficiency of the treatment of the water. Exceeding the value of their parameter is tolerated without prejudice to what is set out in article 14 of the Decree and can be regarded as “non-observance” of the value of the parameter.

For the micro-biological parameters, as opposed to the chemical parameters, the European Directive 98/83/CE establishes analytical reference methods. However it also provides the Member States with the possibility of additional methods at least equivalent to the reference methods established in Appendix III, part 1, to be used as an alternative to those indicated by the law, identified in compliance with specific procedures and statistical processing of the data. In this context the results of the comparative laboratory study organised by Methods Sub-commission II of the Micro-biological and Biological Methods Group of the Upper Health Institute have shown that, as well as the method indicated in Appendix III (ISO 9308-1), the methods described below can also be used as an official reference method for the coliform bacteria parameter at 37°C.
1. Field of application

The ISS A analytical procedures [001A rev. 00; 001B rev. 00; 001C rev. 00] are used to detect the coliforms in water intended for human consumption, in swimming pools and in treated water and in the standard dialysate when dealing with water and dialysis solutions.

2. Analysis methods

2.1 Method – ISS A 001A rev. 00

2.1.1 Principle of the method

Multi test tube MPN (Most Probable Number) method. The method enables the concentration of coliform bacteria at 37°C in a particular volume of water to be determined. It applies to the analysis of water with low or medium contamination, even disinfected and in swimming pools, and in any case to water containing damaged coliforms.

The method enables the concentration of coliform bacteria at 37°C and *Escherichia coli* to be determined simultaneously and directly in samples of water by means of a statistical estimate calculated according to the number of positive and negative test tubes obtained by adding 100 mL of sample to the substrate being grown. The result can be obtained from a suitable pre-prepared table (Table 1). After an incubation period of about 18 hours at 36 ± 1 °C the results can be read. The presence of coliforms is shown by the yellow colouring that appears in the test tubes due to production of o-nitrophenol (yellow) released by the hydrolysis of the ONPG (Orthonitrophenyl-β-D-galactopiranoside) catalysed by the β-D-galactosidase enzyme, characteristic of the coliforms. At the same time *Escherichia coli* can be detected which produces fluorescence in the yellow test tubes if exposed to an ultraviolet lamp, after the hydrolysis of the 4-methylumbellipheryl-β-D-glucuronide (MUG).


2.1.2 Instrumentation and glassware

As well as the normal laboratory equipment (Appendix) the following are needed to carry out the analysis:
- Quanti-Tray™ multi test tube envelopes
- Quanti-Tray™ reference comparator
- Plastic bottles with anti-foam (100 mL) or glass bottles with Schott screw tops that can be sterilised in sterile autoclaves
- Quanti-Tray™ automatic heat sealer
2.1.3 Volume to be analysed

The volume of sample to be analysed is equal to 100 mL, whether the sample is neat or diluted. This is to be determined however according to the type and quantity of water to be examined.

2.1.4 Culture media and reagents

Please go to www.iss.it for complete report.

2.1.5 Procedure

2.1.5.1 Mixing the sample

Add the dry medium to a volume of 100 mL of the sample to be analysed. Mix carefully and, after the powder is completely dissolved, wait for a few minutes. Pour the solution obtained into a Quanti-Tray™ multi test tube envelope. Seal the envelope by inserting it into the Quanti-Tray™ automatic heat sealer. The envelope is sealed in 15 seconds. Incubate at 36 ± 1 °C for 18 hours (up to a maximum of 22 hours). Confirmation tests are not required.

2.1.6. Interpretation of the results

After incubation count the number of yellow test tubes comparing the colour with the comparator and calculate the MPN value by referring to the relative Table 1.

For quality checks it is advisable to use separate suspensions or a mix of micro-organisms: positive control: E. coli NCTC 9001, Klebsiella aerogenes NCTC 9528 or K. pneumoniae ATCC 33186; negative control: Pseudomonas aeruginosa NCTC 10662.

As an alternative however certified reference cultures can be used: positive controls, E. coli and Klebsiella aerogenes; negative control: Pseudomonas aeruginosa.

2.1.7. Expressing the results

Express the results obtained as MPN/100 mL: consider dilution if the sample has not been analysed undiluted.
In the event that there are no positive test tubes (result obtained based on Table 1: <1/100 mL) the analysis is considered statistically equivalent to a P/A (Presence/Absence) test. In this case express the result therefore as 0/100 mL.

If a non-integer value is obtained – a case not considered by the law – it should be considered whether the result should be obtained by rounding to the nearest integer, down when the value of the decimal part is ≤ 0.5, otherwise up.

2.1.8. Performance characteristics of the method

The evaluation of the performance of the method, as part of a trial programme between 4 laboratories, has produced the following results:
- Sensitivity: 100%
- Specificity: 98%
- Recovery (*E. coli* NCTC 9001, *Klebsiella aerogenes* NCTC 9528): 110% *Klebsiella pneumoniae*: Repeatability = 0.008; Reproducibility = 0.007
- Intervals of confidence: corresponding to those calculated based on the formula $\text{MPN} = N \times \ln \frac{N}{N-X}$ (where $N =$ total number of test tubes with positive results) of Table 1

The evaluation of the equivalence of the method (ISO 17994), as part of a programme of tests among 9 laboratories, provided the following results:
- Normality (Rankit plots – Wilk-Shapiro): 97%
- No. of colonies subject to confirmation 4263 (n % of z = 90%); confirmation rate 89%
- Average difference compared to minimum significance of the confirmed counts with respect to UNI EN ISO 9308-1: +65%. 
DETECTION OF GIARDIA CYSTS AND CRYPTOSPORIDIUM OOCYSTS

0. General and definitions

The summary set out below refers to the Giardia cysts and Cryptosporidium oocysts parameter detected with the ISS A methods [001A rev. 00; 001B rev. 00; 001C rev. 00].

In note 2 of Appendix 1 (part C) of Legislative decree no. 31 of 2 February 2001 it was established that if samples of water that come from or are influenced by surface water show positive for the presence of Clostridium perfringens (including spores) the health authority must check that there are no potential dangers due to the presence of pathogenic micro-organisms such as, for example, Cryptosporidium. In the subsequent Legislative Decree no. 27 of 2 February 2002 that brought changes and additions to Legislative Decree no. 31 it was established that in expressing the results reference must be made to a volume of 100 L in terms of the search for the Protozoa.

The methods for searching for cysts and oocysts of pathogenic protozoa, Giardia and Cryptosporidium, respectively will be described below. The significance for the detection of parasitic protozoa in water results from the consideration that, in recent years, some protozoa initially considered to be agents of zoonosis by acquiring wider infecting capacity, have also been recognised as direct human pathogens and that the World Health Organisation has included the pathogenic protozoa Giardia and Cryptosporidium among the emerging pathogens of priority interest.

Giardia lamblia (or intestinalis) is a flagellated protozoa, recognised as pathogenic for humans from the mid 60’s. It has a monoxenous cycle that includes the trophozoite stage and that of cysts. The organisms belonging to the genus Cryptosporidium are protozo coccidea and C. parvum has been recognised as pathogenic for humans from 1976. However in the past few years other species belonging to the genus have been shown to be pathogenic for humans. Even Cryptosporidium has a monoxenous cycle, in which the sexual and asexual reproduction take place in the same host; through a series of stages the oocyst is produced, with a form of resistance in the environment and infectious in humans and animals.

The Giardia cysts and the Cryptosporidium oocysts are the infectious stages and are introduced into the environment with the faeces from infection reservoirs that can be represented by humans, but also by numerous animals whether wild, farmed or domestic. The diffusion of the cysts and the oocysts in the environment is encouraged by the low host specificity of these parasites, as well as the considerable resistance of these structures to environmental stresses.

Giardiasis and cryptosporidiosis are pathologies with fecal-oral transmission that can develop into an asymptomatic form or lead to self-resolving gastro-enteritis in immune competent subjects. With depressed immunity, and particularly AIDS, however, above all with infection from Cryptosporidium, it can become chronic causing persistent diarrhoea with severe consequences that can even lead to death.

The means of infection for both parasites are represented by the consumption of contaminated water or food, from personal contact and from animals that act as reservoirs. However water has been recognised as the main vehicle for the transmission of these parasites, the presence of which has been detected in untreated water, above all surface water, and in drinking water. In fact surface water can be contaminated easily by domestic or farm waste discharges, surface washing and irrigation with fertiliser. On the other hand cysts and oocysts are known to resist environmental stresses and the chemical and physical treatments commonly used in treating drinking water that are not therefore able to guarantee to remove parasites from contaminated water.
1. Field of application

The ISS A analytical procedures [017A rev. 00; 017B rev. 00; 017C rev. 00; 017D rev. 00] are used to detect *Giardia* and *Cryptosporidium* in water intended for human consumption and in swimming pools. They can be used to evaluate the presence and distribution of protozoa in water reserves, to identify the source of the contamination and to analyse the efficiency of the drinking water treatment process. Suitable methods are described below for searching for cysts and oocysts in water intended for human consumption containing low concentrations of suspended solids that enable the number of protozoa if present to be quantified. Certain molecular techniques (PCP, RT-PCR) are also described.

2. Analysis methods

2.1 ISS A 017A rev. 00 method

2.1.1 Principle of the method

The method refers to the ISO/CD15553 standard. It provides for filtration on a capsule with 1 μm nominal porosity, elution of the cysts and oocysts with a washing solution, concentration and purification of the eluate by centrifuging and flotation/immuno-separation and detection and counting with a microscope of the cysts and oocysts by means of direct immuno-fluorescence. Moreover molecular confirmation tests can be carried out by means of PCR and nested PCR reactions (2.3), while information on the state of vitality can be acquired by means of the RT-PCR reaction (2.4).

2.1.2 Instrumentation and glassware

As well as the normal basic laboratory equipment (Appendix) the following are needed to carry out the analysis:
- agitator with arms;
- Sample Mixer MX-1 (*Dynal*) rotating agitator;
- filtration apparatus for membrane filters from 25 mm diameter;
- wet chamber;
- refrigerated centrifuge (around +4°C) with balancing rotor for 50-250 ml containers;
- litre-counter;
- centrifuge containers of 250 ml with conical base or bottle type;
- disposable centrifuge containers of 50 and 15 ml with conical base;
- MPC-1 magnetic device for L10 test tube with plate wall (*Dynal*);
- MPC-S magnetic device for 1.5 ml test tubes (*Dynal*);
- capsule filter in polyethersulphone (1 μm porosity, 6 cm diameter, 21 cm length, 1300 sq.cm. surface area) for volumes of water less than 50 litres of entreated water; capsule filter in polyester (1 μm porosity, 6 cm diameter, 21 cm length, 1300 sq.cm. surface area) for volumes of water more than 50 litres of entreated water or up to 1000 litres of drinking water;
- polycarbonate membrane, 1.2 μm porosity, 25 cm diameter;
- epi-fluorescence microscope with filters for the FITC (450-490 nm excitation filters, 515-520 nm barrier filter) and for the UV (340-380 nm excitation filter, 420 nm barrier filter), 20, 40 and 100x lenses and eyepiece with linear micrometer. Phase contract must be provided and if possible differential interference contrast (DIC) for the 100x lens;
2.1.3 Volume to be sampled

This method enables variable volumes of water (10-1000 L) to be sampled according to the cloudiness, using different types of cartridge (polyethersulphone or polyester) or by adding the appropriate volume to the number to be filtered. If the water is chlorinated add sodium thiosulphate (2.1.4.1) (250 ml of sodium thiosulphate at 2% for every 100 L of sample).

Please go to www.iss.it for complete report.

2.2 ISS A 017A rev. 00 method

2.2.1 Principle of the method

The method refers to the ISO/CD15553 standard.

It is based on the filtration of known volumes of water, by means of filters consisting of compressed foam, on the elution of the discs themselves by means of repeated compression and decompression movements in an eluent swab and on the concentration of the eluate by drying with a vacuum pump. The concentrate is subsequently subjected to clarification by means of flotation on a cushion of saccharose or by means of immuno-separation. The Giardia cysts and the Cryptosporidium oocysts are detected by microscopic analysis of the sample by means of direct immuno-fluorescence. Moreover molecular confirmation tests can be carried out by means of PCR and nested PCR reactions (2.3), while information on the state of vitality can be acquired by means of the RT-PCR reaction (2.4).

2.2.2 Instrumentation and glassware

As well as the normal basic laboratory equipment (Appendix) the following are needed to carry out the analysis:

- **Filtre-Max Automatic Wash Station (IDEXX)** complete with equipment (housing for the filter, machine, piston, elution tube, concentration tube, base of the concentration tube, cover with magnetic bar, Allen keys, steel tube, rubber stoppers, nylon envelopes, o-ring seals);
- **Filtre-Max filter modules (compressed foam discs, IDEXX)**, 1 μm nominal porosity;
- cellulose nitrate membrane, 73 mm diameter, 3 μm porosity (IDEXX);
- magnetic plate;
- tweezers;
- manual vacuum pump.

2.2.3 Volume to be sampled

The volumes of water to be sampled by means of filtration vary according to their origin and cloudiness. The method enables up to 1000 litres of drinking water and 50 litres of waste and untreated surface water to be sampled, by concentrating the sample to a final volume of about 25 ml.